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***IN VITRO* WOUND HEALING EFFECTS OF
SINGLE AND COMBINED AFRICAN HERBAL
MEDICINES**

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P. R. KRISHNAN

PhD. THESIS

2005



16 JAN 2006

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Statement

I, the undersigned hereby state that the work contained in this thesis is wholly original and has not been submitted to any other institution, neither in whole nor in part.

..... (Paul Krishnan)

Abstract

Impaired and aberrant wound healing imposes a huge financial burden in the developed world and is an insurmountable problem in the undeveloped one. Many new approaches, such as gene therapy and tissue engineered skin have met with limited success.

The application of plant-based medicines provides, in principle, a cost effective therapy; a major criticism of herbal medicines, however, is that they are not subjected to the rigours of their pharmaceutical counterparts. This work scientifically investigates the effects of four African herbal medicines used generally in the treatment of wounds; the behaviour of normal human dermal fibroblasts (NHDF) is known to be crucial to the onset of wound healing disorders and culture of this cell type was the means by which effects were gauged.

The herbs are *Cissus rotundifolia* leaf extract (*C. rotundifolia*(L)), *Cassia abbreviata* bark extract (*C. abbreviata*(B)), *Zanthoxylum chalybeum* bark extract (*Z. chalybeum*(B)) and *Zanthoxylum chalybeum* leaf extract (*Z. chalybeum*(L)).

Aqueous extractions of herbal medicines were used in all experiments.

Single and paired combinations of the herbal medicines were assessed. The effects of combinations were compared to a theoretical additive (obtained by the summation of individual herbal extract effects); thus, combination behaviour was defined in terms of being additive, greater than additive or less than additive

The effects of single and combined herbal extracts on cultured NHDF were assessed with respect to the following wound healing parameters:

1. Growth of NHDF in basal media (to unequivocally establish the extent of effects)

and basal media / 2%FBS (to simulate impaired healing).

2. Growth of NHDF in basal media / 10% FBS + 70 µg / ml of insulin-like growth factor (to simulate fibrotic healing)
3. NHDF exposed to H₂O₂ / FeSO₂ oxidant environment (to simulate impaired healing). NHDF were either pre-incubated with herbal extracts for 12 h (to assess protective effects) or exposed to oxidants and extracts simultaneously (to assess the ability of extracts to neutralise oxidants)
4. NHDF production of pro-collagen type I carboxypeptide (PICP) and keratinocyte growth factor (KGF)

For growth related effects and the extent to which NHDF were damaged by exposure to oxidants, the MTS assay was used as an indirect determination of viable cell number.

The production of biomolecules (PICP and KGF), was measured by enzyme linked immunosorbent assay (ELISA).

C. rotundifolia(L) and *C. abbreviata*(B) enhanced the growth of NHDF in basal media and basal media / 2% FBS.

Combinations of these herbal extracts, when applied in basal media, produced less than additive effects when the *C. rotundifolia*(L) proportion exceeded 1:1 but greater than additive effects at *C. rotundifolia*(L) proportions of 1:1 or less.

In the case of *C. rotundifolia*(L) / *C. abbreviata*(B) combinations applied in basal media / 2% FBS, the effects were greater than additive at all proportions.

Z. chalybeum(B) had antiproliferative effects on the NHDF fibrotic model. When combined with *Z. chalybeum*(B) in proportions greater than 1:1, *C. rotundifolia*(L)

reduced the extent to which *Z. chalybeum*(B) was able to inhibit growth; when present in proportions of 1:1 or less, *C. rotundifolia*(L) was not able to influence the effects of *Z. chalybeum*(B).

Neither single nor combined herbal extracts had any effect on the production of biomolecules such as PICP and KGF. The apparent changes corresponding to the application of *C. rotundifolia*(L), *C. abbreviata*(B) and *Z. chalybeum*(B) were due entirely to their growth-related effects.

In terms of antioxidant effects, none of the herbal extracts (single or combined), when incubated with NHDF prior to oxidant addition, were able to protect cells from damage.

C. rotundifolia(L), *Z. chalybeum*(L) and *Z. chalybeum*(B), in descending order of potency, demonstrated an ability to neutralise the effects of oxidants when oxidant and herbal extracts were added simultaneously.

Combinations of *C. rotundifolia*(L) and *Z. chalybeum*(B) produced effects which were additive at all proportions.

Combinations of *C. rotundifolia*(L) and *Z. chalybeum*(L) produced effects which were additive at *Z. chalybeum*(L) proportions greater than 1:1 but super-additive at *Z. chalybeum*(L) proportions of 1:1 or less.

Combinations of *Z. chalybeum*(B) and *Z. chalybeum*(L) were less than additive at all proportions.

The work has provided an appropriate platform for the broader study of the four herbal medicines and it is reasonable to conclude that there is a scientific basis for the

application of these herbal extracts to the treatment of wounds and wound healing disorders.

All of the herbal extracts have influenced at least one of the selected wound healing aspects, whether singly or in combination; in particular, *C. rotundifolia*(L) and *Z. chalybeum*(L) have demonstrated growth related and antioxidant properties.

The further investigation of effective herbs, in terms of other skin cell types (keratinocytes or endothelial cells) or more involved study (organ culture, human trials) would be of merit.

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Chapter 1 – Introduction

1.1 The skin: an overview of structure and function

The skin is the largest and most complex organ of the body and its functions are both protective and regulatory. Together with its derivative structures, it is also known as integument and forms a barrier against physical, chemical and infectious insult; structural and chemical adaptation of this integumentary system exert control over fluctuations in temperature and water content, together with responding to external physical stimuli such as pressure.¹

Skin has a surface area of one point five to two square metres, represents up to fifteen percent of total bodyweight and is the organ most affected by direct environmental influence.¹

The integumentary system is composed of three distinct functional layers, which are themselves multilayered. An outer epidermis provides the main protective function, the dermis contains the capillary network responsible for nutrient provision and toxic waste removal, and a basement membrane serves as an adhesive and communicative junction between the two.^{2,3}

Although distinct, these layers never function independently and their respective physical and biochemical characteristics result in the function of the skin as a complete organ.³

1.1.1 Epidermis

This tissue layer is formed from squamous stratified epithelium and, in common with



other examples of epithelia, is avascular. It is the first line of defence against physical and chemical insult and is responsible for the prevention of excess water loss or gain. In cellular terms, epidermis consists predominantly of keratinocytes (up to ninety five percent) but also included are melanocytes (pigmentation), Langerhans cells (immune response) and Merkel cells (nerve conduction).⁵

1.1.2 Dermis

Responsible for most of the structural strength of the skin, the dermis is largely connective tissue with fibroblasts, a few adipose cells and macrophages. Collagen is the main connective tissue fibre but elastin is also present.⁵

The dermis is subdivided into the papillary layer (adjacent to the epidermis) and a deeper reticular layer.⁶

Although lacking the stratification of epidermal tissue, the dermis is structurally complex and able to interact physically with the epidermis.

1.1.3 Basement membrane

The cutaneous basement membrane zone is located between the epidermis and the dermis and is also known as the dermal-epidermal junction. The junction separates these two cellularly distinct compartments and provides adhesion and a dynamic interface between them, thus governing the overall structural integrity of the skin.⁷

The basement membrane restricts the transit of molecules between the epidermis and the dermis on the basis of size and charge, thereby maintaining correct dermal and epidermal composition.⁸

1.2 The normal response of the skin to injury

1.2.1 Acute wound healing

In terms of the skin, wounding is defined as being a disruption of the structure and function of the epidermis, dermis and basement membrane; cutaneous wound healing is the process by which continuity of these tissues is restored, together with physiological function.⁹

Healing proceeds via unimpaired progression through four overlapping phases: haemostasis, inflammation, proliferation and remodelling; each phase is characterised by the infiltration into the wound site of specific cell types,¹⁰ all of which interact and communicate, by chemical signals, to optimise repair.

1.2.1.1 Haemostasis

The immediate purpose of this phase is the prevention of further tissue loss from damaged blood vessels. This is achieved, upon platelet adhesion and aggregation, by the synthesis of insoluble fibrin; adhesive molecules such as fibronectin and vitronectin are deposited onto the fibrin mesh. The formation of the fibrin based clot within damaged blood vessels facilitates cessation of hemorrhage whereas discharge from blood vessels into surrounding tissue provides a provisional matrix over which cells responsible for repair can migrate.

In the final stages of haemostasis, fibrin degradation products, together with platelet derived growth factors, are attractants for inflammatory cells.

1.2.1.2 Inflammation

In this phase, a variety of leukocytes are released from blood vessels in response to cellular signals. Inflammation is traditionally separated into an early and late stage, each of which is characterised by the predominance of particular leukocytes. The early stage features wound debridement, facilitated by the phagocytosis and eventual destruction of bacteria and cell debris by neutrophils whereas the late stage consists of macrophage predominance and the eventual infiltration into the wound site of lymphocytes.

Macrophages initially assist and ultimately supercede neutrophils, in addition to releasing several potent growth factors and chemoattractants whereas lymphocytes appear to represent a regulatory function of the immune system on wound healing, albeit a poorly understood one.

1.2.1.3 Proliferation

Stimulated growth and migration of neighbouring dermal and epidermal cells to the site of injury is the hallmark of this phase. Cellular production of biological molecules further contributes to restoration of lost tissue components. Proliferation and migration occurs by means of autocrine and paracrine signalling cascades.

Fibroblasts and endothelial cells construct connective tissue rich in collagen, abundantly populated by fibroblasts and highly vascularised, due to the formation of new blood vessels; collectively, this is known as granulation tissue.

Formation of new epidermis is due to keratinocyte migration from the wound margins, followed by stimulated proliferation and differentiation in a process known

as re-epithelialisation.

1.2.1.4 Remodelling

Remodelling occurs in tandem with proliferation i.e as soon as new tissue is produced it is remodelled. Traditionally, it has been treated as a separate phase, primarily because it continues for up to two years after injury, long after the proliferation phase has ceased.

This aspect of cutaneous healing denotes an alteration of the composition and hence structure of granulation tissue, to the end of approximating the prior, undamaged state by the formation of scar tissue; however, despite the length of time afforded to remodelling, the extracellular matrix of a mature scar never attains the physical properties of uninjured skin.

Further tissue reorganisation is brought about by the realignment of collagen fibres by internally generated cellular forces and direct cellular degradation and synthesis of biomolecules.

1.3 Wound healing : a 21st century perspective

1.3.1 Chronic wounds

Multiple local disturbances and systemic disease can result in impaired wound healing;¹¹ the resulting prolonged inflammation and toxic microenvironment are the main reason for transition from the acute to chronic state.

Pressure ulcers, diabetic foot ulcers and venous leg ulcers are common examples of

chronic wounds.¹²

1.3.2 Fibrotic healing disorders

Extreme cellular activity results in the healing process progressing beyond the point at which tissue structure and function are restored.¹³ This results in excess scar formation which, in addition to being cosmetically undesirable, disturbs the structure and function of skin; the most common examples of such fibrotic disorders are keloids and hypertrophic scarring.¹⁴

1.4 Plants : a natural resource in wound therapy

In developing countries such as Africa, injury to the skin is common and a general lack of medical resources often results in a chronic state of healing.¹⁵

Chronic wounds are also a problem in the developed world; the management of these wounds and their associated morbidity/mortality places an enormous drain on healthcare resources. Estimates of the financial burden imposed by chronic wounds in the United Kingdom were reviewed recently and are as follows¹⁶ : the overall cost of wounds to the National Health Service is about one billion pounds a year.

Foot ulceration is the most common complication of diabetes that requires hospitalisation and accounts for around twenty four thousand admissions a year, costing some seventeen million pounds. The annual costs of treating venous leg ulceration and pressure ulcers were estimated at four hundred and three hundred million pounds respectively.¹⁶

Keloid and hypertrophic scars have affected patients for centuries and remain among

the most challenging problems for physicians and surgeons.^{17,18}

Development of tissue culture techniques and animal models has revolutionised the understanding of wound healing in terms of the processes involved and such scientific methodologies have been used in the development of novel approaches to chronic wound treatment.¹¹ Prominent examples are growth factor therapy and tissue engineered skin equivalents.¹⁹ However, a recent report published in the British Journal of Surgery indicated that there has been a generally disappointing clinical outcome from growth factor trials,²⁰ tissue engineered skin has shown promise but there are still concerns regarding host-graft interactions.²¹

Research into keloids and hypertrophic scars has been less fruitful and they remain an ongoing clinical challenge, requiring multimodal therapy to achieve only partially successful results. Until such time as more effective treatment becomes available, clinicians must utilise existing regimes while continuing to experiment with new approaches.²²

In recent years, attention has been turned to the investigation of cost effective, accessible alternatives. Traditional forms of medicine practiced for centuries in Africa and Asia are being scientifically investigated for their potential in the treatment of wounds and related disorders.^{23,24}

1.4.1 Traditional herbal medicine and wound healing

The treatment of injury or disease by plants or plant material, either in the crude or processed state, is known as traditional herbal medicine.²⁵

Wound care can be traced back to early civilisations and most treatments were based on the use of indigenous plants or phytomedicines.²⁴ Today, in developing countries,

up to eighty percent of the population depend mainly on medicinal plants for their primary health care.²⁶ Consequently, largely due to lack of resources, there remains a significant role in wound management for plant-based medicine^{15,27} and it has been estimated that approximately one-third of all traditional herbal medicines are used for the treatment of wounds or skin disorders.²⁸

In developed countries such as the United Kingdom and United States, a conventional medical system prevails and phytomedicines are considered to be alternative, unproven forms of therapy. However, the use of herbal remedies in these countries to treat a wide variety of ailments has increased exponentially over the last two decades,²⁹ this popularity affords a timely opportunity to scientifically investigate the potential for traditional herbal medicines as a cost effective treatment of wound healing disorders.

1.4.2 Bioprospecting.

The establishment of traditional medicine systems has resulted in comprehensive repositories of information regarding the use of medicinal plants in the treatment of injury and disease. Such indigenous knowledge, together with the vast biodiversity typical of developing countries, represents a genuine pharmacological resource for those searching for novel drugs and therapies.³⁰

Bioprospecting is the exploration, extraction and scientific assessment of biological diversity and indigenous knowledge for commercially valuable resources.³¹ The premise behind successful bioprospecting is the screening of plant material by virtue of low-cost bioassays which reproduce pharmacological properties *in vitro*.³²

Bioprospecting methodologies fall into two contrasting categories, each of which is

determined by the search strategy.³³

1.4.2.1 High throughput screenings (HTS).

Plant extracts are screened for pharmacological activity with respect to a particular disease as represented by an appropriate bioassay. Large numbers of compounds are screened very quickly and such a methodology is favoured by many drug companies because it results in a greater sample diversity.³⁴

A major disadvantage, however, is that, due to the high throughput nature, only one bioassay can be used to assess the activity of each plant; consequently, plants having genuine *in vivo* activity but not exhibiting the particular *in vitro* effect are liable to be overlooked.³²

1.4.2.2 Targeted screenings

This approach is dependent on cooperation between scientists and traditional healers. Searches are directed by the application of obtained indigenous knowledge and have the advantage that the choice of bioassay is thus determined by traditional use of the plant as opposed to a disease chosen arbitrarily. Sample collection, extraction and screening generally takes place over a longer period than for HTS and more time and analysis is devoted to samples selected in this way.³³

Although lacking the expediency and sample diversity of HTS, a targeted search strategy is, at least in principle, more efficient, due to the investigated samples already having reported effects in man.³⁵

1.5 Screening of wound-healing plants

1.5.1 General cell culture-based studies of wound healing

Cultured cells have been used to investigate cellular aspects of tissue repair such as proliferation and production of growth factors,³⁶ damage due to excess toxins produced during inflammation,³⁷ and synthesis of wound healing biomolecules.³⁸

These *in vitro* models permit the examination of isolated components of the wound healing process, an undertaking which is difficult in the more heterogeneous *in vivo* situation. Cell culture techniques also enable the preliminary assessment of materials for their potential to promote wound repair.³⁹

Pharmacological agents, dissolved in growth media, are applied to monolayer cultures of cells which have been derived from skin. The cells are incubated for a specified period prior to the measurement of a pharmacological response synonymous with wound healing. Dermal fibroblasts, epidermal keratinocytes and dermal endothelial cells are the most studied wound healing effectors.⁴⁰

1.5.2 Cell culture-based studies of wound healing herbal medicines

1.5.2.1 General procedures

Extraction of plant material

Plant material is macerated and extracted into hot water. Subsequent extractions of pharmacologically active herbs are carried out using organic solvents but aqueous extractions can be most easily reconstituted in growth media. The resulting herbal

extract solutions are lyophilised and stored in the dark at 4°C. Solutions for cell culture-application are diluted from stock on the day at which experiments begin.

Assessment of Proliferation

The wound healing process requires the proliferation of a variety of reparative cells such as fibroblasts, keratinocytes and endothelial cells.

The extent to which herbal extracts influence the growth of these cells is an indication of their potential for wound healing. In studies of this nature, cell growth media is supplemented with a variety of factors which facilitate optimum cell culture; to simulate the differing wound microenvironments characteristic of particular healing disorders, cells are cultured in media containing differing proportions of supplements prior to the addition of herbal extracts.

In addition to a preliminary assessment of wound healing activity, proliferation assays can also provide information as to the limiting cytotoxic concentration.

Assessment of antioxidant effects

Prolonged inflammation is common to chronic wounds such as pressure ulcers, diabetic ulcers and venous leg ulcers. Part of the function of inflammation is the preparation of the wound environment for infiltration by reparative cells such as fibroblasts. During the inflammatory phase, bacteria are destroyed by the release of oxygen free radicals.¹⁰ Prolonged inflammation results in a toxic excess of these species and impairs the healing process.^{10,12} The addition to cell cultures of oxygen free radicals provides an approximation of such a wound microenvironment and enables the antioxidant ability of herbal extracts effects to be gauged.

Assessment of collagen-producing effects

Fundamentally, a wound represents a loss tissue which must be replaced by the healing process. The extent to which cultured fibroblasts produce biomolecules such as collagen is a factor crucial to both impaired and excessive healing. The ability to influence collagen production by cultured cells is therefore another indication of the wound healing-potential of traditional herbal medicine extracts.

1.5.2.2 Examples of cell culture-based investigation of wound healing traditional medicines

Centella asiatica is a plant growing abundantly around the Indian Ocean and has been used traditionally for several centuries in India and Oriental countries as a treatment for wounds. A drug derived from the plant has been developed in Europe, under the name of "Titrated Extract from *Centella asiatica*" (TECA). The extract is a reconstituted mixture of asiatic acid, madecassic acid and asiaticoside⁴¹ and has been investigated in terms of its effects upon fibroblast proliferation and collagen production, where it was found to enhance the latter.⁴²

Eupolin, an ointment used in Vietnam to treat burns, consists of an aqueous extract from the leaves of *Chromolaena odorata* suspended in petrolatum. Due to the lack of resources in Vietnam, considerable interest has been expressed in the clinical application of this traditional remedy.²⁷ Aqueous and organic extracts of this herb have exhibited effects upon fibroblast, endothelial cell and keratinocyte proliferation^{27,43} in addition to protection of fibroblasts and keratinocytes from oxygen free radicals.⁴⁴

Scophularia nodosa, an Irish folk medicine, is still used today in the treatment of

wounds. Methanolic extracts of the plant have recently shown the ability to enhance fibroblast proliferation and further study is ongoing.⁴⁵ Similar effects were evident in studies of extracts from the leaves of *Buddleja globosa*, used in Chile and Argentina for topical wound treatment.⁴⁶

It is common in Traditional Chinese medicine to apply aqueous extractions of several herbs in combination. Aqueous solutions of Burn healing liquid (BHL) have been used for centuries in China as a topical treatment of burns. BHL is comprised of four components : *Radix sanguisorbae*, *Rhizoma Bletilae*, *Caulis Ionicerae* and *Rhizoma Polygoni Cuspidati*. The proliferative effects of burn healing liquid on fibroblasts and keratinocytes have been demonstrated.⁴⁷

The traditional history of turmeric in India and other Asian countries is for its use in food flavouring and medicine, wound healing in particular. This yellow powder is obtained from the tropical plant *Curcuma longa*. An active wound healing ingredient of *curcuma longa*, curcumin, was identified by virtue of its demonstrated protection of fibroblasts from oxidants.⁴⁸

1.6 Reasons for undertaking the study

*“There is no alternative medicine. There is scientifically proven, evidence-based medicine supported by solid data, or unproven medicine for which scientific evidence is lacking”.*⁴⁹

This statement underlines the essential difference between alternative medicines, such as herbal drugs, and their modern pharmaceutical counterparts. Although a significant number of the latter trace their origin to plants, they must meet officially decreed, stringent requirements for purity, safety and efficacy prior to public distribution or clinical application. This discrepancy has resulted in plant-based medicines being

viewed as unknown quantities, to be denied acceptance as bona fide medicines until subjected to the same rigours as modern drugs.²⁵

These observations provide a general impetus for the current work: a desire to determine if the traditional use of herbal medicines can be rationalised scientifically.

In more specific terms, it is intended to investigate the potential of certain wound healing herbal medicines as a cost effective therapy in both the developed and the developing worlds.

1.7 Aim of the study

Name of Herb	Origin	Traditional use
<i>Cissus rotundifolia</i> leaves	Kenya	The leaves are applied directly to burned and scalded skin
<i>Zanthoxylum chalybeum</i> bark.	Kenya	Used traditionally on skin diseases and as an anti-inflammatory.
<i>Zanthoxylum chalybeum</i> bark.	Kenya	Used traditionally on skin diseases and as an anti-inflammatory.
<i>Cassia abbreviata</i> bark	Malawi	Powdered bark is used for dressing ulcers.

Table 1.1 Name, origin and traditional use of the herbal medicines examined in the current study.

The current study involves the assessment of the wound healing activity of four herbs, used traditionally in Africa for the treatment of wounds and / or skin disorders, which are described briefly in Table 1.1

Nutralife UK, a nutritional supplement company, are sponsors of the current work who have entered into a partnership with traditional healers in Malawi and Kenya. Generally, such an arrangement involves the targeted screening of plant extracts used

in African traditional medicine; there is anecdotal evidence of their efficacy but they have not been investigated scientifically and the rationale for their use is based upon generations of indigenous knowledge which in turn is ultimately derived from trial and error.

Cultured cell monolayers will be employed to determine the effects of aqueous herbal extracts on cell proliferation, cellular response to oxidants, collagen production and wound-related growth factor production.

There are no published studies on the wound healing activity of any of the African herbal medicines and, more generally, there is hitherto no documented work on the effects of any herbal extracts on growth factor production.

The use of multiple herbs is common in traditional Chinese medicine and the combinatorial nature of burn healing liquid⁴⁷ suggested the idea of investigating the African herbs in such a manner. This approach affords an opportunity to determine the pharmacological profile of herbal extracts in combination, as compared with their individual profile.

When drugs are studied in this way, the possibility arises that the measured effects of combined drugs may be greater or less than the sum of effects derived individually. Such deviations of combined drugs from purely additive behaviour reflect changes in drug potency; thus, an alternative description of the phenomenon is that a greater or lesser amount of a drug is required to produce a particular effect in combination than is required individually;⁵⁰ when used with appropriate statistical tools, comparison between theoretical additive and combination behaviour have been described as the 'gold standard' of such pharmacological investigations⁵¹

The overall aim of this study is the *in vitro* examination of the wound healing effects

of four African herbs, used traditionally for treatment of wounds, which have not been previously studied. Additionally, it is intended to examine such effects in terms of single and combined herbs, by the application of proven statistical techniques, as a means of determining the extent of pharmacological interaction.

Chapter 2 – Chronic and fibrotic healing

2.1 Chronic wound healing

In the absence of complications, most wounds tend to heal within a few weeks. Chronic wounds, in contrast, either require a prolonged time to heal, do not heal, or recur frequently. These wounds tend to occur when the normal wound healing process has been compromised, for example, by infection, metabolic disturbances, or an underlying disease.⁵²

Chronic wounds inflict a huge cost upon society, impairing the quality of life for millions worldwide and, irrespective of the underlying disease, are invariably characterised by a relative inability to generate new tissue.^{53,54}

2.1.1 Pressure ulcers

Pressure ulcers are one of the major causes of morbidity in older people and the most important problem in nursing home residents, dramatically increasing the cost of medical and nursing care. In particular, pressure ulcers of the foot are very common and difficult to heal among elderly immobilised patients.⁵⁵

Commonly referred to as pressure sores, bedsores, decubitus ulcers, or simply decubitus, pressure ulcers can develop when a sustained load, friction, or shear is applied to localised areas of the body, leading to degeneration of the skin and underlying soft tissues.⁵⁶

There are various explanations regarding the etiology of pressure ulcers, with most experts adhering to the theory that they result from chronic occlusion of capillary blood flow, leading to alternating periods of ischemia and reperfusion. This process is

associated with repetitive formation of reactive oxygen species and concomitant tissue necrosis.⁵⁷

Although ischaemia has long been studied in the chronic pressure ulcer, combined ischaemia / reperfusion events have only recently been directly considered in its pathogenesis.⁵⁸

Excessive production of proteases and further production of reactive oxygen species by the newly-recruited neutrophils exacerbates the condition.⁵⁹

2.1.2 Venous ulcers

Venous leg ulceration is a difficult clinical problem to treat. It occurs in around one percent of the adult population, and its incidence is five times higher among people over sixty. The treatment of these ulcers, involving regular application of dressings, is lengthy and costly.⁶⁰

Venous ulcers constitute the majority of all leg ulcers, accounting for up to eighty percent and, in Europe, account for up to two percent of the healthcare budget.⁶¹

The suggested pathomechanics involve the development of venous hypertension or chronic venous insufficiency by the dysfunction of valves of the superficial, deep, and communicating veins due to congenital or acquired incompetence, deep venous outflow obstruction / muscle dysfunction and calf muscle pump failure.^{62, 63, 64}

The resulting forces generated are sufficient to cause intermittent capillary closure and subsequent ischaemia / reperfusion injury,⁶⁵ leading to a sequence of events resulting in venous leg ulceration; specifically, these generate toxic amounts of proteolytic enzymes such as collagenase and elastase, together with inflammatory cytokines and free radicals.^{62, 63, 64, 66}

Alternatively, recent investigations have focused attention on the involvement of inflammatory cells in the development of venous ulcers; large numbers of activated leukocytes have been shown to occlude capillary loops in affected legs and the release from these cells of toxic and degradative species is thought to be a major factor in prolonged tissue necrosis.⁶⁷

2.1.3 Diabetic ulcers

A reduced capacity to carry out general tissue repair processes is a common complication of diabetes mellitus and diabetic healing is characterised by reduced tensile strength of wounds due to defective matrix production.⁶⁸

Fifteen percent of people with diabetes develop a foot ulcer during their lifetime; such chronic wounds remain the most common reason for diabetes-related hospitalisation and recurrence rates in patients with previous foot ulcers are in excess of fifty percent. Diabetics are ten to fifteen times more likely to have a lower limb amputation than individuals without the disease, and the rate of major amputation continues to rise.⁶⁹

Foot problems in particular develop in diabetic patients for the following reasons :

1. Hyperglycaemia-induced oxidative stress results in decreased nerve conduction velocity and decreased endoneural blood flow, both of which are neuropathic precursors.⁶⁹
2. Neuropathy, leading to altered sensation and vulnerability to trauma and atrophy of the skeletal muscles in the feet is often associated with a subsequent derangement of the bony anatomy of the foot.^{70, 71, 72}

Ischaemic complications may also arise from peripheral arterial disease. These underlying problems are not mutually exclusive and have been known to coexist.^{70,71,72}

As is the case for pressure ulcers and venous leg ulcers, oxidant stress results from ischaemia / reperfusion and the generation of reactive oxygen species.⁷³

2.1.4 Factors which perpetuate chronic wounds

2.1.4.1 Generation of free radicals

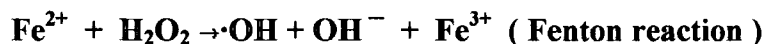
A free radical is any species capable of independent existence which contains one or more unpaired electrons.⁷⁴ The unpaired electron alters the chemical reactivity of the molecule / atom, making it more reactive than the corresponding non-radical form. Oxygen derived free radicals are collectively termed 'reactive oxygen species' and the two major examples of biological importance are the superoxide and hydroxyl radical⁷⁴ and are generated by neutrophils as part of the inflammatory response to injury.

Neutrophil activity such as phagocytosis of bacteria, secretion of proteolytic enzymes and immunomodulatory agents are accompanied by a 'respiratory burst' caused by activation of the plasma membrane-bound NADPH oxidase.^{74,75} :

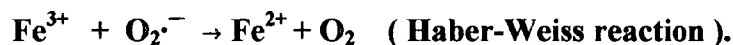


O_2^- undergoes rapid dismutation to form hydrogen peroxide (H_2O_2) in a reaction catalysed by superoxide dismutase.⁷⁶ Hydrogen peroxide alone is not reactive enough to cause damage to macromolecules; it can, however, form the highly reactive

hydroxyl radical via two interdependent reactions.^{76,77} :



The resulting ferric iron can be reduced to ferrous iron as follows :



The ferrous iron produced from the Haber-Weiss reaction provides further reactant for the Fenton reaction; thus, in the presence of iron, physiological generation of superoxide during the respiratory burst culminates in hydroxyl radical generation. Among reactive oxygen species, the hydroxyl radical is the most potent damaging species, able to react with proteins, nucleic acids, carbohydrates and lipids. The high reactivity of the radical results in the disruption of the DNA molecule via formation of protein cross-links, single and double strand breaks and damage to constituent bases; general cell damage is a consequence of lipid peroxidation and protein fragmentation.^{78,79}

Ischaemia / reperfusion injury is a common pathophysiological factor in the three types of chronic ulcer and the phenomenon is worthy of further explanation. When depleted of their blood supply for a significant amount of time, tissues may reduce their metabolism to preserve function.⁸⁰

Reperfusion and the accompanying oxygenation result in elevated amounts of oxygen free radicals; inherent mechanisms which enable tissues to neutralise these species are compromised due to the reduced metabolic rate and they are elevated to toxic levels.⁸¹

Ischaemia-reperfusion events are usually repetitive and the deleterious effects are potentiated, eventually becoming sufficient to cause ulceration.⁸²

In venous ulcers, the ischemic event occurs when the normal arterial-venous pressure

gradient is no longer present. There is blood stasis and effective loss of circulation. When the leg is elevated, circulation is restored, and the inflammatory changes that occur with reperfusion exacerbate the injury. For pressure ulcers, the ischaemic event is the result of a prolonged application of force sufficient to prevent tissue perfusion and, when the force is finally removed, the reperfusion injury occurs.⁸²

In diabetic ulcers, oxidative stress is compounded by the persistent hyperglycaemia of the underlying diabetes, causing increased production of oxygen free radicals through auto-oxidation of glucose.⁸³

There have been relatively few studies of the oxidant stress in chronic or acute wound fluids. One study determined the allantoin : uric acid percentage ratio of chronic wound exudates as a marker of oxidative stress *in vivo*.⁵⁹ A significant elevation of the ratio was found in wound fluid from chronic leg ulcers compared to acute surgical wound fluid.⁵⁹ Other studies have indicated that antioxidant characteristics are enhanced in the chronic wound environment compared with that of acute wounds.^{84,85}

The highly damaging hydroxyl radical is produced by the reduction of iron in the Fenton reaction.⁷⁶ Biological systems are normally protected from this toxic consequence of free iron by virtue of protein complexes such as haem and transferrin, which bind and transport iron respectively; iron is unreactive when combined with these complexes until it is reduced, mobilised and thereby rendered available for redox reactions.^{86,87}

Elevated amounts of free iron in chronic wound exudates have recently been demonstrated;^{88,89} a resulting increase in hydroxyl radicals via the Fenton reaction has been proposed to explain the persistent inflammation and resulting tissue destruction for which the hostile microenvironment of chronic leg ulcers is responsible.^{88,89}

Chronic wound healing is, therefore, typified by a delayed transition between the

inflammation and proliferation phases; the damage incurred by reparative cells as a consequence means that extracellular matrix modelling is also compromised and does not occur to the same degree of efficiency as for acute wounds.⁹⁰

2.1.4.2 Excess proteolytic activity.

MMPs (matrix metalloproteinases) are a family of structurally related enzymes which degrade proteins. About twenty different members of the family have been identified and are produced by a variety of cells including fibroblasts, endothelial cells, macrophages, neutrophils and keratinocytes.⁹¹ In general, MMPs are not normally expressed in skin but are induced temporarily in response to the elevated growth factor levels, cell-matrix interactions and altered cell-to-cell contacts characteristic of tissue injury.⁹²

Only four inhibitors of metalloproteinases (TIMPs) are currently known and the activity is tightly controlled by TIMP-1 and TIMP-2.^{93,94}

Matrix degradation is an essential component of the normal wound repair process and is evident during inflammation, proliferation and remodeling; however, a combination of excess proteolytic activity by MMPs and reduced amounts of their corresponding inhibitors is thought to contribute to the inability of chronic wounds to heal.⁹⁵

Research has shown that chronic wounds contain an abundance of neutrophils, which secrete MMPs and elastases, leading to the inactivation of wound-healing growth factors and excessive protein degradation.^{96,97,98,99}

The production of chemical wound healing-factors by platelets, inflammatory cells and reparative cells is continuous throughout all phases of acute wound healing; the wound healing-factors of major significance are shown in Table 2.1.^{100,101,102}

The stability of peptide growth factors exposed to fluids from healing surgical wounds and from non-healing chronic wounds was examined in vitro.⁹⁶ Transforming growth factor-beta (TGF- β) or platelet- derived growth factor (PDGF) was incubated with fluids from healing surgical wounds and fluids from venous stasis or pressure ulcers; incubation with fluids from the chronic wounds resulted in significant degradation of these growth factors and these results offer a reasonable explanation for the relative lack of success from growth factor therapy.⁹⁶

Wound fluid from chronic ulcers contained extensively degraded vitronectin and fibronectin (adhesive molecules essential for cell migration during wound healing), with a corresponding reduced capacity for cell adhesion and it was hypothesised that these observations were due to excessive protease activity.⁹⁷ Subsequent work demonstrated elevated amounts of neutrophil elastase in these fluids (five to ten-fold) compared to those of acute wound fluid.^{98,99} and examination of fluid from acute surgical wounds and from non-healing pressure ulcers revealed levels of MMP-2 and MMP-9 elevated to more than ten and twenty five times respectively, in the chronic wound fluids.¹⁰³

A more comprehensive consideration of matrix metalloproteinase behaviour involved measurement of the concentrations of MMP-1, MMP-2, MMP-8, MMP-9 and TIMP-2 in extracts of the biopsy homogenates of diabetic ulcers and non-diabetic traumatic wounds.⁹² The concentration of MMP-1 was increased sixty five-fold in biopsies of diabetic foot ulcers compared with similar analysis of traumatic wound tissue.

Furthermore, MMP-2 levels were increased six-fold, MMP-8, twofold and MMP-9, fourteen-fold. These results were associated with a two-fold reduction in the expression of TIMP-2.⁹²

Continued studies in this area demonstrated higher levels of MMP-1, MMP-8 and

Table 2.1. Common chemoattractants, mitogens and growth factors produced by cells from cutaneous wound healing phases^{94,112,132}

Growth factor	Biological source	Cellular target and biological effect
Platelet derived growth factor (PDGF)	Platelets, macrophages, keratinocytes	Chemoattractant for neutrophils Chemoattractant and activator for macrophages. Chemoattractant and mitogen for fibroblasts. Stimulation of production of ECM components. Required for angiogenesis
Tumour necrosis factor-alpha (TNF- α)	Macrophages, mast cells and lymphocytes	Extravasation of neutrophils and monocytes Early activator of growth factor expression in macrophages, fibroblasts and keratinocytes.
Interferons (IFN- α , IFN- β and IFN- δ)	Lymphocytes and Fibroblasts	Activate macrophages. Inhibits fibroblast proliferation.
Interleukin-1 (IL-1),	Neutrophils, macrophages, mast cells, Lymphocytes and keratinocytes	Activates macrophages. Activates endothelial cells. Stimulates proliferation and migration of keratinocytes. Enhances the effects of TNF- α .
Interleukin-2 (IL-2),	Neutrophils, macrophages, mast cells, lymphocytes and keratinocytes	Activates macrophages, Activates T lymphocytes Activates natural killer cells, and Stimulates proliferation and differentiation of activated B lymphocytes.
Interleukin-8 (IL-8)	Neutrophils, macrophages, mast cells, Lymphocytes and keratinocytes	IL-8 - Enhances neutrophil adherence, chemotaxis and granule release.

Growth factor	Biological source	Cellular target and biological effect
Epidermal growth factor (EGF)	Platelets, macrophages, keratinocytes	Mitogenic for keratinocytes, fibroblasts and endothelial cells
Transforming growth factor alpha (TGF- α)	Platelets, macrophages, T-lymphocytes and keratinocytes.	Similar to EGF but exhibits greater mitogenic effect for endothelial cells.
Transforming growth factor beta 1 (TGF- β 1)	Platelets, neutrophils, macrophages, lymphocytes and fibroblasts	Chemoattractant for monocytes, lymphocytes and fibroblasts
Fibroblast growth factor-1 or acidic fibroblast Fibroblast growth factor-1 or acidic fibroblast growth factor (FGF-1 or aFGF)	Fibroblasts and endothelial cells	Mitogenic for fibroblasts, keratinocytes and endothelial cells.
Fibroblast growth factor-2 or basic fibroblast growth factor (FGF-2 or bFGF)	Fibroblasts and endothelial cells	Mitogenic for fibroblasts, keratinocytes and endothelial cells.
Fibroblast growth factor-7 or keratinocyte growth factor (FGF-7 or KGF)	Fibroblasts only	Induces migration, proliferation and differentiation of keratinocytes
Insulin-like growth factor-1 (IGF-1) .	Most tissues Macrophages and fibroblasts	Mitogenic for fibroblasts and endothelial cells
Vascular endothelial growth factor (VEGF)	Macrophages, fibroblasts and keratinocytes	Endothelial cell proliferation

Table 2.1 (continued) . Common chemical wound healing-factors produced by cells from cutaneous wound healing phases^{94,112,132}

correspondingly lower levels of TIMP-2; additionally, MMP-1 and MMP-8 were inactive during the final phases of healing in acute wounds whereas, in chronic leg ulcers, both were present in their active forms.⁹⁵

Interestingly, there is some evidence of unification between excessive protease activity and oxidative stress. The study discussed in the previous section⁵⁹ established a positive correlation ($r^2 = 0.72$) between elevation of neutrophil-elastase and an increased allantoin : uric acid percentage ratio.⁵⁹

2.1.4.3 Microbial infection

During periods of chronic wound-ischemia, the ability to neutralise bacteria is diminished and bacterial colonies increase in number.⁸²

Human studies have established critical tissue oxygen levels at which the risk of wound infection increases. Lower mean subcutaneous tissue oxygen tensions were documented in general surgery patients who developed wound infections compared to those remaining infection-free.^{104,105}

This increased presence of bacteria is a further stimulant for neutrophil invasion of the wound site and the resultant production of degradative proteases and harmful oxidants.

Swabs taken from chronic leg ulcers revealed the most common microbes to be *Staphylococcus aureus*, mixed coliforms, *Pseudomonas aeruginosa* and anaerobes.¹⁰⁶ *Staphylococcus aureus*, coliforms and anaerobes were associated with delayed healing and *Pseudomonas*-colonized ulcers were significantly larger and of longer duration than uncolonized ulcers.¹⁰⁶

In addition to stimulating inflammatory degradative and toxic species, there is some

evidence that some species of microbes may themselves contribute to the production of degradative species. *Pseudomonas aeruginosa* occurs in about twenty to thirty percent of all chronic ulcers¹⁰⁷ and such colonisation apparently results in differences in ulcer size and duration, prompting research into the possible production of proteinases by the species.^{106,107}

The same microbe, by virtue of elastase expression, induced degradation of fibroblast-produced proteins and proteoglycans *in vitro*, thus mimicking proteolytic activity previously identified in chronic ulcer fluid *in vivo*; additionally, elastase-containing conditioned medium and purified elastase inhibited fibroblast cell growth.¹⁰⁷ These effects, in conjunction with the finding that proteinase production was detected in wound fluid *ex vivo*, suggest that bacterial proteinases play a pathogenic role in chronic ulcers.

2.2 Fibrotic disorders

Continuance of the reparatory processes of wound healing beyond the point at which scar formation is optimised results in the formation of keloids or hypertrophic scars; Unique to humans, these two fibrotic healing disorders are characterised by hypervascularity and hypercellularity¹⁰⁸ together with excessive scarring and an overproduction of extracellular matrix components such as type I collagen.¹⁰⁹

The clinical course and physical appearance of keloids and hypertrophic scars define them as separate entities.¹¹⁰

2.2.1 Keloids

These benign, hyperproliferative growths of dermal collagen result from excessive fibroblast response to skin trauma.¹¹¹

Several cell culture-based observations have been made concerning differences between fibroblasts from keloids and normal scar tissue.^{112,113}

Greater fibroblast densities have been demonstrated in keloid tissue compared with normal granulation tissue, suggesting a greater level of proliferation¹¹² They consist of excessive amounts of thick, densely packed collagen bundles in an irregular pattern; extending beyond the confines of the original wound, keloids do not regress spontaneously, and tend to recur after excision.¹¹³

It has been estimated that fifteen to twenty percent of Africans, Hispanics and Orientals suffer from keloids¹¹⁴ and genetic predisposition coupled with some form of skin trauma are factors which play a major role in development. A variety of different types of skin injury can lead to keloid growth, including surgery, ear piercings, lacerations, abrasions and any process resulting in skin inflammation.¹¹⁵

2.2.2 Hypertrophic scars

Rapidly developing into a genuine concern and clinical challenge, hypertrophic scars are painful, unsightly, and detrimental to skin function and the condition often necessitates reconstructive surgery.¹¹⁶

In contrast to keloids, this type of scarring is only produced by deep, dermal injury,¹¹⁷ may regress spontaneously and rarely recurs after excision.¹¹⁸

Hypertrophic scarring is a fibroblast-associated dermal disorder, sharply demarcated

from the surrounding skin and characterised by raised, red, nodular and inelastic scars that undergo slow and incomplete regression in comparison to normal scars. These scars frequently occur after severe thermal injury of the skin¹¹⁹ and, pathophysiologically, are characterised by excess fibroblast activity in terms of proliferation, collagen synthesis and collagen deposition within the wound.¹¹⁹

The events differentiating hypertrophic and normal healing actually commence during the development of granulation tissue and are apparent between three and five weeks after injury.¹²⁰

One of the most notable differences is the orientation of collagen fibres. In the hypertrophic scar granulation tissue, there is a tendency for the collagen fibres to orient themselves in a whorl-like pattern as opposed to the basket weave motif typical of normal healing.¹²⁰

2.3 Fibroblasts are pivotal to chronic and fibrotic healing

Normal cutaneous wound healing has been shown to require the orchestrated communication and interaction of a variety of inflammatory and reparatory cells and it is not appropriate to focus on one cellular entity which completely represents the process.

Fibroblasts are one of the key cells in wound repair; apart from producing major extracellular components such as collagen, elastin, and proteoglycans, they also generate chemotactic and mitogenic factors for keratinocytes and endothelial cells.¹²¹

As a result of their central role in the synthesis and remodelling of extracellular matrix molecules, and the reduced and excessive production of these materials in chronic and fibrotic healing respectively, attention has been turned to fibroblast behaviour during

the onset of such disorders.

A key question is whether the deviation from normal healing is due to an abnormal subpopulation of fibroblasts or whether they are normal wound-healing cells behaving differently under some chemical or physical influence.¹²²

2.3.1 Fibroblasts and chronic healing

2.3.1.1 Reduced proliferation and premature senescence of chronic wound fibroblasts

When determining the rate of proliferation of diabetic ulcer-derived fibroblasts after forty eight, seventy two and ninety six hours, a significant decrease in the proliferation rate was found in the chronic wound fibroblasts compared to those from uninjured skin.¹²³ This study was developed further to illustrate an association between elevated formation of L-lactate and reduced proliferation by diabetic ulcer-derived fibroblasts,¹²⁴ suggesting that specific metabolic by-products of diabetes contribute to delayed healing.

Further attempts have been made to determine the influence upon growth of the chronic wound microenvironment; fibroblasts from the newborn, known to grow rapidly, were used as a model system to explore the influence of chronic wound fluid on the growth of regenerative fibroblasts.¹²⁵ Results indicated that wound fluid from venous leg ulcers dramatically inhibited growth by interference with the cell cycle; specifically, entry into the S-phase (DNA synthesis-phase) by fibroblasts was prevented.¹²⁵

Fluid samples from non-healing venous leg ulcers were compared with those taken from ulcers that had changed from a non-healing to a healing phase.¹²⁶

There was a significantly reduced proliferative response to non-healing wound fluid

samples compared to healing samples and the pro-inflammatory cytokines interleukin-1, interleukin-6 and tumor necrosis factor-alpha were found present in significantly higher concentrations,¹²⁶ suggesting that healing may be impaired by elevated levels of inflammatory mediators.

Increased pressure upon dermal tissues due to venous hypertension is one aspect of venous leg ulcer pathophysiology.⁶¹ Fibroblasts from normal skin were subjected to hydrostatic pressures twice and three times the magnitude of atmospheric pressure.²⁶⁷ Cells cultured in this way demonstrated proportionately reduced growth rates and abnormal morphologies similar to cultured fibroblasts isolated from venous ulcers. It was proposed that altered fibroblast function and morphology due to pressure elevations resulting from venous hypertension contribute to the delayed wound healing observed in this type of chronic wound.¹²⁷

The growth of fibroblasts from chronic wounds was significantly decreased compared with those from acute wounds and normal dermis and the size and morphology of fibroblasts from chronic wounds resembled *in vitro* aged or senescent fibroblasts.¹²⁸

It has been hypothesized that the percentage of senescent cells in *in vitro* populations of fibroblasts isolated from venous ulcers is directly related to the clinical healing time.¹²⁹ Analysis of fibroblasts proliferation from biopsies of venous ulcers established a positive correlation ($r^2 = 0.81$) between percentage of senescence and ulcer healing time and it was postulated that a value of fifteen percent may be used to identify ulcers which are 'difficult to heal'.¹²⁹

Although senescence has been proposed as an explanation of impaired tissue formation and remodelling by fibroblasts, recent studies comparing venous leg ulcer-derived and *in vitro* senesced fibroblasts illustrated that tissue formation and remodelling in senescent fibroblasts is actually superior to that of fibroblasts from

chronic wounds, as evidenced by attachment to type I collagen and production of MMP-2, indicative that senescence alone is insufficient to explain delayed healing.¹³⁰

2.3.1.2 Altered response of chronic wound fibroblasts to wound-healing factors

In addition to the reduced proliferation and senescent characteristics previously observed,¹²⁸ the mitogenic response of chronic wound fibroblasts to human recombinant platelet-derived growth factor was diminished with ulcer age.¹²⁸

Comparison of normal fibroblasts and those derived from venous ulcers illustrated a complete unresponsiveness of the latter cells to transforming growth factor-beta, in terms of type I collagen production;¹³¹ this was associated with a four-fold decrease in growth factor receptor expression.¹³¹

A similar lack of response to transforming growth factor-beta in terms of venous ulcer derived fibroblasts compared to their acute counterparts has been documented¹³² and the proliferative response of diabetic ulcer-derived fibroblasts was diminished relative to normal controls for epidermal growth factor, insulin-like growth factor, basic fibroblast growth-factor and platelet-derived growth-factor.¹³³

2.3.1.3 Upregulation of proteolytic enzymes

Operating under the premise that excessive degradation by matrix metalloproteinases may contribute to impaired healing, the expression of these proteins in dermal and epidermal cells from acute and chronic venous ulcers was investigated.¹³⁴ Collagenase-3 (MMP-13) was expressed abundantly by fibroblasts deep in the chronic ulcer bed but was not detected in any acute wound tissue, suggesting that this protease

is only upregulated by fibroblasts derived from chronic wounds.¹³⁴

The hypothesis that the defective extracellular matrix remodelling characteristic of chronic wounds is associated with differences in the activity of the matrix metalloproteinases and their inhibitors (TIMPs) was studied in chronic wound and patient-matched normal fibroblasts in three-dimensional collagen lattice systems.¹³⁵

The chronic wound fibroblasts exhibited significantly reduced levels of MMP-2, a finding which was associated with a corresponding increase in TIMP-1 and TIMP-2 levels.¹³⁵

Although diabetic foot ulcers are characterized by elevated levels of MMPs, it was not known whether this was due to the underlying diabetic condition or a function of diabetic wound healing. In an attempt to gain insight into the distinction, cells were taken from unwounded diabetic and non-diabetic patients,¹³⁶ when compared with the non-diabetic controls, diabetic fibroblasts produced elevated amounts of MMP-2 and precursor molecules of MMP-3, which suggests that diabetes alone is able to disrupt the proliferation and remodelling phases.¹³⁶

2.3.2 Fibroblasts and fibrotic healing disorders

2.3.2.1 Structure of keloids and hypertrophic scars

The collagen nodule, absent from normal scars, is the structural unit of all hypertrophic scars and keloids and it is reasonable to assume that study of the fine structure of the nodule might reflect the origin of the hypertrophic and keloid scar.¹³⁷

Nodules were shown to be composed of a marked increase of unidirectional collagen fibrils aligned in a highly stressed orientation. There is also a marked increase in the

number of fibroblasts and, although relatively avascular, the nodule is surrounded by microvessels and it was suggested that the origin of the hypertrophic scar and keloid is related to wound revascularisation.¹³⁷

In addition to the observation that collagenous nodules are common to both disorders, keloids and hypertrophic scars exhibited high levels of occluded microvessels and microvascular comparisons suggested keloids may be more similar to mature scars than to hypertrophic scars.¹³⁸ When examined by transmission electron microscopy the collagen fibrils of keloids were larger, more irregular and more densely packed than in hypertrophic scars. The occurrence of the irregular fibrils in keloids may reflect a significant difference in terms of collagen synthesis, fusion or breakdown. It was also suggested that the essential difference between keloids and hypertrophic scars may be in the volume of microvessels injured, and, hence, the amount regenerated.¹³⁸

Morphological examination of fifty six post-injury granulation tissues, taken from five cases of varying age post-injury, indicated that nodules are of varying shape and size due to fusion of adjacent microvascular collagen masses between lateral branches.¹³⁹ Granulation tissue was implanted into nude mice and although several proceeded to develop scar, nodules only developed when the implants contained lateral microvascular branches. It was proposed that hypertrophic scars and keloids are a product of granulation tissue elements, the most important of which are primed active fibroblasts and excessive microvascular regeneration.¹³⁹

2.3.2.2 Proliferation of keloid and hypertrophic scar-fibroblasts

Biopsies of tissue from normal skin, keloids and hyperproliferative scars were

assessed and compared in terms of fibroblast density and proliferation.¹⁴⁰

Generally, the mean density of dermal fibroblasts was significantly higher in keloids and hypertrophic scars than in normal skin, with keloid fibroblasts exhibiting the greatest density. The same trend was observed for proliferating activity¹⁴⁰ and confirmed in a later study.¹⁴¹ It was proposed that the higher fibroblast density and proliferative activity of keloids may explain how they continue to increase their volume and invade the surrounding tissue, while hypertrophic scars, with lower fibroblast density and proliferating activity, show a tendency towards spontaneous regression.^{140,141}

2.3.2.3 Response of keloid and hypertrophic scar fibroblasts to growth factors

The ability of fibroblasts isolated from lesions of hypertrophic scars, keloids, normal skin, or normal scars in contracting the provisional wound matrix was compared and analysed.¹⁴² Hypertrophic scar fibroblasts showed a consistently higher level of fibrin matrix gel contraction than other fibroblasts. This heightened level of contractility was attributed partially to the autocrine effect of transforming growth factor-beta 1. Normal and keloid fibroblasts exhibited similar basal rates of gel contraction, and both responded to platelet-derived growth factor and transforming growth factor-beta by increasing contraction between two and three-fold.¹⁴²

However, forty five percent of the transforming growth factor-beta-induced increase in gel contraction by keloid fibroblasts was mediated by the autocrine production of platelet-derived growth factor.¹⁴² The observed differences in fibrin matrix gel contractility and the underlying growth factor-mediated contraction were proposed to contribute to the different pathologies of keloids and hypertrophic scars.

In normal cutaneous wound healing, apoptosis is believed to mediate the decrease in cellularity during the transition between granulation tissue and scar and disruptions to apoptotic pathways may contribute to the onset of fibrotic healing disorders,¹⁴³ hence, the resistance of normal skin-derived, keloid-derived and hypertrophic scar-derived fibroblasts to Fas-mediated and staurosporine-induced apoptosis was assessed.¹⁴³

In contrast to fibroblasts from normal skin and hypertrophic scars, keloid-derived fibroblasts were significantly resistant to both Fas-mediated and staurosporine-induced apoptosis.

Addition of transforming growth factor-beta-1 significantly inhibited Fas-mediated apoptosis in hypertrophic scar-derived and normal skin-derived fibroblasts; furthermore, neutralisation of autocrine transforming growth factor-beta1 with its antibody abrogated the resistance of keloid-derived fibroblasts. Anti-apoptotic activity was not observed with transforming growth factor-beta-2.¹⁴³

The stimulating effects of exogenous transforming growth factor-beta 2 on cultured fibroblasts from keloids, burn-hypertrophic scars and normal skin were examined in terms of contraction of a collagen lattice and whether such effects can be suppressed with the growth factor antibody.¹⁴⁴ Keloid fibroblasts were most affected by the addition of the growth factor and, in contrast with normal fibroblasts, initial contraction by cells from both disorders was significantly increased; antibody addition inhibited the function of keloid and burn hypertrophic scar fibroblasts and also reversed the increased contraction of all the treated groups;¹⁴⁴ implicating an altered cellular response to transforming growth factor-beta 2 in the etiology of keloid and burn-hypertrophic scars.¹⁴⁴

As a means of biochemically distinguishing between burn and non-burn hypertrophic scars, proliferative scar specimens were implanted into athymic, splenic nude rats;

upon successful establishment, scars were injected with varying doses of transforming growth factor-beta 2 or control for five consecutive days and then again on days ten, fifteen, and twenty.¹⁴⁵

The specimens were measured weekly during the treatment period and a biopsy was acquired on days thirty and sixty. Fibroblasts from the explanted biopsies and the original scars were grown in cell culture, and proliferation studies were performed. From the original scar specimens, keloid scars demonstrated the greatest cell proliferation kinetics. After treatment with the growth factor, both keloids and burn hypertrophic scars showed an increase in their cell proliferation kinetics which was not exhibited by non-burn hypertrophic scars.¹⁴⁵

In addition to establishing a common pathophysiology between keloids and burn hypertrophic scars, this study served to demonstrate that the nature of injury exerts an influence on the biochemical characteristics of hypertrophic scars in general.

The role of transforming growth factor-beta 1 was further investigated in terms of the rate of collagen synthesis by fibroblasts from normal, keloid and hypertrophic scars.¹⁴⁶

Fibroblasts were cultured in fibrin-gel matrices in the presence or absence of the growth factor or growth factor antibody and collagen production was measured after forty eight hours. Keloid fibroblasts increased their rate of collagen production by almost threefold in response to transforming growth factor-beta 1, whereas fibroblasts derived from hypertrophic scars and normal skin were unaffected; the antibody reduced the rate of collagen synthesis of keloid fibroblasts by forty percent but did not suppress normal collagen production by the other fibroblast types.¹⁴⁶ It was concluded that, although similar in that they both overproduce collagen, keloid and hypertrophic scars differ in their sensitivity to transforming growth factor-beta 1, which is abundant

during the proliferation phase of wound healing.¹⁴⁶

Although the biological activities of transforming growth factor-beta 1 had been extensively studied, its regulation remained obscure. An enzyme-linked immunosorbent assay (ELISA) revealed a greater than twofold increase in production of the growth factor in conditioned medium obtained from insulin-like growth factor-treated cells compared to that from untreated controls.¹⁴⁷ These results were associated with a greater than twofold increase in transforming growth factor-beta 1 mRNA in response to insulin-like growth factor-1, an effect which persisted for at least forty eight hours after removal of the latter from the culture medium.¹⁴⁷ Furthermore, almost sixty percent of the increase in transforming growth factor-beta 1, caused by insulin-like growth factor-1, could be blocked by the addition of transforming growth factor antibody to the culture medium, suggesting that auto-induction of transforming growth factor-beta 1 may be involved;¹⁴⁷ these findings suggest a regulatory role for insulin-like growth factor-1.

The ability of keloid fibroblasts to resist apoptosis was found to correlate with over-expression of insulin-like growth factor-1 receptor;¹⁴⁸ insulin-like growth factor may inhibit apoptosis by induction of transforming growth factor-beta 1, which is also associated with such resistance.¹⁴³

In addition to its potential role in regulation, insulin-like growth factor-1 induces enhanced mRNA expression of procollagen I and III¹⁴⁹ and reduced production of collagenase¹⁵⁰ by hypertrophic scar-derived fibroblasts.

The *in vitro* migration rate of keloid fibroblasts is used to represent the clinically-observed outgrowth of tissue beyond the wound margin.¹⁵¹ Experiments using such a methodology have demonstrated greatly enhanced migration which is associated with both over-expression of insulin-like growth factor-1 receptor¹⁵¹ and measured

amounts of the growth factor itself.¹⁵²

2.3.2.4 Production of collagen by keloid and hypertrophic scar fibroblasts

Quantification of general collagen production after forty eight hours by cultured fibroblasts from normal skin, keloid and hypertrophic scars demonstrated the production by keloid and hypertrophic scar fibroblast of twelve and three times respectively that of normal cells,¹⁵³ supporting the assumption that excessive collagen production is one aspect of the etiology of fibrotic disorders.

There is, however, evidence that the relative proportion of collagen types, as opposed to a general overproduction of collagen, may be more explanatory of clinical aspects of abnormal scar structure. The ratios of types I and III collagen produced by fibroblasts from normal, keloid and hypertrophic scars were examined.¹⁵³ Although both disorders demonstrated increased type I : type III ratios compared with normal scar fibroblasts, the increase was almost threefold for keloid fibroblasts whereas hypertrophic scar fibroblasts exhibited an increase of only thirty percent. Furthermore, the increase in type I collagen for keloid fibroblasts was associated with an increase in the expression of procollagen I (a precursor molecule to type I collagen) of sixfold that produced by normal scar cells; expression of procollagen III was not similarly elevated in keloid fibroblasts. In hypertrophic scar and normal scar fibroblasts, expression of procollagens I and III was unaffected.¹⁵³

Similar work compared the synthesis of procollagen I and III by cultured fibroblasts from normal granulation tissue, keloids and hypertrophic scars.¹⁵⁴ It was found that type I procollagen production by fibroblasts from keloids and hypertrophic scars was significantly higher than for granulation tissue fibroblasts; however, in contrast to the

previous study, hypertrophic scar fibroblast production of procollagen I was more than sixty percent greater than that of keloid fibroblasts.¹⁵⁴ Synthesis of type III procollagen by abnormal scar fibroblasts was significantly reduced in comparison with their granulation tissue counterparts (forty six and seventy three percent of granulation tissue values for keloids and hypertrophic scars respectively). Interestingly, the type I : type III collagen ratios for keloids and hypertrophic scars were comparable (thirty two point four and thirty three point four respectively) compared with twelve point two for granulation tissue.¹⁵⁴

Although some aspects of the latter study are contradictory of the former, factors common to both are the elevated production of procollagen I and increased type I : type III collagen ratios relative to fibroblasts from normal tissues.

4.3.3 Conclusion

The chronic wound environment is directly associated with some underlying disease or condition. The venous hypertension and physical occlusion of blood vessels, aetiological to venous leg ulcers and pressure ulcers respectively, cause ischaemia which can increase the risk of microbial infection and potentiate the inflammatory response; in addition, due to eventual tissue reperfusion, a toxic excess of reactive oxygen species are generated which perpetuates inflammation.

Diabetes can generate reactive oxygen species by glucose oxidation, which, in addition to tissue toxicity and inflammation, is a causative factor in the neuropathy characteristic of diabetic foot ulcers. In general, these events conspire to render the subsequent proliferation and remodelling phases inefficient, resulting in improper wound closure and scar formation. Although a variety of cells are subjected to the

deleterious effects of the underlying pathology and prolonged inflammation of a particular chronic wound, there is considerable evidence that these conditions alter the behaviour of dermal fibroblasts in particular.

In the case of fibrotic disorders, there are pronounced behavioural differences between fibroblasts from keloids, hypertrophic scars and normal wound tissue in terms of structure, proliferation rates, programmed cell death, response to wound-healing factors and synthesis and remodelling of collagen.

Although less understood than chronic wounds, there is evidence that both keloids and hypertrophic scars are a consequence of the genetic predilection of fibroblasts for an altered phenotype in response to injury.

Chapter 3–Materials and Methods

Due to their pivotal position in chronic and fibrotic wound healing, the work described here used fibroblasts as the cellular representative of such disorders.

It was not possible to obtain tissue from pressure ulcers, venous ulcers, diabetic ulcers, keloids or hypertrophic scars. The cell culture environment of normal human dermal fibroblasts will be modified as a means of simulating the behaviour of fibroblasts from these disorders.

Normal human dermal fibroblasts will hereafter be referred to simply as NHDF and it was decided to assess the effects of single and combined African herbs on the following wound healing aspects :

1. NHDF proliferation
2. Proliferation of an NHDF-based model of fibrotic disorder.
3. Exposure of NHDF to hydroxyl radicals
4. Production of procollagen type I carboxy peptide (PICP) by NHDF.
5. Production of Keratinocyte Growth Factor (KGF) by NHDF

3.1 Materials

3.1.1 Normal Human Dermal Fibroblasts (NHDF)

NHDF were purchased from Biowhittaker UK Ltd, Woking, England.

The product code was CC–2511, the batch number, 1F 1618 and the tissue acquisition number, 8560.

Cells were donated by a 31 year old female Caucasian and were supplied in 1.0 ml frozen cryovials which always contained between 500,000 and 525, 000 cells.

The stated viability, as determined by Trypan Blue exclusion, was always in excess of 80%.

3.1.2 Cell culture and cell handling equipment

Incubator

A LEEC controlled temperature mammalian culture cabinet was used for both the incubation of NHDF and reagent equilibration.

Flow cabinet

A Microflow Class II laminar flow cabinet was used for the sub-culturing of cells and preparation of cell culture media and reagents.

Centrifuge

Centrifugation was carried out using a Phillip Harris Scientific Centrifuge (Sigma Laboratories).

3.1.3 Cell culture media and reagents

Fetal Bovine Serum (FBS), Fibroblast Basal Media (FBM), Gentamycin / Amphotericin B (1000 units), Trypsin, Trypsin Neutralising Solution (TNS) and Hanks Balanced Salt Solution (HBSS) were purchased from Biowhittaker UK Ltd, Woking, England

Hydrogen peroxide (5 M, 30 % wt / wt), Trypan Blue (0.4 %) and Iron (II) sulphate were purchased from Sigma Aldrich

3.1.4 Cell proliferation and ELISA kits

3.1.4.1 MTS assay kit

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] 'MTS cell titre one solution' assay kits were purchased from Promega UK, Southampton, England.

3.1.4.2 PICP assay

Enzyme Linked Immunosorbent Assay (ELISA) kits for quantification of procollagen type I carboxy peptide (PICP) were purchased from Takara, Tokyo, Japan by way of Biowhittaker UK Ltd, Woking, England.

3.1.4.3 KGF assay

Enzyme Linked Immunosorbent Assay (ELISA) kits for quantitation of Keratinocyte Growth Factor (KGF) were purchased from R and D Systems, Oxford, England.

3.1.5 Glassware and plastiware

3.1.5.1 Glassware

An improved Neubauer haemocytometer and 20 ml stoppered tubes were purchased from Fisher Scientific, Leicestershire, England.

3.1.5.2 Plastiware

25 cm culture flasks, 10.0 ml sterile pipettes, 35 mm culture plates, 96-well flat-bottomed microtitre plates and 15.0 ml conical centrifuge tubes were purchased from Fisher Scientific, Leicestershire, England

3.1.6 Liquid handling

Single channel pipettes, ranging from from 1-1000 μL and a Fisherbrand eight channel multipipette (20-300 μL) was purchased from Fisher Scientific, Leicestershire, England.

3.1.7 Measurement of optical densities

Absorbance changes of biological reagents were determined using a Dynex MRX automated plate reader.

For proliferation / cytotoxicity assays, plates were read at 490 nm.

For quantification of procollagen type-I carboxypeptide (PICP) and keratinocyte

growth factor (KGF), plates were read at 430 nm.

3.1.8 Plant Material

Samples of *Cissus rotundifolia* leaves, *Zanthoxylum chalybeum* bark and leaves and *Cassia Abbreviata* bark were provided by Dr Philip Cheung of the Department of Health, University of Durham, Durham, England.

3.2 Methods

3.2.1 Sub-culturing of NHDF

All reagents were allowed to equilibrate in the incubator prior to use with the cells.

All handling of NHDF occurred in a Class II laminar flow cabinet.

NHDF were grown to approximately 80 % confluence.

Media was aspirated from the flask and the cells rinsed with 5 ml HBSS.

2 ml of Trypsin / EDTA was added and NHDF monitored microscopically for 3 min.

After the majority of cells had been detached from the flask by Trypsin / EDTA solution, 4 ml of Trypsin Neutralising Solution was added and the resulting cell suspension transferred to a 15 ml conical centrifuge tube. The flask was rinsed with a further 2 ml of HBSS to collect residual cells and this was added to the tube. The cell suspension was centrifuged at 100 g for 5 min and the resulting pellet was re-suspended in 4 ml of fibroblast basal media / 10 % FBS by slow pipetting using a 10 ml pipette. Depending on requirements, re-suspended NHDF were either distributed

equally into four 25cm² flasks containing 10 ml fibroblast growth media / 10 % FBS or counted using a haemocytometer, diluted and known quantities dispensed (seeded) into culture vessels for experiments.

Cells from between the third and fifth passages were used in all experiments.

3.2.2 Cell counting using a haemocytometer

3.2.2.1 Background

A haemocytometer consists of a thickened glass slide into which a small, perfectly square counting chamber has been cut. The volume of the square is 0.1 mm³ (10⁻⁴ ml). The floor of the chamber is divided into nine sections and the type of cell determines which sections are used for counting.

3.2.2.2 Counting of NHDF

50 µ L of cell suspension is mixed with an equal amount of Trypan Blue (0.4 %) and 9 µ L of the mixture was pipetted into the haemocytometer.

In the case of NHDF, only the four outer squares were used for counting. The mean cell number, 'C_{av}', was used to determine the cell number per ml, 'C_{ml}' as shown in the following equation :

$$C_{ml} = 2C_{av} \times 10^4 \quad (5.1)$$

The factor of 10⁴ was necessary to express the volume (counted in 10⁻⁴ ml) in terms of

1.0 ml and a factor of 2 was incorporated to account for the dilution in Trypan Blue.

3.2.3 Aqueous extraction of plant material : experimental rationale

3.2.3.1 Nature of extractant

The extraction of plant material is the first step in the scientific investigation of herbal medicines. A fundamental of the screening strategy employed when bioprospecting for new medicines is the isolation of a single chemical entity which is believed to be responsible for a particular effect. To this end, a variety of solvents and techniques are used to extract specific chemical groups from raw plant material. However, the extraction and isolation of a single chemical entity has no merit unless sole responsibility for an attributed effect can be demonstrated; there is a school of thought which contends that the efficacy of medicinal plants is a consequence of the interaction between the many chemical components present and that isolating particular components reduces the overall effect.^{155,156}

It is intended in the current study to determine the degree of interaction between herbs in combination, as opposed to isolating and identifying active herbal ingredients believed to be responsible for effects. Therefore, in terms of the extraction of plant material, the premise is that traditional application of the whole plant can be best represented in vitro by aqueous extraction. Although admittedly, this fraction will not reproduce exactly the proportions found in undisturbed plant materials, it will, by virtue of water being the universal solvent, contain a significant number of chemicals present.

3.2.3.2 Aqueous extraction of plant material : method

Leaves from *Cissus Rotundifolia* and *Zanthoxylum Chalybeum* (5 g) were chopped into small pieces of about 1 mm².

Herbal medicine	Brief description of aqueous solution
<i>Cissus rotundifolia</i> leaf extract (<i>C. rotundifolia</i> (L))	Pale yellow solution required filtering twice
<i>Zanthoxylum chalybeum</i> bark extract (<i>Z. chalybeum</i> (B))	Light brown solution required filtering four times.
<i>Zanthoxylum chalybeum</i> leaf extract. (<i>Z. chalybeum</i> (L))	Pale yellow solution required filtering three times
<i>Cassia abbreviata</i> bark extract <i>C. abbreviata</i> (B))	Light brown solution required filtering three times.

Table 5.1. Description and abbreviation of aqueous herbal extract solutions.

Bark from *Xanthoxylum chalybeum* and *Cassia abbreviata* (5 g) was sawn into small pieces of about 2 mm².

All plant materials were heated separately at 80°C in 500 ml of distilled water for 1 hour and descriptions of the resulting herbal solutions are given in Table 5.1.

The solutions were freeze dried and kept in the dark at 4°C until required for use.

3.2.4 Assessment of NHDF proliferation : experimental rationale

3.2.4.1 Representation of the wound microenvironment

NHDF growth media consists of a basal media (which maintains cells in a healthy but

quiescent state) to which can be added a source of growth factors in varying proportions. A common form of such an additive is Fetal Bovine Serum (FBS), which is replete with the growth factors listed in Table 2.1.

The use of basal media supplemented with 10 % FBS is a recurrent factor in the normal maintenance of NHDF cell lines¹⁵⁷ and construction of in vitro models of the skin.¹⁵⁸ Additionally, it has been shown to be necessary for the healing of whole skin organ culture.^{159,160} Adjustment of the proportion of growth factors in culture medium presents an elegant means by which the microenvironment of wound-related processes may be simulated at the cell culture level. Depleted levels of growth factors such as those found in chronic wound fluid are generally represented by greatly reduced amounts of FBS (usually 0.5 %) or even serum free media. Conversely, the addition to growth media of factors known to promote excessive proliferation facilitates the modelling of fibrotic disorders in vitro.

It is conceivable that the use of even small amounts (0.5 %) of FBS could produce artefacts by interacting with herbal constituents in a growth enhancing manner and, therefore, the use of a media devoid of growth factors has the advantage of establishing unequivocally the contribution of herbal components to cell proliferation. Even allowing for the growth factor reduction discussed previously caused by excessive matrix metalloproteinase activity, chronic wounds present a physiological milieu in which herbal drugs will, to some degree, come into contact with various mitogens. Given the assertion that the wound healing microenvironment can be modelled by varying the proportion of FBS, an attempt was made to represent this inevitability.

In the study of the proliferative effects of an aqueous extract from *C. odorata*, the activity of the extract in basal media supplemented with 0.5 % FBS was compared

with that in basal media supplemented with 2 % FBS.²⁷ Although a rationale for the selection of the latter FBS percentage was not given, it has been demonstrated that 2 % is the minimum serum supplementation which maintains the cell viability of cultured whole skin but results in an overall impairment of wound healing.^{159,160}

The use of basal media supplemented with 2 % FBS is thus appropriate in the modeling of a physiological environment in which growth factors are reduced but not absent.

Large amounts of IGF-1 are present in the dermis of keloids and hypertrophic scar;¹⁶¹ although other growth factors, notably members of the TGF- β family have been implicated in the aetiology of fibrotic disorders,^{143,144,146} IGF-1 has been shown to influence the expression of at least one TGF- β isoform.¹⁴⁷ Consequently this growth factor has been used in conjunction with NHDF to construct a representative model of these disorders to study the anti-fibrogenic effects of drugs.¹⁶¹ It was felt that such a model was appropriate for similar *in vitro* assessment of the herbal extracts.

3.2.4.2 Incubation period of NHDF with herbal extracts

Several cell culture-based studies of the proliferative effects of herbal extracts on NHDF determine cell numbers at various points after the application of herbal extracts, as opposed to a single measurement.^{27,42,46,47,162,163}

Enumeration of NHDF at several points after herbal extract addition has the advantage of establishing a time course for effects; also, insight is given as to the length of time taken for effects to be manifest. Realistically, 24 hours after addition of extracts is the earliest point at which such determination can, and should, be made.

The performance of assays or cell counts at multiple time points requires that one well batch of cells be used for each point; however, assessment of the separate and combined effects of several herbal extracts on a variety of wound healing processes necessitates some economy of time and resources.

A graphical depiction of how the effect of the most potent dose, determined after one incubation period, varies with time, presents a means to incorporate a temporally-based assessment while minimising overuse of reagents and biological material.

Regarding the length of the incubation period, a choice was made based on physiological reasoning, rather than arbitrarily; it has been shown in whole skin organ culture, that dermal fibroblasts proliferate for a period of three days prior to migration into the wound site on the fourth day post -wounding.¹⁶⁴

Prolonged inflammation and its deleterious effect via free radicals and proteases would therefore reduce the growth rate of NHDF during this period. A three day incubation period of NHDF with herbal extracts prior to enumeration, with cells grown in both types of reduced media, would best represent such a situation.

In the absence of data pertaining to time periods for which fibroblasts are most active during fibrotic disorders, the same three day period may be used to assess the effects of herbal extracts on an NHDF-based model. Thus a symmetry is maintained in the assessment of proliferative and antiproliferative effects.

Once the extracts are added, the desired result of a controlled, self contained model of wound healing does not reconcile with the invasive nature of media changes and it was thus felt that effects should be judged on a single application basis.

3.2.4.3 Methods of NHDF enumeration

Direct counting of cells is the only unequivocal means of determining changes in cell number and hence the extent of proliferation.

However, when a range of concentrations of several potentially stimulatory or inhibitory agents are being investigated, counting microscopically with a haemocytometer is time consuming and would reduce the breadth and scope of a study such as the current one. Relatively inexpensive assays which lend themselves to repeated assessment of large samples are an advantage in terms of both time and resource conservation.

The application of these assays to the current study is that changes in NHDF proliferation may be assessed quantitatively by changes in the absorbance signals of cells exposed to herbal extract. The simplest way of expressing the extent of such changes is by indexing the absorbances of NHDF treated with herbal extracts to those of untreated controls. The majority of studies involving the effects upon NHDF proliferation of herbal extracts have employed the MTT assay;^{27,38,47,162,163} others have used the neutral red assay.^{45,46}

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay

First described in 1983, the MTT reagent is a pale yellow dye and may be added directly to the media in which cells are cultured.¹⁶⁴

The MTT assay is based on the reduction by living cells of the dye; specifically, the tetrazolium ring of the dye is reduced to formazan by the succinate-tetrazolium-reductase system, which is active only in viable cells, to produce a purple formazan product.¹⁶⁵ The reaction is believed to occur in mitochondria and is directly

proportional to viable cell number. The formazan product is insoluble in culture media, precipitating as crystals and must be extracted into an organic solvent such as acidified sodium dodecyl sulphate. The optical density of the resulting organic formazan solution is determined at 570 nm.¹⁶⁶

Neutral red assay

The neutral red assay is a dye inclusion method for the determination of cell viability. The cationic neutral red dye diffuses through the cell membrane, accumulating in the cytoplasm, where it is incorporated into, and bound by, the lysosomes of viable cells in a manner which is directly proportional to cell number.¹⁶⁷ Media must be removed prior to addition of the neutral red solution. After incubation, the neutral red solution is discarded, the cells washed and a solution such as acetic acid in ethanol is used to elute any neutral red which has been taken up by the cells. The absorbance of the neutral red solution is measured at 540 nm.¹⁶⁸

In any cell culture-based assay, disturbance of cells prior to measurement must be minimised. The major difference between the two assays is that, after incorporation into cells, neutral red is chemically unchanged, merely residing in lysosomes. It follows that any neutral red solution on the exterior of cells will be eluted when the organic extractant is added and, if not removed, contribute to absorbance readings; this necessitates several invasive washing steps which often remove cells and also reduce assay sensitivity.¹⁶⁹

In the case of the MTT assay, the only measurable product is produced by, and retained within, cells, thus abrogating the need for washing.

Furthermore, the metabolic activity of NHDF, as measured by the MTT assay, has been used as a means of demonstrating the efficacy of tissue engineered skin products

in several clinical studies.^{170,171,172} This assay, therefore, is able to reflect metabolic changes of NHDF which may occur even in the absence of proliferation, an insight which would not be provided by the neutral red assay.

It was decided to use a modified form of the MTT assay, namely, the MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt].

Bio-reduction of MTS reagent yields a formazan product which is soluble in culture media, thus eliminating the potentially error-prone extraction step. The solution can be applied without prior or subsequent cell culture invasion and exhibits more rapid colour development than MTT.^{173,174}

Tetrazolium-based assays such as MTT and MTS develop optical densities via cell the metabolism; hence, drugs affecting the metabolic rate at which the tetrazolium ring is reduced to formazan will produce artefacts.¹⁷⁵ Indirect assays of cell growth, reliant on measurements of cellular processes, must in some way be correlated with cell number.

The construction of a time course has been discussed and graphical depictions of absorbance versus time were proposed for the most effective herbal extract concentrations. Data from such considerations would comprise half of the criteria for MTS assay correlations with cell number and it would simply remain to perform manual counts upon parallel sets of plates.

The data obtained would enable cell number versus time and absorbance versus cell number to be plotted. A linear correlation in the latter case denotes that changes in absorbance are depicting changes in cell number; depending on the extent, deviation from linearity would be indicative of either absorbance changes being completely due to metabolic effects or a combination of metabolic and proliferative effects.

3.2.5 Effects of herbal extracts on NHDF proliferation : method

3.2.5.1 Preliminary work and general information

MTS assay.

For colourimetric determinations, 20 μ L MTS reagent was added to each well and the plate incubated for 2 hours prior to optical density measurement at 490 nm.

Determination of IGF-1 concentration for NHDF fibrotic model

IGF-1 solutions of 10, 20, 30, 40 50, 60, 70, 80, 90 and 100 μ g / ml were prepared from frozen stock in basal media / 10 % FBS. Upon preparation, solutions were drawn through a 0.2 μ m filter for sterilisation; all culture media contained Gentamycin / Amphotericin B (1000 units)

NHDF (3×10^3) were seeded into replicate wells of a 96 well plate in 100 μ L basal media / 10 % FBS and allowed to attach for 24 hours. The media was aspirated and replaced with basal media / 10% FBS containing the specified concentrations of IGF-1. A set of cells grown in basal media / 10 % FBS was used as a normal control.

The plate was incubated for 3 days and the MTS assay performed.

The dose response curve for this experiment, plotted on logarithmic axes, is shown in Figure 3.1

The maximum effect of 52% above controls corresponded to a IGF-1 concentration of 70 μ g / ml and this concentration was selected for use in the NHDF fibrotic model.

Solutions of herbal extracts

Herbal extract solutions of 10^{-3} , 10^{-2} , 10^{-1} , 1.0, 10, 100 and 1000 μ g / ml were

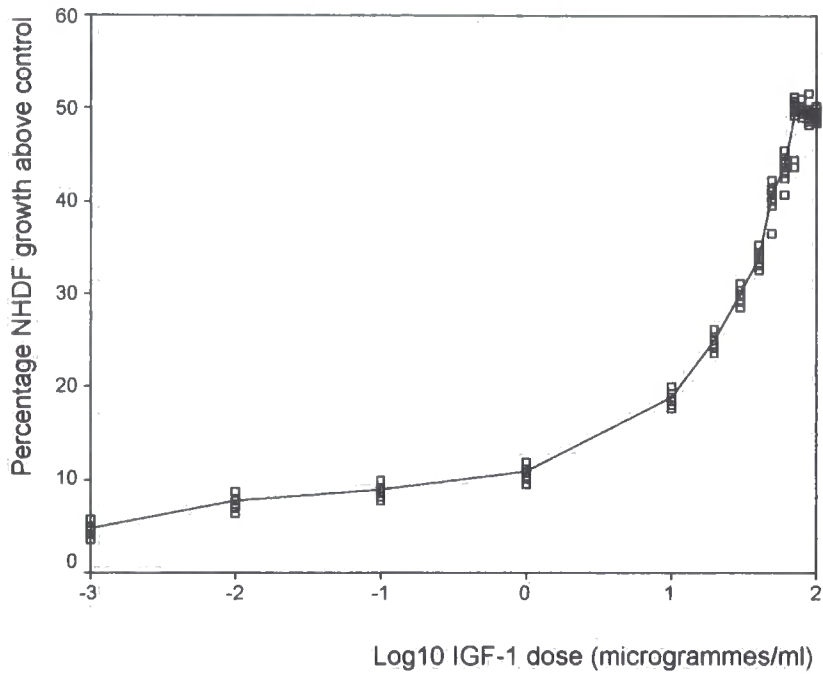


Figure 3.1. Dose response relationship between NHDF grown in basal media / 10% FBS + IGF-1

prepared in 15 ml tubes by serial dilution of refrigerated stock on the day of the experiment. Extract solutions were created in basal media, basal media / 2 % FBS and basal media / 10 % FBS containing 70 μ g / ml IGF-1. Upon preparation, all solutions were drawn through a 0.2 μ m filter to remove microorganisms; all culture media contained Gentamycin / Amphotericin B (1000 units).

Assessment of herbal extract interference with MTS reagent.

The possibility existed that the herbal extracts alone may interact with the MTS reagent and alter the optical densities of different groups. To establish the extent of

any interference, herbal extract solutions (100µL of 1000µg / ml), diluted in the different media types, were incubated with 20µL of MTS reagent for 2 hours. Controls consisted of the corresponding media type without herbal extracts.

There was no statistically significant difference between the control groups and those which included herbal extract solutions and it was concluded that the extracts did not interfere with the MTS assay.

Assessment of the optical densities of herbal extract solutions.

The herbal extract solutions may themselves contribute to optical densities in a dose dependent fashion, thus distorting the true nature of herbal effects on proliferation.

To investigate this, herbal extract solutions (100µL of 1000µg / ml), diluted in the different media types, were incubated for 2 hours, after which absorbances were measured at 490 nm, reference wavelength 630 nm. Controls consisted of the corresponding media type without herbal extracts.

There was no statistically significant difference between the control groups and those which included herbal extract solutions and it was concluded that the extracts alone did not contribute to optical densities in at 490 nm.

3.2.5.2 Assessment of herbal extracts in simulated chronic wound environment

NHDF (3×10^3) were seeded into 96-well plates in 100µL basal media / 10 % FBS and allowed to attach for 24 hours. The media was aspirated and replaced with basal media or basal media / 2 % FBS.

NHDF were allowed to equilibrate for 24 h, the media was aspirated and the herbal

extract solutions, diluted in either basal media or basal media / 2 % FBS, were added in the specified concentrations. Controls consisted of NHDF grown in basal media and basal media / 2 % FBS. Additionally, a set of cells grown in basal media / 10 % FBS was used as a positive control.

The cells were incubated with the extracts for a 3 day period and the MTS assay was performed.

3.2.5.3 Assessment of herbal extracts in a simulated fibrotic environment

NHDF (3×10^3) were seeded into 96-well plates in 100 μ L basal media / 10 % FBS and allowed to attach for 24 hours. Media was aspirated and replaced with basal media / 10% FBS supplemented with 70 μ g / ml IGF-1.

NHDF were allowed to equilibrate for 24 h, the media was aspirated and the herbal extract solutions, diluted in basal media / 10 % FBS supplemented with 70 μ g / ml IGF-1, were added in the specified concentrations. Controls consisted of a set of cells grown in basal media / 10 % FBS. The cells were incubated with the extracts for a 3 day period, after which the MTS assay was performed.

3.2.5.4 Assessment of cytotoxicity

In all experiments, ten wells were allocated as a reference for cytotoxicity assessment. Upon seeding of NHDF into wells, the cells were allowed to equilibrate for one hour, after which time the MTS assay was performed.

The corresponding absorbances of these cells were taken as a baseline and herbal extract concentrations which produced absorbances of less than this minimum level were assumed to be toxic.

3.2.5.5 Correlation of cell number with optical density

Although dose response curves were constructed for herbal extracts which produced changes in optical density measurements, their effect upon proliferation was yet to be concretely established. To this end, the herbal extract concentrations which gave the greatest change in optical density were selected for correlation.

NHDF (3×10^4) were seeded into 35 mm culture plates in 1.0 ml basal media / 10 % FBS and allowed to attach for 24 hours. The media was aspirated and replaced with basal media basal media / 2 % FBS or basal media / 10 % FBS supplemented with 70 $\mu\text{g} / \text{ml}$ IGF-1

NHDF were allowed to equilibrate for 24 h and the media was aspirated. The herbal extract solutions, diluted in either basal media, basal media / 2 % FBS or basal media / 10 % FBS supplemented with 70 $\mu\text{g} / \text{ml}$ IGF-1, were added in the specified concentrations. Depending on the wound related effect being examined, controls consisted of basal media, basal media / 2 % FBS or basal media / 10 % FBS supplemented with 70 $\mu\text{g} / \text{ml}$ IGF-1.

For each herbal extract concentration, one set of plates was used for the MTS assay and another (parallel) set was used for direct haemocytometer cell counts. The MTS assay and the parallel cell counts were performed 1, 3, 5, 7 and 9 days after addition of herbal extracts to provide both a time course and correlation of changes in MTS response and cellular growth.

3.2.6 Antioxidant effects of herbal extracts : experimental rationale

3.2.6.1 Protective and scavenging effects

In all studies of this nature, NHDF were exposed to predetermined concentrations of oxidants and effects were gauged in the context of being protective or scavenging.

Protective effects

The assessment of protective effects was based on the extent to which incubation with herbal extracts prior to oxidant exposure reduced subsequent damage.

Scavenging effects

Scavenging effects involved a determination of the degree to which herbal extracts reduce toxic effects by interacting directly with oxidants in the extra-cellular environment.

The incubation period for which cells are cultured with herbal extracts prior to exposure to oxidants is a potentially confounding factor. Assuming an extract has an effect upon cell growth, the number of viable cells will be increased or decreased during the incubation period, which in turn will conceal the true nature of extract effects on NHDF or oxidants. This problem can be obviated by selecting a period which precedes the point at which growth is induced by a particular herbal extract.

3.2.6.2 Representation of oxidant environment

All studies of this nature involving NHDF exposure to an inflammatory environment

employed hypoxanthine-xanthine oxidase and / or hydrogen peroxide as a means of generating oxygen free radicals.^{37,44,46,48,176}

The antioxidant studies which applied hydrogen peroxide most likely generated the hydroxyl radical by the Fenton reaction. The harmful effects of the superoxide radical, generated from xanthine / hypoxanthine oxidase are believed to be indirect, resulting from its conversion to hydroxyl radical.¹⁷⁷

The Fenton reaction and the Haber-Weiss reaction, discussed in are interdependent and a consideration of the relative amounts of Fe^{2+} , Fe^{3+} and H_2O_2 required in each has given insight into the molar ratios required for maximum cytotoxicity.

The continuation of the Fenton reaction is dependent upon the generation of sufficient Fe^{2+} from the Haber-Weiss reaction and it has been shown that cytotoxicity is maximal when Fe^{2+} and Fe^{3+} are in equal ratio,^{178,179,180} a condition which is satisfied when Fe^{2+} and H_2O_2 are in a 2 : 1 ratio.¹⁸¹

Preliminary work to establish a suitable oxidant environment consists of NHDF exposure to various amounts of H_2O_2 and Fe^{2+} in a fixed ratio of 1 : 2 and iron II sulphate (FeSO_4) is to be used as the source of Fe^{2+} .

3.2.6.3 Culture media in which hydrogen peroxide and herbal extracts are diluted

For protective experiments, NHDF were cultured in basal media supplemented with 10 % FBS and herbal extracts were added to cells in this media.

Oxidants were diluted in HBSS prior to addition to NHDF.

For scavenging effects, NHDF were cultured in 10 % FBS prior to the addition of either oxidants diluted in HBSS (controls) or oxidants and various concentrations of herbal extracts diluted in HBSS (treatment groups).

The species used for the purposes of replicating the effects of prolonged inflammation are, in physiological conditions, powerful oxidants. The obverse of this is that they are easily reduced. Were the oxidants to be diluted in basal media, they would be rapidly reduced by the carbohydrates, lipids and amino acids necessary to support growth at the basal level. Addition of oxidants to media containing any proportion of FBS would simply compound this problem. To an extent, such components would act as scavengers for oxidants and greatly ameliorate their effects, thus masking any ability of herbal extracts to protect or scavenge oxidants.

HBSS does not contain iron salts, will not disrupt the equilibrium of the proposed Fenton reaction-based model and hence provides a temporary culture medium in which oxidant environments may be represented.

3.2.6.4 Method of cell viability determination

The merits of the MTS assay have been previously discussed and this assay was selected for cell viability determination.

3.2.7 Antioxidant effects of herbal extracts : methods

3.2.7.1 Preliminary work and general information

Solutions of herbal extracts and H₂O₂ / FeCl₂

H₂O₂ solutions of 10⁻⁶, 10⁻⁵, 10⁻⁴, 10⁻³, 10⁻², 10⁻¹ and 1.0 M were prepared in 15 ml centrifuge tubes from refrigerated stock; HBSS was the diluent. FeSO₄ was

immediately added and the tubes lightly vortexed. The overall ratio of $\text{FeSO}_4 : \text{H}_2\text{O}_2$ was 2 : 1 for each solution. Unless stated otherwise, all $\text{H}_2\text{O}_2 / \text{FeSO}_4$ solutions shall hereafter be referred to as oxidants or oxidant solutions.

Herbal extract solutions of 10^{-3} , 10^{-2} , 10^{-1} , 1.0, 10, 100 and 1000 $\mu\text{g} / \text{ml}$ were prepared in 15 ml centrifuge tubes by serial dilution of refrigerated stock on the day of the experiment.

For assessment of protective effects, herbal extract solutions were prepared in basal media / 10 % FBS.

For assessment of scavenging effects, herbal extracts were added to HBSS which contained oxidants in such a way that the resulting solutions were comprised of herbal extracts in the specified concentrations together with the required $\text{FeSO}_4 : \text{H}_2\text{O}_2$ molarity ratio of 2 : 1; these solutions were prepared immediately prior to their use with cell cultures.

MTS assay

Generally, for colourimetric determinations, 20 μL MTS reagent was added to each well and the plate incubated for 2 hours prior to optical density measurement at 490 nm, reference wavelength 630 nm

Determination of oxidant concentration

NHDF (3×10^3) were seeded into wells of a 96-well microplate in basal media / 10 % FBS. Cells were allowed 24 hours for attachment after which the media was aspirated and the cells washed twice with physiologically buffered saline (PBS). Concentrations of oxidants, diluted in HBSS, in the specified concentration range and molarity ratio, were added and the plate incubated for 1 hour. Controls consisted of cells incubated in HBSS only. At the end of the incubation period, the oxidant

solutions were aspirated, replaced with basal media / 10 % FBS and the MTS assay was performed.

An oxidant concentration consisting of 10^{-2} M H_2O_2 / 2×10^{-2} $FeSO_4$, which reduced viable cell numbers to 32 % of untreated controls, was felt to adequately reduce the number of viable NHDF whilst allowing a sufficient potential recovery, as shown in Figure 3.2.

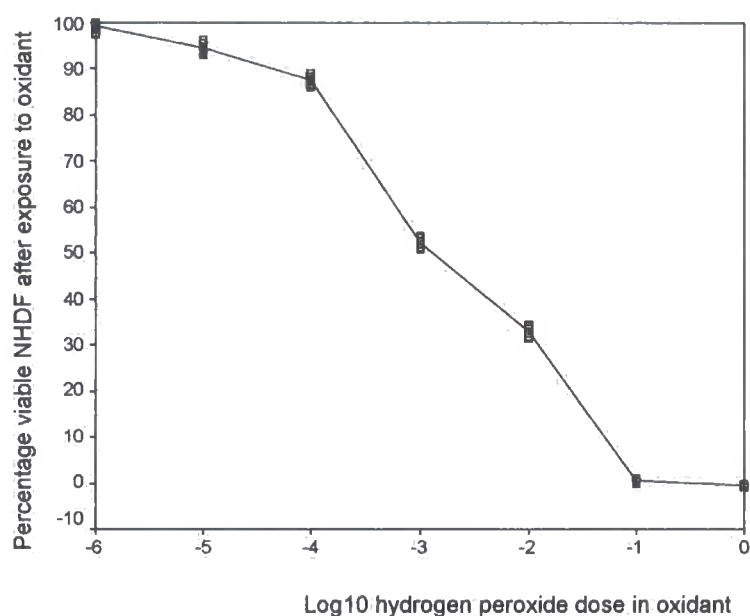


Figure 3.2. Dose response relationship between percentage NHDF viability and oxidant dose

Determination of 'protective' incubation period of NHDF with herbal extracts

NHDF (3×10^3) were seeded into 96-well microplates in basal media / 10 % FBS. Cells were allowed 24 hours for attachment after which the media was aspirated and the herbal extract solutions, diluted in basal media / 10 % FBS, were added in the specified concentrations. The plate was incubated for a period of 12 hours and the MTS assay was performed.

There were no effects upon NHDF proliferation of any herbal extract during this period and a 12 hour incubation was selected for the assessment of protective effects.

3.2.7.2 Protective effects of herbal extracts

NHDF (3×10^3) were seeded into 96-well microplates in 100 μ L basal media / 10 % FBS. Cells were allowed 24 hours for attachment after which the media was aspirated and the herbal extract solutions, diluted in basal media / 10 % FBS, were added in the specified concentrations.

NHDF were incubated with the extracts for a period of 12 hours, after which the media was aspirated and the cells washed twice with PBS.

Oxidant solutions (10^{-2} M H_2O_2 / 2×10^{-2} $FeCl_2$), diluted in HBSS, were added and the plates incubated for 1 hour. After incubation, oxidant solutions were aspirated, replaced with basal media / 10 % FBS and the MTS assay was performed.

3.2.7.3 Scavenging effects of herbal extracts

NHDF (3×10^3) were seeded into 96-well microplates in 100 μ L basal media / 10 % FBS and allowed to attach for 24 hours, after which the media was aspirated and cells were washed twice with PBS.

Solutions consisting of 10^{-2} M H_2O_2 / 2×10^{-2} $FeSO_4$ and the specified herbal extract concentrations were added and the plates incubated for 1 hour. After incubation, oxidant solutions were aspirated, replaced with basal media / 10 % FBS and the MTS assay was performed.

3.2.8 Effects of herbal extracts on collagen production: experimental rationale

3.2.8.1 Collagen quantification

Radioimmunoassay-based measurement of collagen production

A common method of collagen determination is based on the measurement of the amount of hydroxyproline, which accounts for approximately ten percent of the collagen molecule. Quantification may be performed by radioimmunoassay, the principle of which is the uptake by NHDF of radioactively labelled proline, which in turn is incorporated into hydroxyproline prior to collagen synthesis.¹⁸² This method has been used to measure collagen-producing effects of extracts of *Ginkjo biloba*.³³ and *Centella asiatica*.^{41,42} A problem with the direct quantification of collagen, as attempted in the radioisotope assay, is that it is generally insoluble in aqueous solutions such as culture media. This necessitates the several digestion, extraction, separation and purification steps^{38,41,42} and a major criticism for such treatment is loss of analyte due to the number of times the original collagen source is processed.¹⁸²

Procollagen type-I carboxy peptide (PICP) assay

Type I collagen is one of several collagen types which are synthesized as precursor molecules. These collagens are first synthesized in the form of procollagen α -chains, each of which has a carboxy-terminal extension peptide that aids in triple-helix formation during the assembly of the procollagen molecule.¹⁸³

Soluble procollagen molecules are released into the extracellular space, and thereafter mature into insoluble collagen fibres by removal of the soluble carboxy-terminal extension peptide outside the fibroblasts.¹⁸⁴

Thus, levels of procollagen type I carboxy-peptide (PICP) in cell culture supernatants may be used to reflect ongoing collagen synthesis.¹⁸⁵

The earliest example of collagen determination by quantification of PICP was based on radioimmunoassay.¹⁸⁶ However, the advent of self contained enzyme linked immunosorbent assay (ELISA) kits based on cell culture in microtitre plates has resulted in PICP quantitation by virtue of colourimetric measurement.

The principle of such measurement, as described by the manufacturers,¹⁸⁷ is as follows :

The system is based on a sandwich-type enzyme linked immunoassay (ELISA), which utilizes two monoclonal antibodies, each recognising a different epitope on the PICP molecule. The assay kit comprises a 96-well microtitre plate which has been precoated with one clone and an antibody- peroxidase conjugate solution (POD-conjugate).

In the presence of both the pre-coated and POD-conjugated forms of the antibody, PICP samples or standards are bound to each at the respective epitope.

The addition to the microtitre plate of a buffered solution comprising hydrogen peroxide and tetramethyl benzidine initiates a reaction between this reagent and POD.

The resulting colour intensity is proportional to the amount of PICP present in samples or standards and can be measured spectrophotomerically. Furthermore, accurate sample concentrations can be determined by reference to a standard curve.

The assay procedure requires several washing steps to remove potentially confounding species such as unbound PICP which remains in the cell culture supernatant and, regarding potential loss of analyte, is ostensibly open to the same criticisms as the radioimmunoassay. The major difference, however, is that the

analyte remains bound to the microtitre plate and is not subjected to further chemical treatment once the reaction has been stopped.

The relative self containment of the assay is primarily due to the fact that PICP is merely a polypeptide sequence having primary, but not secondary or tertiary structure. Consequently, it is non-fibrillar, readily soluble in aqueous solution and hence culture media.

It was decided that the virtue of measuring collagen directly in the radioisotope assay is offset by the potential analyte losses; additionally, the variety of different chemical treatments to which the collagen source is subjected, make it a lengthy procedure.

The PICP assay lends itself readily to the expedient screening of several drugs for their effects on collagen production by cultured NHDF and was, therefore, selected for this purpose.

3.2.8.2 Culture media in which herbal extracts were applied

In previous studies of herbal extracts, cells were seeded in basal media / 10 % FBS and allowed to attach to the plate prior to replacement with serum-free media supplemented with ascorbic acid (50 µg/ml).^{38,42,43}

For the purposes of assessing collagen production in a simulated chronic wound environment, the virtues of changing to serum-free media are as follows :

1. Avoidance of cross reaction

Animal-derived serum can contribute artefacts to collagen quantification, particularly in the case of the PICP assay, where the antibodies in the ELISA kit cross react with

animal derived PICP.¹⁸⁷

2. Effects on collagen production are attributed to herbal extracts only

The only way of emphatically attributing effects upon NHDF collagen production to herbal extracts is to expose the cells to herbal extracts in the absence of growth factors present in FBS. It is conceivable that extracts may act synergistically with growth factors and hence distort their true contribution.

Although the stimulatory effect of ascorbic acid on collagen production has been demonstrated,^{188,189} studies of the effects of drugs or chemicals have been made in its absence.^{190,191} It was felt that ascorbic acid was a potentially confounding factor in exactly the same way as FBS and the substance was omitted from culture media.

3.2.9 Effects of herbal extracts on collagen production : methods

3.2.9.1 Preliminary work and general information

MTS assay.

For colourimetric determinations, 20 μ L MTS reagent was added to each well and the Plate incubated for 2 hours prior to optical density measurement at 490 nm, reference wavelength 630 nm.

Procollagen type-I carboxy peptide (PICP) assay

Upon completion of a 3 day incubation period, cell culture supernatants were collected by aspiration and the PICP assay was performed in the following steps :

1. 100µL antibody-POD conjugate was added to each well of the pre-coated microtitre plate.
2. Cell culture supernatants and standard PICP solutions (20µL) were added immediately after step 1.
3. The plate was covered and incubated at 37° C for 3 hours
4. The contents of each well was aspirated and discarded.
5. Wells were washed four times each with 100 µ L of physiologically buffered saline (PBS).
6. 100µL Buffered solution (hydrogen peroxide / tetramethyl benzidine) is added to each well and the plate incubated at room temperature for 15 min.
7. 100µL of 1 N H₂SO₄ is added to stop the reaction and the absorbances were recorded at 430 nm.

Solutions of herbal extracts

Herbal extract solutions of 10⁻³, 10⁻², 10⁻¹, 1.0, 10, 100 and 1000 µg / ml were prepared in 15 ml tubes by serial dilution of refrigerated stock on the day of the experiments. Extract solutions were created in basal media and basal media / 10 % FBS supplemented with 70 µg / ml IGF-1 and drawn through a 0.2µm filter for sterilisation; all culture media contained Gentamycin / Amphoterecin B (1000 units).

3.2.9.2 Assessment of herbal extract effects on PICP production by NHDF grown in basal media

3 x 10³ NHDF were seeded into 96 well plates in 100µL basal media / 10 % FBS and allowed to attach for 24 hours. The media was aspirated and replaced with basal media.

NHDF were allowed to equilibrate for 24 h, the media was aspirated and the herbal extract solutions, diluted in basal media, were added in the specified concentrations.

Controls consisted of NHDF grown in basal media.

The cells were incubated with the extracts for a 3 day period and the PICP assay was performed.

To assess the extent to which altered NHDF collagen production was due to changes in proliferation, the MTS assay was used; aspirated supernatants were replaced with basal media and the MTS assay was performed, as a means of distinguishing from genuinely affected collagen production or production due to altered cell numbers.

3.2.9.3 Assessment of herbal extracts on PICP production by NHDF-based fibrotic model

NHDF (3×10^3) were seeded into 96-well plates in 100 μ L basal media / 10 % FBS and allowed to attach for 24 hours. Media was aspirated and replaced with

basal media / 10% FBS supplemented with 70 μ g / ml IGF-1.

NHDF were allowed to equilibrate for 24 h, the media was aspirated and the herbal extract solutions, diluted in basal media / 10 % FBS supplemented with 70 μ g / ml IGF-1, were added in the specified concentrations. Controls consisted of a set of cells grown in basal media / 10 % FBS. The cells were incubated with the extracts for a 3 day period, after which the PICP assay was performed.

Herbal extract concentrations which appeared to inhibit PICP production coincided with concentrations having similar effects on the proliferation of the NHDF-based fibrotic model. In these cases, aspirated supernatants were replaced with basal media

and the MTS assay was performed, as a means of distinguishing from genuinely affected collagen production or production due to altered cell numbers.

3.2.9.4 Assessment of cytotoxicity

In all experiments, ten wells were allocated for cytotoxicity assessment. Upon seeding of NHDF into wells, the cells were allowed to equilibrate for one hour, after which time the MTS assay was performed. The corresponding absorbances of these cells were taken as a baseline and herbal extract concentrations which produced absorbances less than this minimum level were assumed to be toxic.

3.2.10 Effect of herbal extracts on KGF production : experimental rationale

3.2.10.1 Reasons for selecting KGF for study

Most of the growth factors produced by NHDF affect the proliferation of a wide variety of cellular targets, stimulating the proliferation of cells derived from mesodermal, ectodermal and endodermal tissue; additionally, they can act as neurotrophic and angiogenic factors *in vivo*.¹⁹²

Clearly, these growth factors have a very broad mitogenic spectrum. As a result of this ambiguity, an emphatic casual relationship between the effects of drugs, herbal or otherwise, on the production of such growth factors and enhancement of wound healing cannot be established.

The existence of keratinocyte growth factor (KGF), in the cell culture supernatant of a human lung fibroblast cell line, was first reported in 1989.¹⁹³ In contrast to most of

the other NHDF growth factors, KGF was thought to be a highly specific mitogen for different types of epithelial cells and was named as a result of observed predominant activity on keratinocytes.^{193,194}

More recent studies have reinforced the specific nature of this cytokine; in studies of whole skin organ culture, expression of KGF has been detected only in the dermis¹⁹⁵ and the receptor for KGF is expressed by epithelial cells only.¹⁹⁶ This growth factor is thus doubly specific, in terms of the cellular source and target cells.

Biopsies of incisions from patients undergoing plastic surgery were found to contain KGF levels which were three fold that of uninjured skin¹⁹⁷ and the growth factor has been shown to protect cultured epidermal keratinocytes from reactive oxygen species.¹⁹⁸

The current study is restricted to the investigation of the effects of herbal extracts on dermal cells; however, an investigation into the effects of herbal extracts on the production of keratinocyte growth factor by NHDF may give an insight into how the extracts may influence, albeit indirectly, epidermal cells such as keratinocytes.

3.2.10.2 KGF measurement by enzyme linked immunosorbent assay (ELISA)

There has been very little work pertaining to the production of KGF by cultured NHDF; only three articles were found in which the production by these cells of the growth factor was quantified, none of which involved herbal extracts. Two studies compared KFG production by human oral and dermal fibroblasts^{199,200} whereas the other investigated the effects of hyaluronic acid on production.²⁰¹

All studies employed enzyme linked immunosorbent assay (ELISA) kits for KGF quantitation.^{199,200,201}

The principles behind the determination of KGF in this way are similar to those for PICP but there are procedural differences.²⁰² In the case of the PICP assay, the antibody POD-conjugate is added to the microplate prior to addition of samples or standards; whereas in the KGF assay, the order of addition is reversed; binding of KGF to the antibody coated onto the plate occurs first, which in turn necessitates extra washing steps prior to the addition of the conjugated version of the antibody.

This was the procedure for the original ELISA kit for the PICP assay,¹⁸⁷ prior to an overall improvement. The ELISA kit described for KGF quantification has, thus far, not been similarly upgraded.

The resulting colour intensity is proportional to the amount of PICP present in samples or standards and can be measured spectrophotometrically. Furthermore, accurate sample concentrations can be determined by reference to a standard curve.

3.2.10.3 Culture media in which herbal extract solutions were created

Regarding the studies which compared oral and dermal fibroblasts, one assessed KGF production in basal media / 10 % FBS,²⁰⁰ the other employed serum-free media.¹⁹⁹

When fibroblasts of different origin are being compared, as opposed to an investigation of the effects of a test substance and a control, it could be argued that the inclusion in culture media of FBS is reasonable because the two cell types can be assessed in terms of an optimal growth environment.

The serum free assessment was felt to be superior, however; commercially available serum undoubtedly contains significant amounts of KGF and, even allowing for the fact that one group of cells may exhibit measurably different production than another, the true amount secreted into culture medium is impossible to gauge.

Regarding the assessment of hyaluronic acid,²⁰¹ there was no virtue in the addition to NHDF of the biopolymer in anything other than serum free media. As is the case in studies which involve proliferation and collagen producing effects, only the test substance should be present, to achieve an unequivocal determination of its contribution in isolation. Given the myriad of growth factors which comprise FBS, KGF production due solely to interaction between hyaluronic acid and FBS components, cannot be discounted; such an occurrence would still result in a level of KGF production greater than that for controls.

It was thus decided to apply herbal extracts to NHDF in basal media only.

3.2.11 Assessment of herbal extract effects on KGF production : methods

3.2.11.1 Preliminary work and general information

MTS assay

For colourimetric determinations, 20 μ L MTS reagent was added to each well and the plate incubated for 2 hours prior to optical density measurement at 490 nm, reference wavelength 630 nm.

Keratinocyte growth factor (KGF) assay

Upon completion of a 3 day incubation period, cell culture supernatants are collected by aspiration and the KGF assay is performed in the following steps :

1. 100 μ L samples and standards were added to the wells.
2. The plate was covered and incubated at 37° C for 3 h.

3. The contents of each well were aspirated and the wells washed four times each with 100 μL of physiologically buffered saline (PBS).
4. 200 μL antibody-POD conjugate was added to each well of the pre-coated microtitre plate.
5. The plate was covered and incubated for 1.75 h.
6. The contents of each well were aspirated and washed four times each with 100 μL of physiologically buffered saline (PBS).
7. 200 μL buffered reagent solution (hydrogen peroxide / tetramethyl benzidine) was added to each well.
8. The plate is covered and incubated for 30 min.
9. 50 μL of 2 N H_2SO_4 was added to stop the reaction and the absorbances are recorded at 490 nm.

Solutions of herbal extracts

Herbal extract solutions of 10^{-3} , 10^{-2} , 10^{-1} , 1.0, 10, 100 and 1000 $\mu\text{g} / \text{ml}$ were prepared in 15 ml tubes by serial dilution of refrigerated stock on the day of the experiments. Extract solutions were created in basal media and drawn through a 0.2 μm filter for sterilisation; all culture media contained Gentamycin / Amphoterecin B (1000 units).

3.2.11.2 Determination of the effects of herbal extracts on KGF production

NHDF (3×10^3) were seeded into 96 well plates in 100 μL basal media / 10 % FBS and allowed to attach for 24 hours, after which time the media was aspirated and replaced with basal media.

NHDF were allowed to equilibrate for 24 hours, the media was aspirated and the herbal extract solutions, diluted in basal media, were added in the specified concentrations.

Controls consisted of NHDF grown in basal media.

The cells were incubated with the extracts for a 3 day period and the KGF assay was performed.

To assess the extent to which altered KGF production by NHDF was due to changes in proliferation, the MTS assay was used; aspirated supernatants were replaced with basal media and the MTS assay was performed, as a means of distinguishing from genuinely affected collagen production or production due to altered cell numbers.

3.2.11.3 Assessment of cytotoxicity

In all experiments, ten wells were allocated for cytotoxicity assessment. Upon seeding of NHDF into wells, the cells were allowed to equilibrate for one hour, after which time the MTS assay was performed. The corresponding absorbances of these cells were taken as a baseline and herbal extract concentrations which produced absorbances less than this minimum level were assumed to be toxic.

3.3 Pharmacological assessment of herbal extract performance

3.3.1 General pharmacological principles

3.3.1.1 Dose response relationships

Analysis of the effects of drugs, whether the drugs are herbal or otherwise, ultimately relates effect with concentration. In the case of studies involving several drugs having similar effects, a standard means of representation and comparison is required.

Physiological responses are due to the activation of corresponding receptors and the agents which are capable of binding to these receptors (and producing effects) are known as agonists; since there are a finite number of receptors, the pharmacological behaviour of a drug tends toward the attainment of some maximum effect.²⁰³

Studies of drug-receptor interactions (on the basis of the drug being a single chemical entity) often result in the generation of concentration-effect curves and the behaviour of particular drugs may be compared; an example is given in Figure 3.3a²⁰³ The geometry of the curves corresponds to a rectangular hyperbola and the general equation describing the relationship is :

$$E = \frac{E_{\max} * C}{C + EC_{50}} \quad (3.1)$$

Where 'E' is the effect resulting from a particular dose, 'C', 'E_{max}' is the maximum possible effect (attained when receptors are fully occupied by agonists) and 'EC₅₀' is the dose at which half the maximum effect is produced (corresponding to half maximal receptor-agonist binding).

It is common pharmacological practice to plot dose-response relationships on logarithmic axes, as a means of determining the dose regime over which changes of effect are linear, as shown in Figure 3.3b.

The rectangular hyperbola is transformed into a sigmoid and is useful for drug comparisons, as shown in Figure 3.4a; the linear parts of the curve are in turn plotted logarithmically so a comparison can be made of two (or more) drugs over their most effective doses (Figure 3.4b).

The potency of a drug may be defined as the amount required to produce a given effect; potency varies inversely with the magnitude of the dose required to produce the effect and is a means by which the behaviour of different drugs may be compared.²⁰⁴

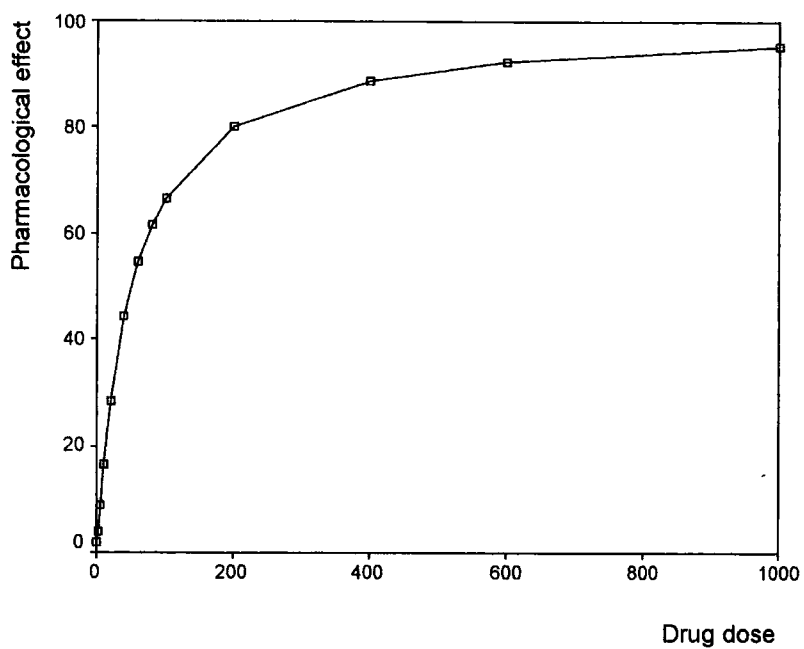
3.3.1.2 Detection and measurement of drug interactions

Two drugs having overtly similar pharmacological properties may be combined for the purpose of investigating whether or not the effects of the combination differ from the sum of individual effects. There are four ways in which drug combination behaviour may be categorised:

1. Additive behaviour

The sum of the effects of a specified ratio of separate components is equal to the effect produced by the same ratio in combination. The implications of this type of behaviour are that the mechanisms of action are independent, drug behaviour in combinations being consistent with individual dose response curves.²⁰⁵

a/



b/

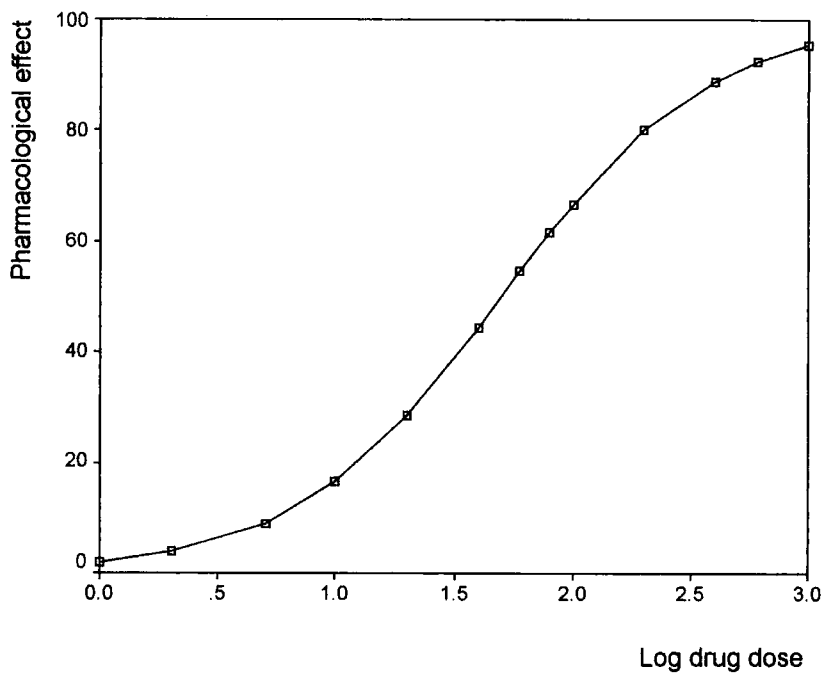
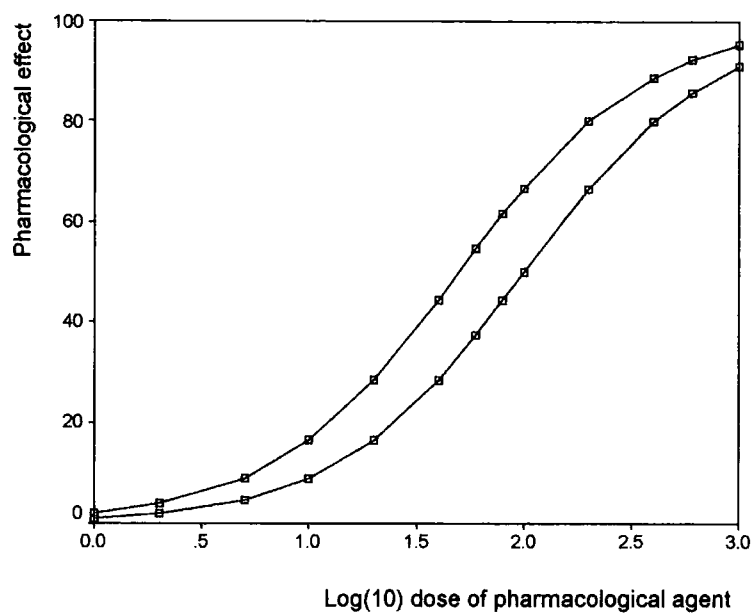


Figure 3.3 Two common methods of representing the dose response relationship.

a/ Rectangular hyperbola resulting from effects being plotted on a linear dose scale

b/ Sigmoid resulting from effects being plotted on a logarithmic dose scale

a/



b/

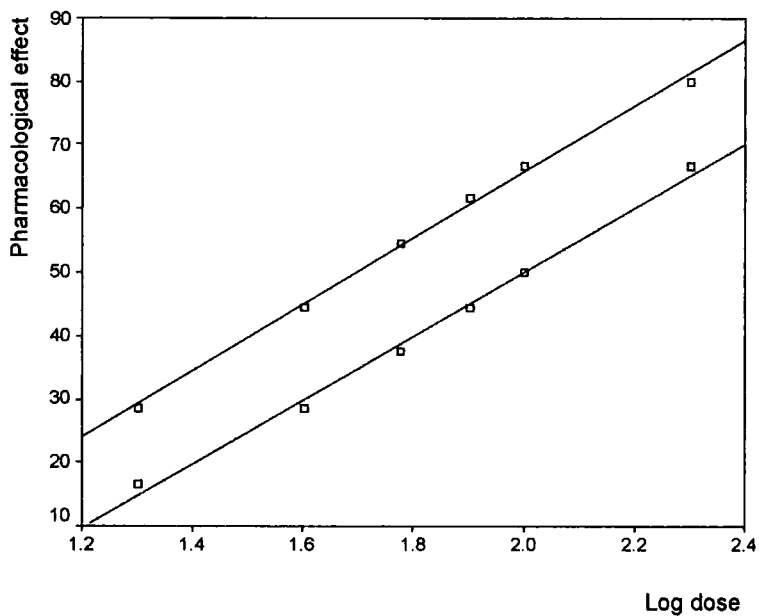


Figure 3.4 Use of logarithmic scales for the purpose of drug comparison; lesser potency drug (blue curve), greater potency drug (black curve).

a/ Sigmoidal plots for all effects

b/ Regression plots of the linear portion of the sigmoid.

2. Super-additive behaviour (synergy)

When combined, drugs may interact chemically or physically; as a result the sum of the effects of a specified ratio of separate components is less than the effect produced by the same ratio in combination. Alternatively, for a given effect, a lesser quantity of the combined dose is required in comparison to the sum of individual doses; pharmacological drug interaction results in an enhanced overall effect.

3. Sub-additive behaviour

Drugs may exert their effects by the same mechanism, binding to the same receptor; in this case, there is competition between them for the binding site and the drug having the more compatible structure is preferentially bound.

In combinations, the less compatible drugs will produce inferior effects in comparison to their individual application.²⁰⁵

4. Antagonistic behaviour

Drugs may or may not act on the same receptor but nevertheless interact or influence one another in such a way that the ability to produce a given effect is reduced for one or all of them; pharmacological drug interaction results in a reduced overall effect.¹⁵⁶

Dose response curves may be added to produce a theoretical additive curve; comparison of the pharmacological behaviour of actual combinations with this model have been used to assess the nature and degree of drug interactions (Figure 3.3).^{206,207}

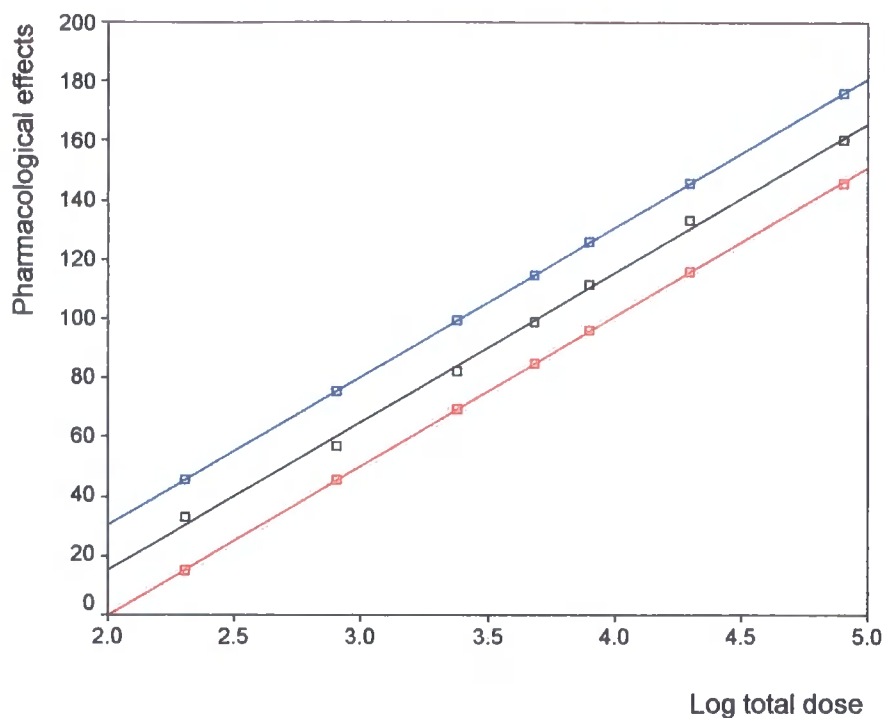


Figure 3.3. Visual comparison of the pharmacological; behaviour of drug combinations in terms of theoretical additivity (black curve), super-additivity (blue curve) and less than additivity (red curve)

3.3.1.3 Analysis of herbal extract interactions: rationale

Preliminary experiments revealed that upon the attainment of a maximum, the effects of several of the herbal extracts declined steadily with increased dose; this deviates from classical pharmacological behaviour. Herbal extracts contain several chemical components (as opposed to the single entity represented in Figure 3.1); it was therefore felt that comparison of herbal extract combinations with theoretical additive curves would be best effected in terms of the complete dose response data, rather than being restricted to the linear portion of the logarithmic plot.

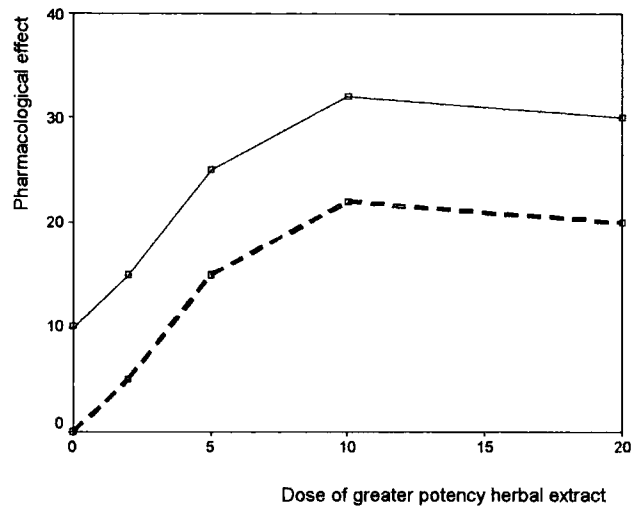
For two herbal extracts demonstrating particular effects, a theoretical additive model was derived from individual dose response data; the model was based on the addition of a fixed quantity of one extract to another and the added drug was of lesser potency. Figure 3.4a shows the hypothetical dose response curve of Drug 1; 10 μg of Drug 2 (corresponding to a pharmacological effect of 10 units) are added to 2, 5, 10 and 20 μg of Drug 1. Only the more potent drug (Drug 1) is shown on the dose axis, enabling the proportion of Drug 1 in the combination to be readily apparent. The original dose response curve undergoes an upward translation of 10 units due to the effects of the added drug.

Figure 3.4b illustrates a hypothetical comparison between the theoretical additive curve from Figure 3.4a and the effects of actual combinations. 10 μg of Drug 2 are added to 2, 5, 10

and 20 μg of Drug 1 to give Drug 2 proportions of 5:1, 2:1, 1:1 and 1:2. For the additive curve, as the proportion of Drug 2 is reduced from 1:1 to 1:2, there is a slight decline in effects; the curve for super-additive behaviour, in addition to demonstrating a greater effect, continues to ascend whereas the curve corresponding to less than additive behaviour exhibits a decline which is more pronounced than for the additive curve.

In addition to demonstrating differences in the magnitude of effects between additive and combination curves, there is the potential for these diagrams to give insight into differences between the pharmacological profiles of actual and theoretical behaviour.

a/



b/

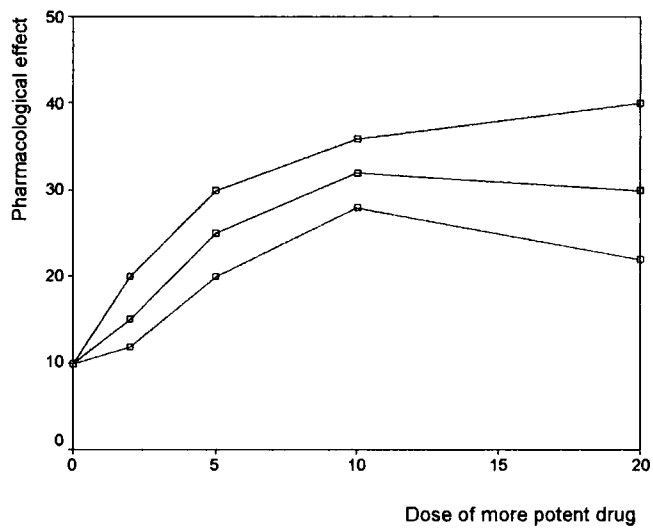


Figure 3.4. Hypothetical derivation and application of theoretical additive curves for the study of drug interactions when 10 μg of Drug 1 is added to 2, 5, 10 and 20 μg of Drug 2

a/ Derivation of theoretical additive curve (unbroken line) from original dose response curve (broken line)

b/ Use of the theoretical additive curve for the demonstration of additive (black), super-additive (blue) and less than additive behaviour (red)

3.3.1.4 Analyses of herbal extract interactions:methodology

Dose response relationships

Dose response relationships and values of EC50, IC50 and Emax were generated with the use of SPSS for Windows (version 11.0)

Upon demonstration of herbal extract effects in terms of NHDF proliferation, response to oxidants, and production of PICP or KGF, the relative absorbances of treatment groups and control groups were used to obtain effects by the following general equation:

$$\text{Effects} = \left[\frac{\text{Absorbance of group treated with herbal extract}}{\text{Absorbance of control group}} \times 100 \right] - 100 \quad (3.2)$$

Effects were thus expressed in terms of a percentage above or below control values.

Herbal extract dose response curves were constructed and plotted on logarithmic axes.

The ascending portion of the dose response curves were fitted to hyperbolic models using curve fitting software.

Analysis of herbal extract combinations

Combinations of two effective herbs were made by adding the less potent herbal extract in the following proportions : 5:1, 2:1, 1:1 and 1:2.

Combinations of one effective herb and one ineffective herb were made by adding the ineffective herbal extract in the following proportions : 5:1, 2:1, 1:1 and 1:2.

Combinations of two non-effective herbs were made by adding herbal extracts in the following proportions : 5:1, 2:1, 1:1 and 1:2 and 1:5.

In all cases, the effects of combinations were derived from Equation 3.2 and compared with the sum of effects from individual dose response data.

Rather than being inferred from the original dose response relationship, the additive data were generated in tandem with combination experiments, as a means of effecting direct comparisons.

Bio-molecule production indexed to growth

In the case of single herbal extracts which affected NHDF growth and appeared to influence bio-molecule production, the use of Equation 3.2 was not sufficient to demonstrate an intrinsic effect; the possibility existed that changes in the production of bio-molecules such as PICP and KGF were solely attributed to changes in cell number as opposed to bio-molecule-producing characteristics.

For this reason, for a given dose, apparent effects on bio-molecule production were compared with established effects upon growth; only statistically significant differences between the groups were considered to genuinely reflect effects on bio-molecule production.

Similar comparisons were made in the case of combinations of growth-affecting herbal extracts which appeared to affect NHDF production of biomolecules.

3.4 Statistical analyses

All statistical operations were performed with the use of SPSS for Windows (version 11.0). Generally, differences at the 1% level were considered to be significant.

3.4.1 Dose response relationships

For the dose response relationship for single herbs, replicates of ten wells of a 96-well

plate were used to represent each herbal extract concentration.

Analysis of variance (ANOVA) was performed to determine the significance of differences between the absorbances of groups treated with herbal extracts and control groups.

To assess the effects of herbal extracts on NHDF production of biomolecules (PICP and KGF), an independent samples t-test was performed to determine differences between apparent effects and growth-related effects.

3.4.2 Correlation of absorbance with NHDF number

For the assessment of the variation of absorbances with NHDF number, replicates of five plates were used. Correlations between absorbances and cell number were established using linear regression.

3.4.3 NHDF number vs time for growth-affecting herbal extracts

For the assessment of differences in NHDF growth under the influence of herbal extracts, replicates of five plates were used.

An independent samples t-test was used to determine the significance of growth related differences between herbal extracts and controls and also differences between two or more herbal extracts.

3.4.4 Comparisons of Combination and additive behaviour

Replicates of ten wells of a 96-well plate were used to represent the concentration of

each additive and combination herbal extracts An independent samples t-test was performed to establish the significance of differences between the effects of combinations and theoretical additives.

In the case of herbal extracts which affected NHDF growth, apparent effects of combinations on biomolecule production were compared with the effects of the same combinations on growth by an independent samples t-test.

Chapter 4-Effects of herbal extracts on NHDF proliferation

4.1 Effects of herbal extracts on NHDF cultured in basal media

4.1.1 Summary of effects

4.1.1.1 Single herbal extracts

Z. chalybeum(B) demonstrated no effects up to a dose of 200 µg/ml, whereupon toxicity was evident (Appendix 1.1).

Z. chalybeum(L), when applied at a dose range of 0.001-1000 µg/ml, demonstrated no effects upon NHDF growth; increased dosages produced no effects and toxicity was eventually observed at a dose of 1100 µg/ml (Appendix 1.1)

C. rotundifolia(L), when applied at a dose range of 0.001-1000 µg/ml, demonstrated an enhancing effect on the growth of NHDF and toxicity was eventually observed at a dose of 1100 µg/ml.

C. abbreviata(B), when applied at a dose range of 0.001-1000 µg/ml, demonstrated an enhancing effect on the growth of NHDF and toxicity was eventually demonstrated at a dose of 1300 µg/ml.

4.1.1.2 Combined herbal extracts

The effect upon NHDF growth was assessed for the following six paired herbal extract combinations:

Combinations of *Z. chalybeum*(B) / *Z. chalybeum*(L) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 and 1:5 produced no effects upon NHDF growth.

Addition of *Z. chalybeum*(B) to *C. rotundifolia*(L) in proportions of 5:1, 2:1, 1:1 and 1:2 did not affect the NHDF growth enhancing ability of *C. rotundifolia*(L).

Addition of *Z. chalybeum*(L) to *C. rotundifolia*(L) in proportions of 5:1, 2:1, 1:1 and 1:2 did not affect the NHDF growth enhancing ability of *C. rotundifolia*(L).

Addition of *Z. chalybeum*(B) to *C. abbreviata*(B) in proportions of 5:1, 2:1, 1:1 and 1:2 did not affect the NHDF growth enhancing ability of *C. abbreviata*(B).

Addition of *Z. chalybeum*(L) to *C. abbreviata*(B) in proportions of 5:1, 2:1, 1:1 and 1:2 did not affect the NHDF growth enhancing ability of *C. abbreviata*(B).

Combinations of *C. rotundifolia*(L) / *C. abbreviata*(B) containing *C. rotundifolia*(L) proportions of 5:1, 2:1, 1:1 and 1:2 produced effects which were super-additive and sub-additive.

4.1.2 Dose response relationships for *C. rotundifolia*(L) and *C. abbreviata*(B)

For *C. rotundifolia*(L), statistically significant effects, up to a maximum, were observed over the dose range 1-80 μ g/ml ($p < 0.001$; $F(12, 117) = 7334.0$).

For *C. abbreviata*(B), statistically significant effects, up to a maximum, were observed over the dose range 1-40 μ g/ml ($p < 0.001$; $F(12, 117) = 2763.0$).

Percentage NHDF growth above controls, plotted on a logarithmic scale, is shown in Figure 4.1a. In the case of *C. rotundifolia*(L), between the first and second dose application, there is a relatively small increase in effects. Application of subsequent doses, up to the maximum effect, resulted in greater increases in effects on a linear basis; in contrast, although the profile of *C. abbreviata*(B) was linear for the first three doses, the increase in effects due to the application of the fourth dose was less than linear.

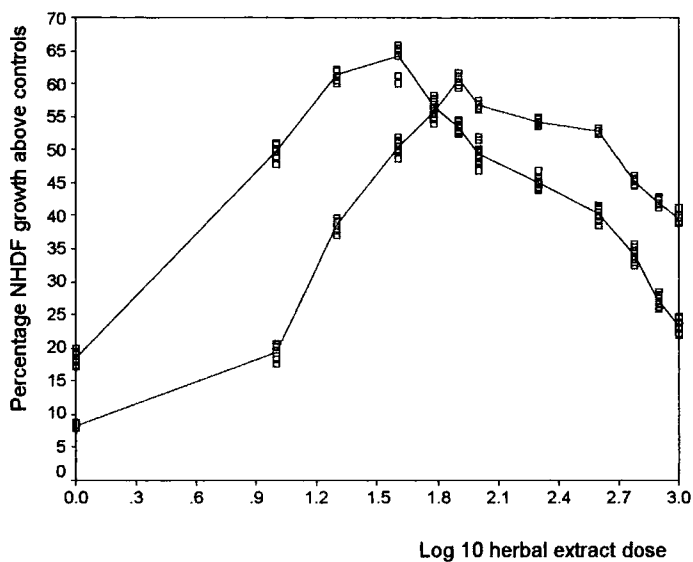
For both herbal extracts, there was a steady decline of effects with dose once a maximum was attained; this decline was more pronounced in the case of *C. abbreviata*(B).

C. abbreviata(B) was the more potent herbal extract and also attained a higher maximum effect (64.25% compared with 60.84%);

The ascending portion of the dose response curves were also plotted on a linear dose scale and fitted to hyperbolic models which are briefly described below. These plots are shown in Figure 4.1b.

For *C. rotundifolia*(L), the value of E_{max} (74.5%) and EC_{50} (24.3 μ g/ml) were reasonable approximations of the actual maximum effect (60.84%) and the dose which results in half the actual maximum (20.0 μ g/ml) respectively.

a/



b/

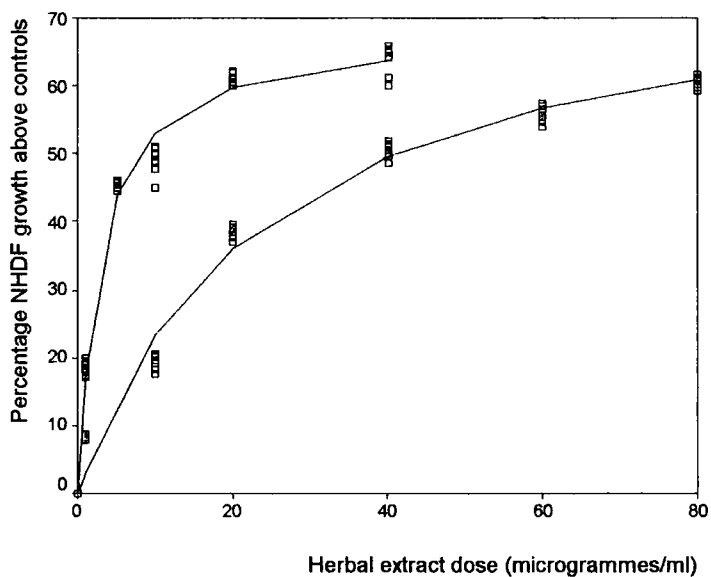


Figure 4.1 NHDF growth versus dose for *C. rotundifolia* leaf extract (blue curves) and *C. Abbreviata* bark extract (black curves).

a/ effects versus dose plotted on a logarithmic scale

b/ effects versus dose (up to maximum effect) plotted on a linear scale and fitted to a hyperbolic model.

The value of r^2 was 0.98, which demonstrated a good correlation of effects with dose for the model, up to the maximum.

For *C. abbreviata*(L), the value of E_{max} (67.1%) was a good approximation of the actual maximum effect (64.25%) whereas the EC_{50} (2.5 $\mu\text{g/ml}$) did not approximate well to the dose which results in half the actual maximum (6.0 $\mu\text{g/ml}$).

The value of r^2 was 0.91, which demonstrated a good correlation of effects with dose for the model, up to the maximum

4.1.3 Correlation of absorbance with NHDF number when cells were cultured in the presence of the most effective herbal extract doses

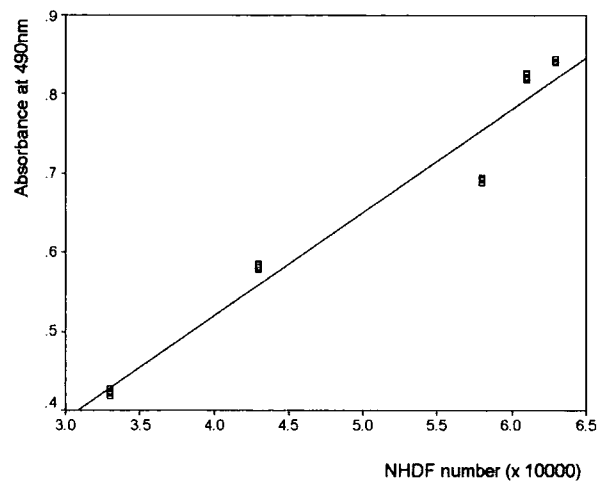
Linear regression was performed for absorbance (490nm) and cell number for NHDF grown in the presence of herbal extracts.

Correlation coefficients pertaining to treatment of cells with 80 $\mu\text{g/ml}$ of *C. rotundifolia*(L) and 40 $\mu\text{g/ml}$ of *C. abbreviata*(B) were 0.92 and 0.91 respectively and the correlation in each case was taken to be linear; a visual representation of the relationship for the application of each herbal extract is shown in Figure 4.2.

4.1.4 Time course for NHDF cultured in the presence of the most effective herbal extract dose

In Figure 4.3, growth rates of NHDF cultured in the presence of 80 $\mu\text{g/ml}$ of *C. rotundifolia*(L) and 40 $\mu\text{g/ml}$ of *C. abbreviata*(B) were compared.

a/



b/

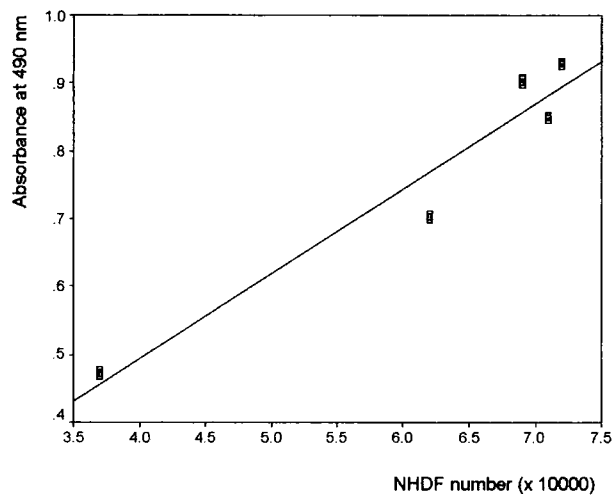


Figure 4.2 Correlation of absorbance with NHDF number for *C. rotundifolia*(L) and *C. abbreviata*(B) Upon addition of a single dose of herbal extract, cells were cultured over a period of 9 days in 35 mm culture plates; cell counts and absorbance readings were performed at 1, 3, 5, 7 and 9 days after herbal extract application.

a/ Correlation for NHDF treated with 80 $\mu\text{g/ml}$ of *C. rotundifolia*(L)

b/ Correlation for NHDF treated with 40 $\mu\text{g/ml}$ of *C. abbreviata*(B)

(Five replicate plates were used for measurement of absorbance and cell number).

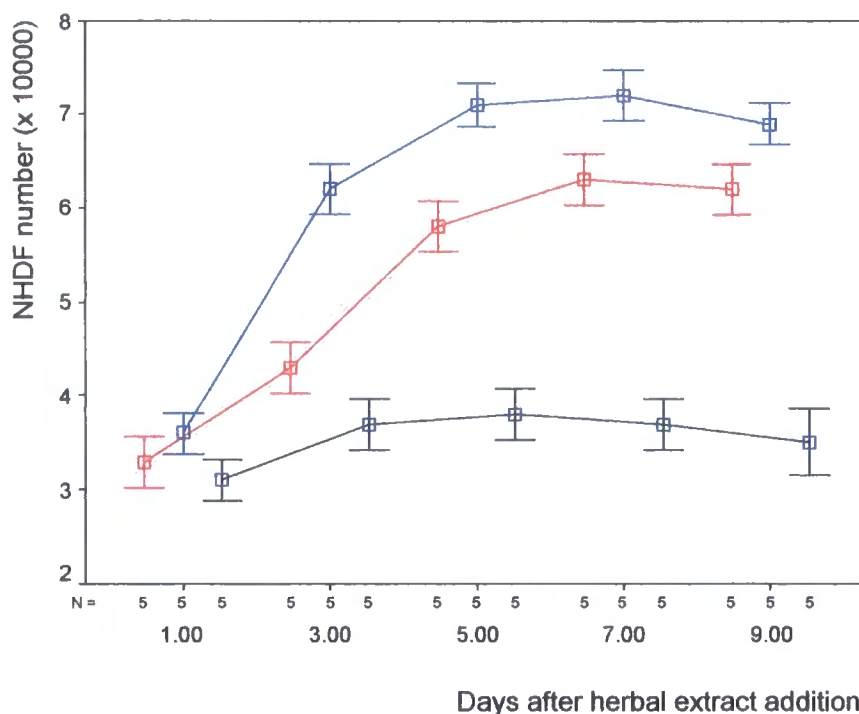


Figure 4.3 NHDF number versus time for NHDF cultured in basal media only (black curve), basal media + 80 µg/ml of *C. rotundifolia* leaf extract (red curve) and basal media + 40 µg/ml *C. abbreviata* bark extract (blue curve).

Upon addition of a single dose of herbal extract, cells were cultured over a period of 9 days in 35 mm culture plates; cell counts were performed at 1, 3, 5, 7 and 9 days after application of 80 µg/ml of *C. rotundifolia*(L) and 40 µg/ml of *C. abbreviata*(B).

(Results are the mean and standard deviation of five replicate plates).

Controls consisted of cells cultured in basal media only.

Generally, differences between control and NHDF treated with herbal extracts only became significant on day 3.

Cell numbers for NHDF grown in the presence of *C. rotundifolia*(L) were superior to that of control cells on day 3 ($t = 5.8, p < 0.001$), 5 ($t = 11.5, P < 0.001$), 7 ($t = 15.0, p < 0.001$) and 9 ($t = 16.0, p < 0.001$).

In the case of NHDF grown in the presence of *C. abbreviata*(B), cell numbers were superior to controls at days 3 ($t = 14.4, p < 0.001$), 5 ($t = 20.9, p < 0.001$), 7 ($t = 20.2, p < 0.001$) and 9 ($t = 18.2, p < 0.001$).

In terms of herbal extract comparisons, cell numbers in the group cultured in the presence of *C. abbreviata*(B) were superior to those of the group treated with *C. rotundifolia*(L); comparisons were made at days 3, 5 and 7 (day 3: $t = 11.0, p < 0.001$; day 5: $t = 8.2, p < 0.001$ and day 7: $t = 5.2, p < 0.01$).

The respective growth rate at corresponding time periods for NHDF cultured in basal media only and in the presence of herbal extracts is shown in Table 4.1

Nature of growth media	Basal medium	<i>C. rotundifolia</i> (L)	<i>C. abbreviata</i> (B)
Growth rate (cells/day for days 1-3)	3000	7000	13000
Growth rate (cells/day for days 3-5)	500	7500	4500
Growth rate (cells/day for days 5-7)	-500	2500	500
Growth rate (cells/day for days 7-9)	-1000	-500	-1500

Table 4.1 Growth rates for NHDF grown in basal media and basal media containing *C. rotundifolia* leaf extract and *C. abbreviata* bark extract.

For NHDF cultured in the presence of *C. rotundifolia*(L), the growth rate between days 3 and 5 was superior to that between days 1 and 3; between days 5 and 7, the growth rate decreased markedly, although NHDF numbers were still increased over this period; between days 7 and 9, the growth rate was unchanged.

For NHDF cultured in the presence of *C. abbreviata*(B), the initial growth rate, between days 1 and 3, was the greatest; between days 3 and 9, growth rates were pro-

gressively reduced and actually became negative between days 7 and 9.

On day 9, there were no significant differences between NHDF numbers between groups treated with herbal extracts.

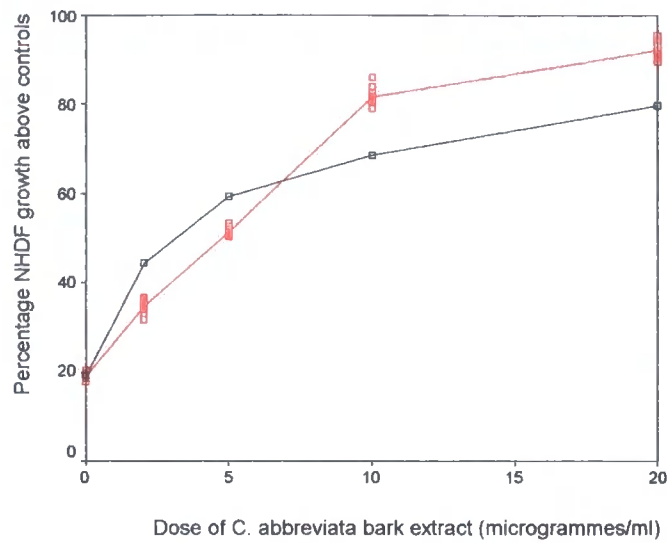
4.1.4 Behaviour of combinations of *C. rotundifolia*(L) and *C. abbreviata*(B)

4.1.4.1 Comparison plots for additive and combination effects

Figure 4.4a shows the effect of adding 10 µg/ml of *C. rotundifolia*(L) to 2, 5 and 10 and 20 µg/ml of *C. abbreviata*(B). Between *C. rotundifolia*(L) proportions of 5:1 and 1:1, the behaviour of combinations was characterised by a roughly linear relationship between effects and *C. abbreviata*(B) dose whereas for additive behaviour, increases in effects were less pronounced.; between *C. rotundifolia*(L) proportions of 1:1 and 1:2, the increase in effects occurred to an equal degree for additive and combination behaviour. At *C. rotundifolia*(L) proportions of 5:1 and 2:1, the effects of combinations were sub-additive ($t = 12.0$, $p < 0.001$) and ($t = 19.8$, $p < 0.001$) respectively; reduction to proportions of 1:1 and 1:2 produced super-additive behaviour ($t = 17.5$, $p < 0.001$) and ($t = 17.1$, $p < 0.001$) respectively. This trend was also observed when a dose of 20 µg/ml of *C. rotundifolia*(L) was added to *C. abbreviata*(B) in similar fixed mass ratios (data not shown).

The addition of 30 µg/ml of *C. rotundifolia*(L) to 6, 15, 30 and 60 µg/ml of *C. abbreviata*(B) is shown in Figure 4.4b. A roughly linear dose-effect relationship was again evident between *C. rotundifolia*(L) proportions of 5:1 and 1:1 for combinations, in contrast to additive behaviour, where the increases in effects became less pronounced. The effects of combinations were sub-additive at *C. rotundifolia*(L)

a/



b/

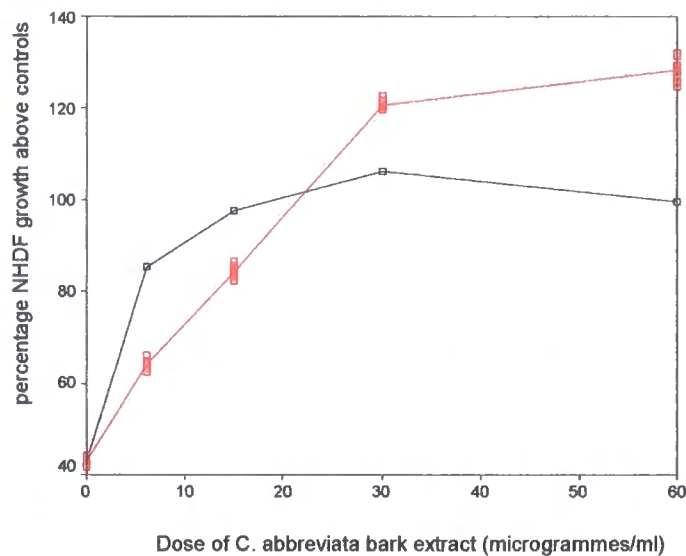


Figure 4.4 Additive behaviour (black curve) compared with combination behaviour (red curve) for *X. chalybeum* bark extract / *C. rotundifolia* leaf extract combination.

a/ Additive and combination behaviour when a dose of 10 $\mu\text{g/ml}$ of *C. rotundifolia* leaf extract is added to the indicated doses of *C. abbreviata* bark extract.

b/ Additive and combination behaviour when a dose of 30 $\mu\text{g/ml}$ *C. rotundifolia* leaf extract is added to the indicated doses of *C. abbreviata* bark extract.



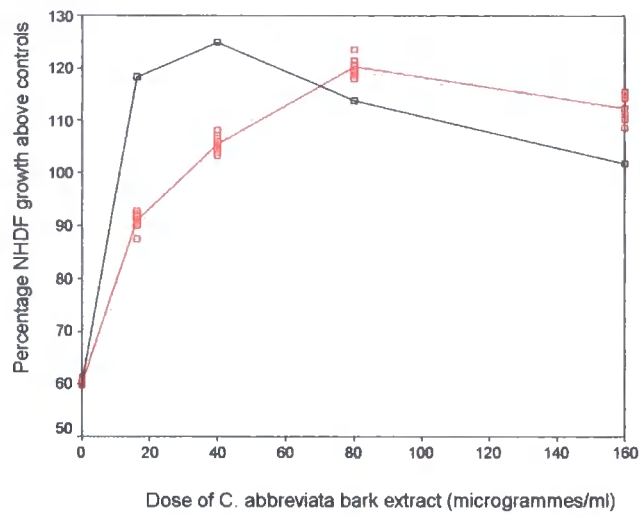
proportions of 5:1 and 2:1 ($t = 38.9$, $p < 0.001$) and ($t = 26.6$, $p < 0.001$) respectively but super-additive when the proportion decreased to 1:1 and 1:2 ($t = 35.3$, $p < 0.001$) and ($t = 36.3$, $p < 0.001$) respectively.

Between *C. rotundifolia*(L) proportions of 1:1 and 1:2, there was a further disparity between the profiles in that the effects of combinations continued to increase as opposed to the slight decrease exhibited in the case of additive behaviour. This trend was also observed when doses of 40, 50, 60 and 70 $\mu\text{g/ml}$ *C. rotundifolia*(L) were added to *C. abbreviata*(B) in similar fixed mass proportions (data not shown).

Figure 4.5a depicts the addition of 80 $\mu\text{g/ml}$ *C. rotundifolia*(L) to 16, 40, 80 and 160 $\mu\text{g/ml}$ of *C. abbreviata*(B). As *C. rotundifolia*(L) proportions are reduced to 1:1, combination behaviour was characterised by an increase in effects whereas additive effects reach a maximum at a *C. rotundifolia*(L) proportion of 2:1. Both additive and combination effects were reduced as the *C. rotundifolia*(L) proportion decreased to 1:2. At *C. rotundifolia*(L) proportions of 5:1 and 2:1, the effects of combinations were sub-additive ($t = 47.0$, $p < 0.001$) and ($t = 34.2$, $p < 0.001$) respectively whereas proportions of 1:1 and 1:2 produce super-additive behaviour ($t = 11.4$, $p < 0.001$) in both cases. This trend was also evident for the addition to *C. abbreviata*(B) of 100 $\mu\text{g/ml}$ (similar fixed mass proportions).

Upon addition of 200 $\mu\text{g/ml}$, although a similar trend was observed, the increase in the effects of combinations (as *C. rotundifolia*(L) proportions are reduced to 1:1) was less pronounced. (data not shown). The effects produced by the addition of 400 $\mu\text{g/ml}$ of *C. rotundifolia*(L) are shown in Figure 4.5b. At *C. rotundifolia*(L) proportions of 5:1 and 2:1, the effects of combinations were sub-additive ($t = 97.3$, $p < 0.001$) and ($t = 17.4$, $p < 0.001$) respectively whereas 1:1 and 1:2 produced superadditive behaviour ($t = 11.7$, $p < 0.001$) and ($t = 15.3$, $p < 0.001$) respectively.

a/



b/

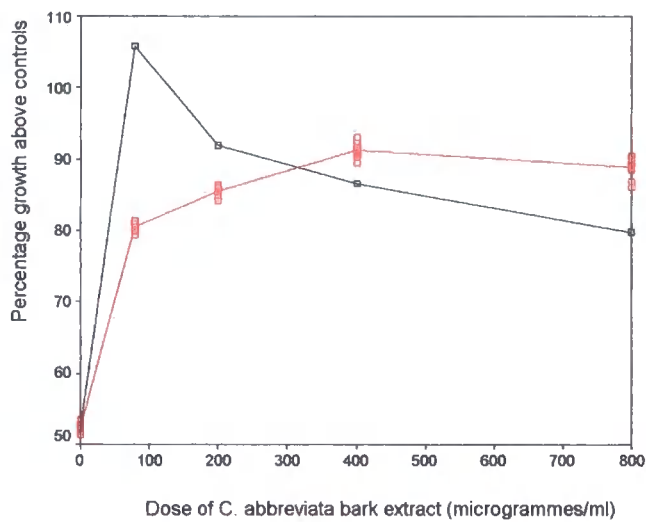


Figure 4.5 Additive behaviour (black curve) compared with combination behaviour (red curve) for *C. abbreviata* bark extract / *C. rotundifolia* leaf extract combination.

a/ Additive and combination behaviour when a dose of 80 µg/ml of *C. rotundifolia* leaf extract is added to the indicated doses of *C. abbreviata* bark extract.

b/ Additive and combination behaviour when a dose of 400 µg/ml *C. rotundifolia* leaf extract is added to the indicated doses of *C. abbreviata* bark extract.

Additive effects reach a maximum at a *C. rotundifolia*(L) proportion of 5:1 and decreased upon reduction of the proportion to 2:1, whereas combination effects continue to increase, attaining a maximum at a *C. rotundifolia*(L) proportion of 1:1; a further reduction of *C. rotundifolia*(L) proportion to 1:2 produced a negligible decrease in the effects of combinations; the decrease in additive effects, however, progressed in a steady fashion.

This trend was also evident when 600, 800 and 1000 µg/ml of *C. rotundifolia*(L) were added to *C. abbreviata*(B) in similar fixed mass proportions (data not shown).

The combinations attained a maximum effect (133.5% NHDF growth above controls) when 20 µg/ml of *C. rotundifolia*(L) was added to 40 µg/ml of *C. abbreviata*(B).

4.1.5 Discussion

4.1.5.1 Individual dose response relationships

Both herbal extracts exhibited common behaviour whereby, upon producing a particular maximum effect, further increases in dosage produced sub-maximal effects which decreased steadily (Figure 4.1a). This behaviour deviates from classic pharmacological behaviour, where, as effects approach a maximum level, further increases in dosage produce negligible changes in effect.

One explanation for this difference is the fact that herbal drugs contain several chemical components which may, in sufficient quantities, displace bound active ingredients from growth receptors.

The curve fitting parameters for the hyperbolic plots of effects versus dose were mostly reasonable approximations of maximum effect values and the doses which produced effects of half the maximum. Differences between software-ascribed and actual values of Emax and EC50 are due the software treating the data as sections of larger hyperbolas and hence extrapolating and interpolating the effects.

It is appropriate, however, to apply the model as a dose response relationship up to the maximum.

4.1.5.2 Correlation of absorbance with NHDF number

The MTS assay is an indirect means of determining changes in cell number on the basis of the measurement of cell metabolism; however, some pharmacological agents may affect metabolic end point of the assay and hence give false indications regarding the extent of growth and a correlation between absorbance and cell number is required.

The good correlations established between absorbance and NHDF number when *C. rotundifolia*(L) and *C. abbreviata*(B) were applied in unsupplemented media provided validation for the pharmacological behaviour of individual and combined herbal extracts.

4.1.5.3 Time course for NHDF grown in the presence of *C. rotundifolia*(L) and *C. abbreviata*(B)

Between days 1 and 3, the growth rate of NHDF treated with *C. abbreviata*(B) was approximately 2.5 times that for cells treated with *C. rotundifolia*(L). This represents

the only period during which the growth rate of NHDF treated with *C. abbreviata*(B) is superior. Thus this initial marked increase in cell numbers appears to be the major reason for the differences in the growth profiles. Possibly, *C. abbreviata*(B) is more rapidly assimilated, bound to receptors and hence able to exert more pronounced effects at an early time point; on the other hand, *C. rotundifolia*(L) may be less readily assimilated, increasing its effect over a longer period.

All media types were applied on the basis of a single dose and the eventual depletion of nutrients is the most likely cause of the decline in the numbers of *C. abbreviata*(B) treated cells.

4.1.5.4 Effects due to combinations of *C. rotundifolia*(L) and *C. abbreviata*(B)

Generally, the effects of combinations were sub-additive when *C. rotundifolia*(L) was present in proportions greater than 1:1, namely 2:1 and 5:1; as the *C. rotundifolia*(L) proportion was reduced to 1:1 and 1:2, combination effects were super-additive.

The observed less than additive behaviour may be due to one of the following:

1. The two herbal extracts may exert their growth enhancing effects by the same mechanism, binding to a similar NHDF receptor; in this case, herbal extracts compete for the binding site and the more structurally compatible active ingredient is bound. In such a scenario, the less compatible herbal extract will produce an inferior effect in comparison to its behaviour individually.

2. The two herbal extracts may or may not act on the same receptor but nevertheless interact or influence one another in a way which disrupts the growth enhancing

activity of one or both of them (antagonism). Given the multitude of components present in herbal extracts, it is possible that such interference may be between components other than the active ingredients.

The super-additivity is the result of increasing the proportion of *C. abbreviata*(B) from 1:5 to 1:1. Regardless of the means by which sub-additivity is produced, the proportion of 1:1 would appear to herald the positive interaction of the two extracts. Super-additivity was also evident when post-maximal effects were declining, which suggests that the eventual displacement of the active ingredient(s) from receptors may be offset when the herbal extracts are combined.

4.2 Effects of herbal extracts applied in basal media / 2% FBS

4.2.1 Summary of effects

4.2.1.1 Single herbal extracts

Z. chalybeum(B) demonstrated no effects up to a dose of 600 µg/ml, whereupon toxicity was evident (Appendix 1.1)

Z. chalybeum(L), when applied at a dose range of 0.001-1000 µg/ml, demonstrated no effects upon NHDF growth; increased dosages produced no effects and toxicity was eventually observed at a dose of 1300 µg/ml (Appendix 1.1)

C. rotundifolia(L), when applied at a dose range of 30 -1000 µg/ml, demonstrated an enhancing effect on the growth of NHDF and toxicity was eventually observed at a dose of 1500 µg/ml.

C. abbreviata(B), when applied at a dose range of 20 -1000 µg/ml, demonstrated an enhancing effect on the growth of NHDF and toxicity was eventually demonstrated at a dose of 1600 µg/ml. The nature of this dose response relationship is described in more detail in section 4.2.2

4.2.1.2 Combined herbal extracts.

The effect upon NHDF growth was assessed for the following six paired herbal extract combinations

Combinations of *Z. chalybeum*(B) / *Z. chalybeum*(L) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 and 1:5 produced no effects upon NHDF growth.

Combinations of *Z. chalybeum*(B) / *C. rotundifolia*(L) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 did not affect the ability of *C. rotundifolia*(L) to enhance NHDF growth

Combinations of *Z. chalybeum*(L) / *C. rotundifolia*(L) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 did not affect the ability of *C. rotundifolia*(L) to enhance NHDF growth.

Combinations of *Z. chalybeum*(B) / *C. abbreviata*(B) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 did not affect the ability of *C. abbreviata*(B) to enhance NHDF growth

Combinations of *Z. chalybeum*(L) / *C. abbreviata*(B) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 did not affect the ability of *C. abbreviata*(B) to enhance NHDF growth

Combinations of *C. rotundifolia*(L) / *C. abbreviata*(B) containing *C. rotundifolia*(L) proportions of 5:1, 2:1, 1:1 and 1:2 produced effects which were superior to the theoretical additive model.

4.2.2 Dose response relationships for *C. rotundifolia*(L) and *C. abbreviata*(B)

For *C. rotundifolia*(L), statistically significant effects, up to a maximum, were observed over the dose range 30-120 μ g/ml ($p < 0.001$; $F(15, 144) = 1276.8$).

For *C. abbreviata*(B), statistically significant effects, up to a maximum, were observed over the dose range 20-70 μ g/ml ($p < 0.001$; $F(12, 117) = 1709.8$).

Percentage NHDF growth above controls, plotted on a logarithmic scale, is shown in Figure 4.6a. In the case of both herbal extracts, there is a less than linear increase of NHDF growth with herbal extract dose and, up to their respective maximum effects, the behaviour of *C. rotundifolia*(L) and *C. abbreviata*(B) is described by a similar profile. For both herbal extracts, the maximum effect was maintained for three subsequent dose applications, after which increased dosages produced sub-maximal

Effects. For both herbal extracts, there was a steady decline of effects once a maximum was attained.

C. abbreviata(B) was the more potent herbal extract and also attained a higher maximum effect (27.6% compared with 20.9%); additionally the decrease in effects with dosage for this herbal extract, once the maximum had been attained, was less pronounced.

The ascending portion of the dose response curves were also plotted on a linear dose scale and fitted to hyperbolic models which are briefly described below. These plots are shown in Figure 4.6b.

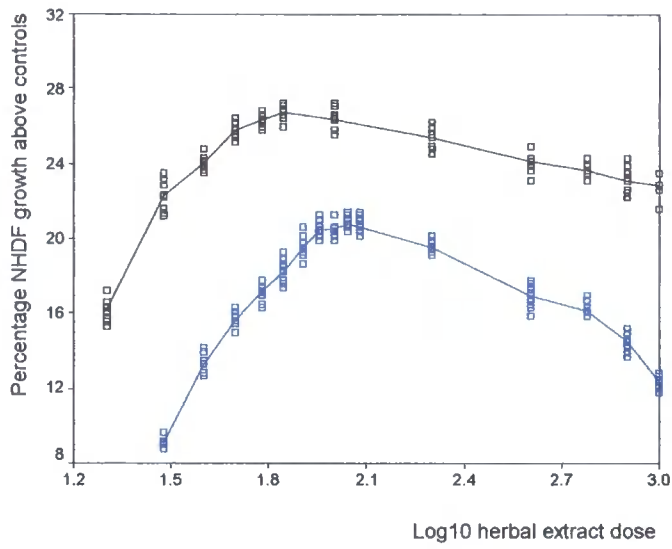
For *C. rotundifolia*(L), the values of E_{max} (42.0%) and EC₅₀ (90.7 µg/ml) were not good approximations of the actual maximum effect (20.9%) or the dose which results in half the actual maximum (30.0 µg/ml); however, the value of r^2 was 0.94, which demonstrated a good correlation of effects with dose, up to the maximum.

For *C. abbreviata*(L), the value of E_{max} (35.6%) was a more reasonable approximation of the actual maximum effect (27.6%); it was not possible to establish from Figure 4.7b the dose which results in half the actual maximum; the value of r^2 was 0.92, which demonstrated a good correlation of effects with dose for the model, up to the maximum

4.2.3 Correlation of absorbance with NHDF number

Correlation coefficients pertaining to treatment of cells with 90 µg / ml of *C. rotundifolia*(L) and 70 µg / ml of *C. abbreviata*(B) were 0.95 and 0.97 respectively and a visual representation is shown in Figure 4.7.

a/



b/

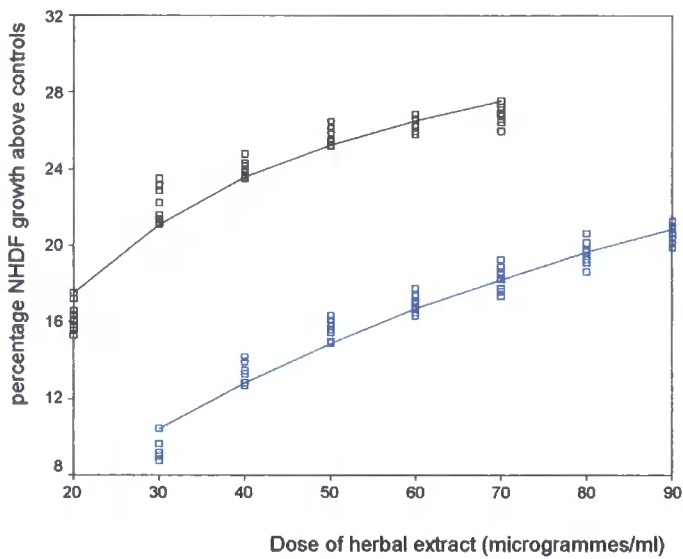


Figure 4.6 NHDF growth versus dose for *C. rotundifolia* leaf extract (blue curves) and *C. Abbreviata* bark extract (black curves).

a/ effects versus dose plotted on a logarithmic scale

b/ effects versus dose (up to maximum effect) plotted on a linear scale and fitted to a hyperbolic model.

4.2.4 Time course for NHDF cultured in the presence of the most effective herbal extract dose

The growth rate of NHDF cultured in the presence of 90 µg / ml of *C. rotundifolia*(L) and 70 µg / ml of *C. abbreviata*(B) were compared with the growth of cells cultured in basal media / 2% FBS; the results of these experiments are shown in Figure 4.8

Generally, differences between control and NHDF treated with herbal extracts only became significant on day 3.

Cell numbers for NHDF grown in the presence of *C. rotundifolia*(L) were superior to those of control cells on day 3 ($t = 5.8, p < 0.001$), 5 ($t = 5.8, P < 0.001$) and 7 ($t = 4.0, p < 0.001$); however, the relative increase in NHDF numbers at each time point was roughly the same for both groups, resulting in similar growth profiles. Between days 7 and 9, there was a decrease in cell numbers which was more pronounced in the case of NHDF grown in the presence of *C. rotundifolia*(L) and there was no significant difference between cell numbers of each group on day 9.

In the case of NHDF grown in the presence of *C. abbreviata*(B), cell numbers were superior to controls at days 3 ($t = 20.9, p < 0.001$), 5 ($t = 14.5, p < 0.001$), 7 ($t = 8.5, p < 0.001$) and 9 ($t = 11.0, p < 0.001$).

Cell numbers in this group were also superior to those of the group treated with *C. rotundifolia*(L) when compared at the same time points (day 3: $t = 14.5, p < 0.001$; day 5: $t = 8.2, p < 0.001$; day 7: $t = 5.2, p < 0.001$; day 9: $t = 8.6, p < 0.001$).

The respective growth rates and corresponding time periods for NHDF grown in basal media / 2% FBS and basal media / 2% FBS plus herbal extract are shown in Table 4.2. When compared with NHDF grown in control media or media containing *C. rotundifolia*(L), NHDF growth under the influence of *C. abbreviata*(B) did not

resemble the profile of either of the other groups; growth was very rapid and far superior over the period between 1 and 3 days whereas between days 3 and 7, the growth rate was inferior to both the other groups, actually remaining stationary between days 5 and 7. Between days 7 and 9, NHDF numbers declined at a rate which was less pronounced than for the group treated with *C. rotundifolia*(L) and similar to the control group.

Nature of growth media	Basal medium + 2% FBS	<i>C. rotundifolia</i> (L)	<i>C. abbreviata</i> (B)
Growth rate (cells/day for days 1-3)	3500	7500	17500
Growth rate (cells/day for days 3-5)	10000	10000	5000
Growth rate (cells/day for days 5-7)	3500	2500	500
Growth rate (cells/day for days 7-9)	-3500	-5500	-2500

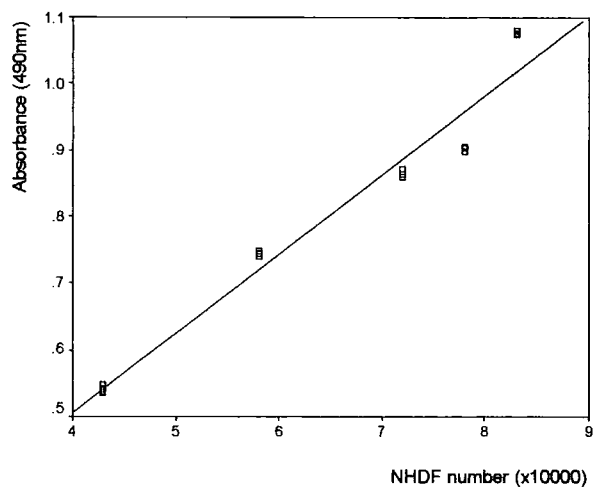
Table 4.1 Growth rate comparison for cells grown in basal media / 2% FBS and basal media / 2% FBS containing *C. rotundifolia* leaf extract and *C. abbreviata* bark extract.

4.2.4 Behaviour of combinations of *C. rotundifolia*(L) and *C. abbreviata*(B)

4.2.4.1 Comparison plots for additive and combination effects

Figure 4.9a shows the effect of adding 40 µg/ml of *C. rotundifolia*(L) to 20, 40 and 80 µg/ml of *C. abbreviata*(B). At all proportions of *C. rotundifolia*(L), 2:1, 1:1 and 1:2 the effects of combinations were greater than additive ($t = 30.8, p < 0.001$; $t = 55.0, p < 0.001$; $t = 54.6, p < 0.001$ respectively). and the shape of the curves for both types of behaviour was similar.

a/



b/

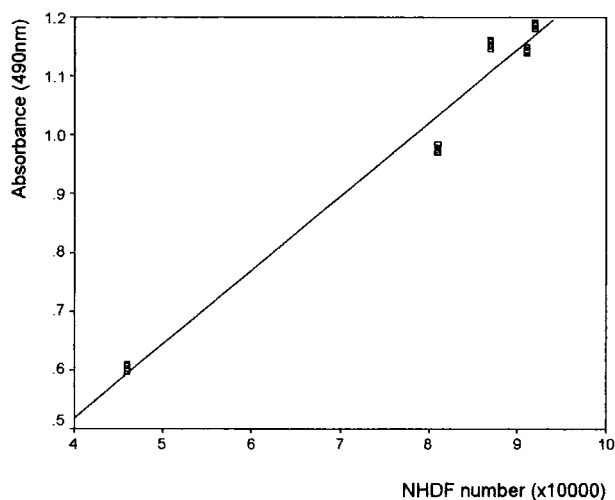


Figure 4.7 Correlation of absorbance with NHDF number for *C. rotundifolia*(L) and *C. abbreviata*(B) Upon addition of a single dose of herbal extract, cells were cultured over a period of 9 days in 35 mm culture plates; cell counts and absorbance readings were performed at 1, 3, 5, 7 and 9 days after herbal extract application.

a/ Correlation for NHDF treated with 90 $\mu\text{g/ml}$ of *C. rotundifolia*(L)

b/ Correlation for NHDF treated with 70 $\mu\text{g/ml}$ of *C. abbreviata*(B)

(Five replicate plates were used for measurement of absorbance and cell number).

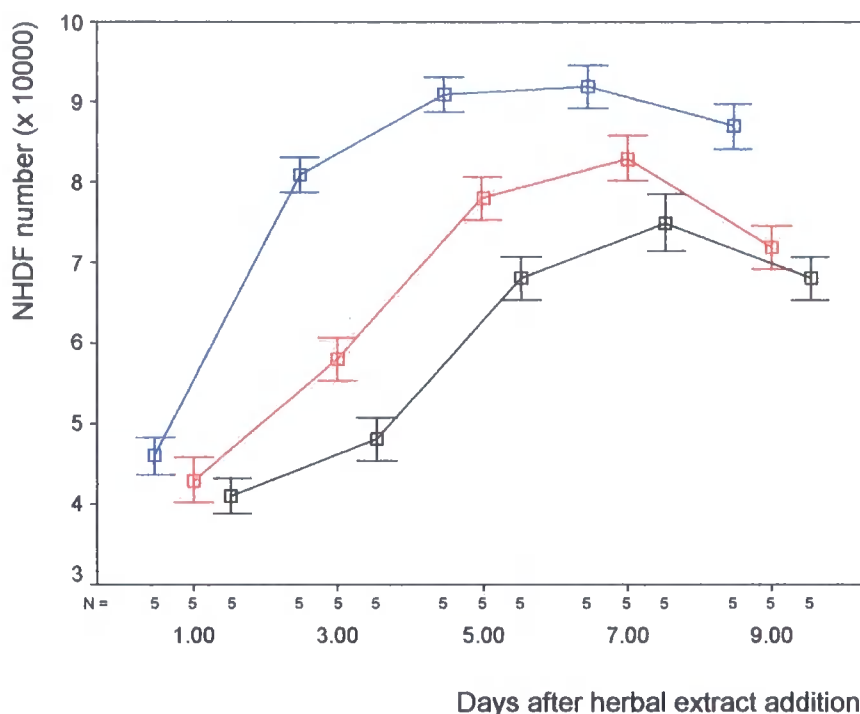


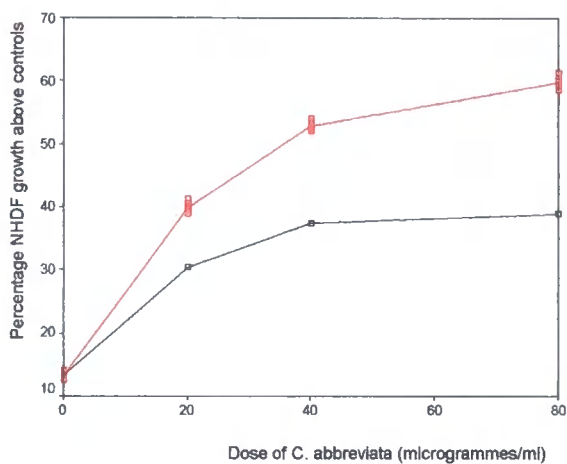
Figure 4.8 NHDF number versus time for NHDF cultured in media containing 2% FBS only (black curve), 2% FBS + 90 µg/ml of *C. rotundifolia* leaf extract (red curve) and 70 µg/ml *C. abbreviata* bark extract (blue curve).

Upon addition of a single dose of herbal extract, cells were cultured over a period of 9 days in 35 mm culture plates; cell counts were performed at 1, 3, 5, 7 and 9 days after application of 80 µg/ml of *C. rotundifolia* and 40 µg/ml of *C. abbreviata*.

(Results are the mean and standard deviation of five replicate plates).

The divergence of combination behaviour from additivity appeared to increase with an increasing proportion of *C. abbreviata*(L); in particular, between proportions of 1:1 and 1:2, the effects of combinations continued to increase whereas those for additivity remained unchanged. Similar behaviour was demonstrated when a dose of 50 µg/ml of *C. rotundifolia*(L) applied in the same fixed mass ratios (data not shown).

a/



b/

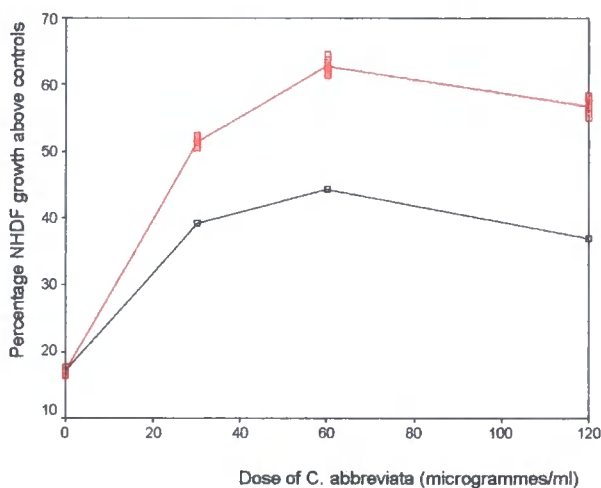


Figure 4.9 Additive behaviour (black curve) compared with combination behaviour (red curve) for *C. rotundifolia* bark extract / *C. rotundifolia* leaf extract combination.

a/ Additive and combination behaviour when a dose of 40 µg/ml of *C. rotundifolia* leaf extract is added to the indicated doses of *C. abbreviata* bark extract.

b/ Additive and combination behaviour when a dose of 60 µg/ml *C. rotundifolia* leaf extract is added to the indicated doses of *C. abbreviata* bark extract.

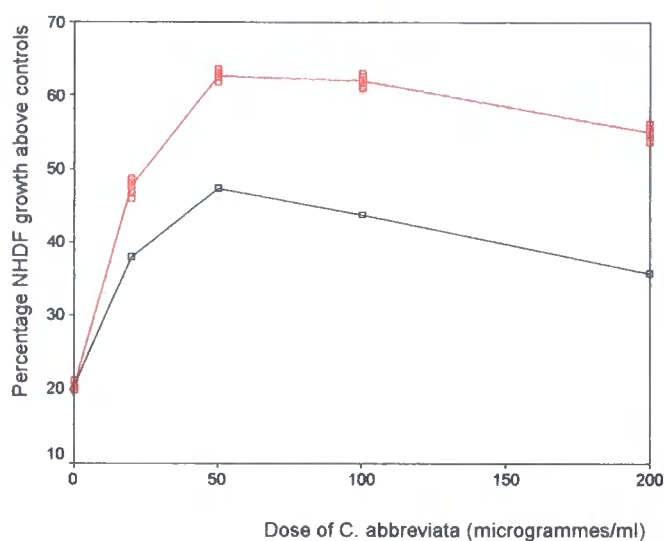
The addition of 60 µg/ml of *C. rotundifolia*(L) to 30, 60 and 120 µg/ml of *C. abbreviata*(B) is shown in Figure 4.9b. Effects of combinations are greater than additive for all proportions of *C. rotundifolia*(L), 2:1, 1:1 and 1:2 ($t = 44.0$, $p < 0.001$; $t = 45.9$, $p < 0.001$; $t = 46.6$, $p < 0.001$ respectively). The most pronounced divergence occurs when the *C. rotundifolia*(L) proportion is reduced from 2:1 to 1:1. Due to the higher doses combined, percentage NHDF growth reached a maximum value beyond which effects began to decline; effects were reduced by similar relative amounts for both curves between *C. rotundifolia*(L) proportions of 1:1 and 1:2. Similar behaviour was demonstrated by the addition of 70, 80 and 90 µg/ml *C. rotundifolia*(L) in the same fixed mass ratios (data not shown)

The addition of 100 µg/ml of *C. rotundifolia*(L) to 20, 50, 100 and 200 µg/ml of *C. abbreviata*(B) is shown in Figure 4.10a. The effects of combinations were greater than additive for all *C. rotundifolia*(L) proportions, 5:1, 2:1, 1:1 and 1:2 ($t = 27.3$, $p < 0.001$; $t = 60.2$, $p < 0.001$; $t = 57.1$, $p < 0.001$; $t = 59.6$, $p < 0.001$ respectively). The divergence between additive and combination behaviour became more pronounced when the *C. rotundifolia*(L) proportion was reduced from 5:1 to 2:1.

Between *C. rotundifolia*(L) proportions of 2:1 and 1:1, the effects of combinations remain at the maximum level, whereas those for the additive model begin to decline. As the *C. rotundifolia*(L) proportion is further decreased to 1:2, both the additive and combination effects decrease by the same relative amount.

When *C. rotundifolia*(L) was added in doses of 200, 400, 600 and 800 µg/ml, common behaviour was exhibited by the combinations, as shown for the latter dose in Figure 4.10b; in this case, 800 µg/ml was added to 160, 400 and 800 µg/ml of *C. abbreviata*(B). At all proportions of *C. rotundifolia*(L), 5:1, 2:1 and 1:1, effects for combinations were greater than additive ($t = 40.6$, $p < 0.001$), ($t = 43.8$, $p < 0.001$) and

a/



b/

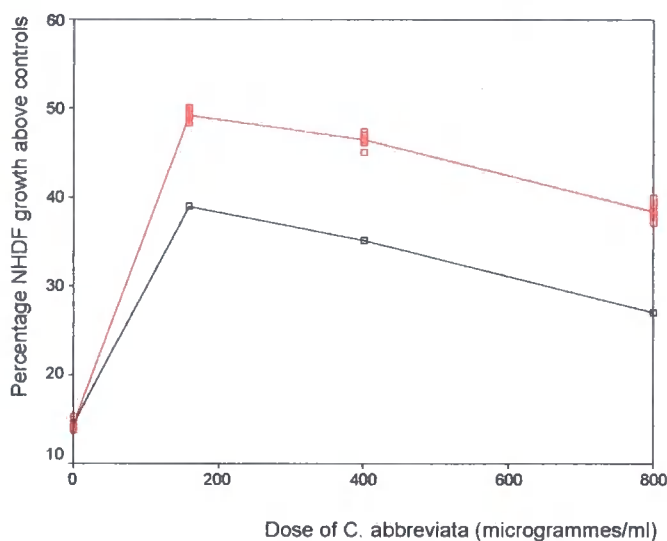


Figure 4.10 Additive behaviour (black curve) compared with combination behaviour (red curve) for *X. chalybeum* bark extract / *C. rotundifolia* leaf extract combination.

a/ Additive and combination behaviour when a dose of 100 µg/ml of *C. rotundifolia* leaf extract is added to the indicated doses of *C. abbreviata* bark extract.

b/ Additive and combination behaviour when a dose of 800 µg/ml *C. rotundifolia* leaf extract is added to the indicated doses of *C. abbreviata* bark extract

($t = 35.4$, $p < 0.001$) respectively); additionally, the decline in effects declined to a similar degree as the *C. rotundifolia*(L) proportion was reduced from 5:1 to 1:1.

The combinations attained a maximum effect (66.04% NHDF growth above controls) when 70 $\mu\text{g/ml}$ of *C. rotundifolia*(L) was added to 70 $\mu\text{g/ml}$ of *C. abbreviata*(B).

4.2.4 Discussion

4.2.4.1 Individual dose response relationships

Both herbal extracts exhibited common behaviour whereby, upon producing a particular maximum effect, further increases in dosage produce only sub-maximal effects which decreased steadily (Figure 4.7a). This behaviour deviates from classic pharmacological behaviour, where, as effects approach a maximum level, further increases in dosage produce negligible changes in effect.

One explanation for this difference is the fact that herbal drugs contain several components which may, in sufficient quantities, displace bound active ingredients from growth receptors.

Interestingly, in the case of experiments performed in media supplemented with FBS, *C. abbreviata*(B) exhibited a lesser decline in effects once the maximum had been attained; the opposite was the case in experiments performed in basal media (Figure 4.1a). Thus, the use of FBS appears to alter the dynamics of herbal extract dose response profiles.

The curve fitting parameters for the hyperbolic plots of effects versus dose were not good approximations of maximum effect values and the doses which produced effects

of half the maximum. Differences between software-ascribed and actual values of Emax and EC50 are due the software treating the data as sections of larger hyperbolas and hence extrapolating and interpolating the effects.

It is appropriate, however, to apply the model as a dose response relationship up to the maximum.

4.2.4.2 Correlation of absorbance with NHDF number

The MTS assay is an indirect means of determining changes in cell number on the basis of the measurement of cell metabolism; however, some pharmacological agents may affect metabolic end point of the assay and hence give false indications regarding the extent of growth and a correlation between absorbance and cell number is required.

Although such a correlation had already been established when herbal extracts were applied in basal media, it was felt the possibility existed that the extracts could combine with 2% FBS to produce effects that were predominantly metabolic.

The good correlations established between absorbance and NHDF number when *C. rotundifolia*(L) and *C. abbreviata*(B) were applied in basal media / 2% FBS provided validation for the pharmacological behaviour of individual and combined herbal extracts.

4.2.4.3 Time course for NHDF grown in the presence of *C. rotundifolia*(L) and *C. abbreviata*(B)

The greater cell densities of NHDF cultured in 70 µg/ml *C. abbreviata*(B) were by

virtue of the superior rate of growth between days 1 and 3; over this period, the growth rate under the action of *C. abbreviata*(B) is approximately three times that for *C. rotundifolia*(L) over this period. Between days 3, 5 and 7, the growth rate for NHDF cultured in media containing *C. abbreviata*(B) was inferior to that for cells cultured in control media and media containing *C. rotundifolia*(L). Differences of this nature were also evident in experiments involving basal media; possibly *C. abbreviata*(B) is more rapidly assimilated, bound to receptors and hence able to exert almost maximum effects at an early time point; on the other hand, *C. rotundifolia*(L) may be less readily assimilated and attains its maximum effect over a longer period.

In the case of NHDF grown in control media and also media containing herbal extracts, cell numbers begin to decline after day 7; of the two herbal extracts, the decline was more pronounced in the case of media containing *C. rotundifolia*(L), which was the opposite of finding resulting from basal media experiments; all media types were applied on the basis of a single dose and the eventual depletion of nutrients is the most likely cause of the decline.

4.2.4.4 Effects due to combinations of *C. rotundifolia*(L) and *C. abbreviata*(B)

Overall, combinations of the two herbal extracts, when applied in media containing 2% FBS consistently produced super-additive effects at all experimental proportions; this is in contrast to their behaviour in basal media, where effects were sub-additive when *C. abbreviata*(B) was present in less than equal proportions and super-additive at equal and greater than equal proportions. It therefore seems reasonable to conclude that 2% FBS enhances the interaction of the herbal extracts which was demonstrated for basal media; additionally, in the case of effective and non-effective herbal

extracts, doses at which NHDF toxicity was observed were elevated in comparison to experiments in basal media, indicative of enhanced tolerance.

Super-additivity was also evident when post-maximal effects were declining, which suggests that the eventual displacement of the active ingredient(s) from receptors is offset due to combining the herbal extracts.

4.3 Effect of herbal extracts on growth of the fibrotic model

Distinction between growth inhibition and toxicity due to herbal extracts

In proliferation assessments of the type discussed here, a decrease in viable cell number relative to controls may be due to inhibition of growth, cell death / damage or some combination of the two.

Thus, at the outset, it is important to establish a baseline measurement of healthy non-growing cells. This value may be expressed as a percentage below that of growing cells and, on a dose response curve, will be represented by a horizontal line; since this value corresponds to the initial number of seeded cells, all effects below this line signify cell damage and the curve is effectively partitioned into areas of reduced growth and cell toxicity.

4.3.1 Summary of effects

4.3.1.1 Single herbal extracts

C. rotundifolia(L), when applied at a dose range of 0.001-1000 µg/ml, demonstrated no effect on the growth of the fibrotic model; increased dosages produced no effects and toxicity was eventually observed at a dose of 1700 µg/ml (Appendix 1.2)

C. abbreviata(B), when applied at a dose range of 0.001-1000 µg/ml, demonstrated no effect on the growth of the fibrotic model; increased dosages produced no effects and toxicity was eventually demonstrated at a dose of 1800 µg/ml (Appendix 1.2).

Z. chalybeum(L), when applied at a dose range of 0.001-1000 µg/ml, demonstrated no effect on the growth of the fibrotic model; increased dosages produced no effects and toxicity was eventually observed at a dose of 1800 µg/ml (Appendix 1.2).

Z. chalybeum(B), when applied at a dose range of 0.001-1000 µg/ml demonstrated inhibitory effects up to a dose of 1600 µg/ml, whereupon toxicity was evident

4.3.1.2 Combined herbal extracts

The effect upon the growth of the fibrotic model was assessed for the following six paired herbal extract combinations

Combinations of *Z. chalybeum*(B) with *Z. chalybeum*(L) in *Z. chalybeum*(L) proportions of 5:1, 2:1, 1:1 and 1:2 did not affect the ability of *Z. chalybeum*(B) to inhibit the growth of the fibrotic model.

Combinations of *Z. chalybeum*(L) / *C. rotundifolia*(L) containing *Z. chalybeum* (B) proportions of 5:1, 2:1, 1:1, 1:2 and 1:5 produced no effects upon the growth of the fibrotic model.

Combinations of *Z. chalybeum*(L) / *C. abbreviata*(B) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 and 1:5 produced no effects upon the growth of the fibrotic model.

Combinations of *C. rotundifolia*(L) / *C. abbreviata*(B) containing *C. rotundifolia*(L) proportions of 5:1, 2:1, 1:1, 1:2 and 1:5 produced no effects upon the growth of the fibrotic model.

Combinations of *Z. chalybeum*(B) / *C. abbreviata*(B) containing *C. abbreviata*(B) proportions of 5:1, 2:1, 1:1, 1:2 did not affect the ability of *Z. chalybeum*(B) to inhibit the growth of the fibrotic model.

Combinations of *Z. chalybeum*(B) / *C. rotundifolia*(L) containing *C. rotundifolia*(L) proportions of 5:1, 2:1, 1:1, 1:2 reduced the ability of *Z. chalybeum*(B) to inhibit the growth of the fibrotic model.

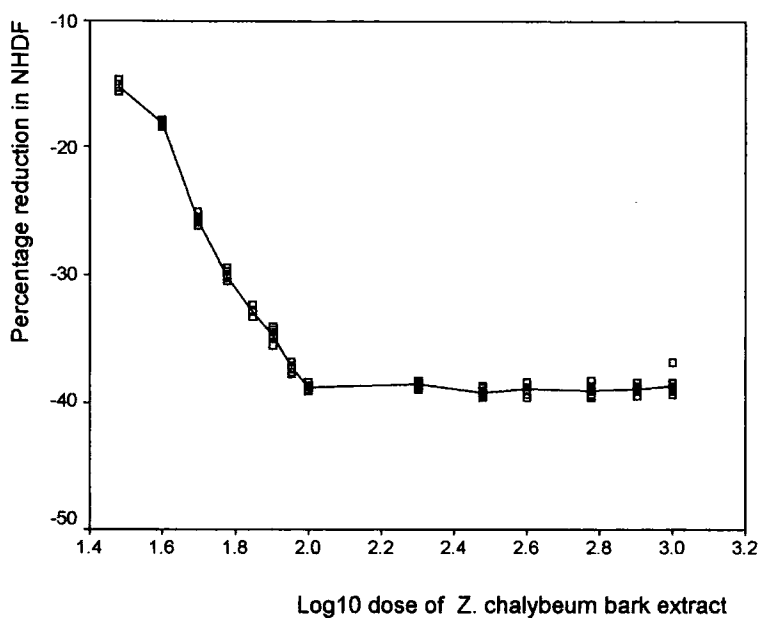
4.3.2 Dose response relationships for *Z. chalybeum*(B).

For the aqueous extract of the bark of *X. chalybeum*, significant effects were observed over a dose range of 30 – 1000 µg/ml ($p < 0.001$, $F(14, 135) = 9681.8$).

Percentage growth below controls versus log₁₀ herbal extract dose is shown in Figure 4.11a. In contrast to the logarithmic plots of *C. rotundifolia*(L) and *C. abbreviata*(B), in both unsupplemented and supplemented media, the maximum effect is maintained and the plot for the *Z. chalybeum*(B) data most closely resembled a sigmoid;

The ascending portion of the dose response curve was plotted on a linear scale and fitted to a hyperbolic model, as shown in Figure 4.11b.

a/



b/

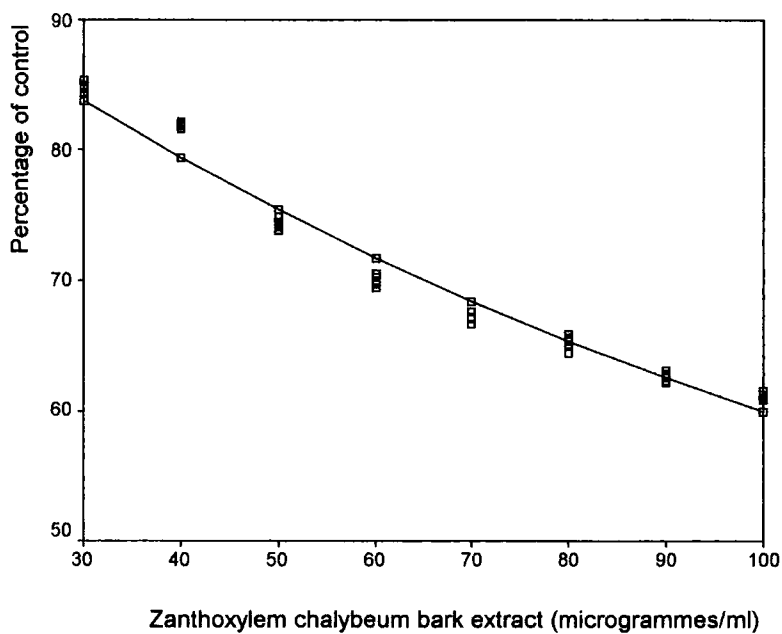


Figure 4.11 Dose response relationships for *Z. chalybeum* bark extract

a/ Percentage growth below controls versus dose plotted on a logarithmic scale

b/ Percentage growth below controls (up to the maximum effect) versus dose plotted on a linear scale and fitted to a hyperbolic model.

For *Z. chalybeum*(B), the value of Emax (109.0%) and IC50 (171.8 µg/ml) did not approximate well to the actual maximum (38.8%) and dose which produced half the actual maximum (40.0 µg/ml) respectively.

The value of r^2 was 0.97, which demonstrated a good correlation of effects with dose for the model, up to the maximum.

4.3.3 Correlation of absorbance with NHDF number

Linear regression was performed for absorbance (490nm) and cell number for NHDF grown in the presence of 100 µg / ml of *Z. chalybeum*(B).

The correlation coefficient was 0.97 and the relationship was taken to be linear; a visual representation of the relationships is shown in Figure 4.12.

4.3.4 Time course for NHDF cultured in the presence of the most effective herbal extract dose

Generally, differences between the groups were significant from day 3 onward.

Cell numbers for NHDF grown in control media were superior to those grown in the presence of control cells on day 3 ($t = 8.5$, $p < 0.01$), 5 ($t = 10.6$, $P < 0.001$), 7 ($t = 10.6$, $p < 0.001$) and 9 ($t = 10.6$, $p < 0.001$).

The growth rates of NHDF grown in control and herbal extract media are shown in table 4.3.

From day 3 onward, there was a clear reduction in growth due to the action of this dose of *Z. chalybeum*(B). There was also a disparity between the growth profiles; from days 1 to 7, the growth rate of control cells decreases with time over this period

whereas the rate for NHDF treated with *Z. chalybeum*(B) was linear. Thus, although the herbal extract reduced the NHDF number, cell growth proceeds at a fixed rate between days 1 and 7. Between days 7 and 9, there is a decline in cell numbers for the control group (due to a negative growth rate) whereas for the treatment group, there was a slight increase in NHDF numbers.

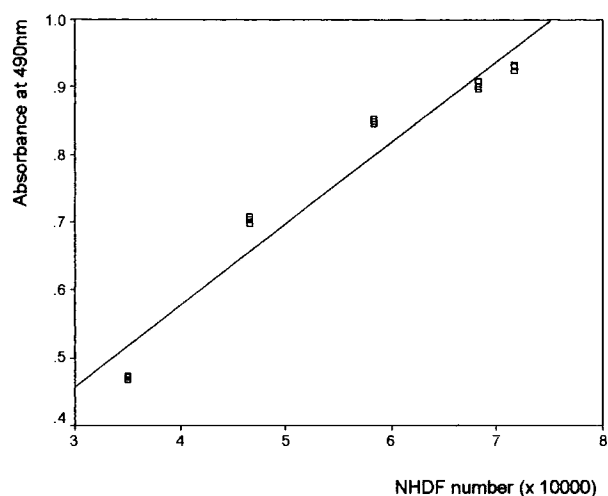
Nature of growth media	Basal medium/10% FBS + IGF-1	<i>Z. chalybeum</i> (B)
Growth rate (cells/day for days 1-3)	15000	5500
Growth rate (cells/day for days 3-5)	8000	5500
Growth rate (cells/day for days 5-7)	5000	5000
Growth rate (cells/day for days 7-9)	-3000	-1500

Table 4.3. growth rates of NHDF cultured in basal media / 10% FBS and Basal media / 10% FBS + 70 µg/ml IGF-1.

4.3.3 Effect of combinations of *Z. chalybeum*(B) and *C. rotundifolia*(L) on NHDF fibrotic model

The addition of 60 µg / ml of *C. rotundifolia*(L) to 30, 60 and 120 µg / ml of *Z. chalybeum*(B) is shown in Figure 4.13a. The additive curve demonstrated linear change of effects with dose as the *Z. chalybeum*(B) proportion was increased to 1:1. Between proportions of 1:1 and 1:2, the change of effects with dose was less pronounced. ($t = 20.2, p < 0.001$); as the proportion is reduced to 1:1, and 1:2, additive behaviour is exhibited; the less than additive behaviour was responsible for the difference in profile of the two curves.

a/



b/

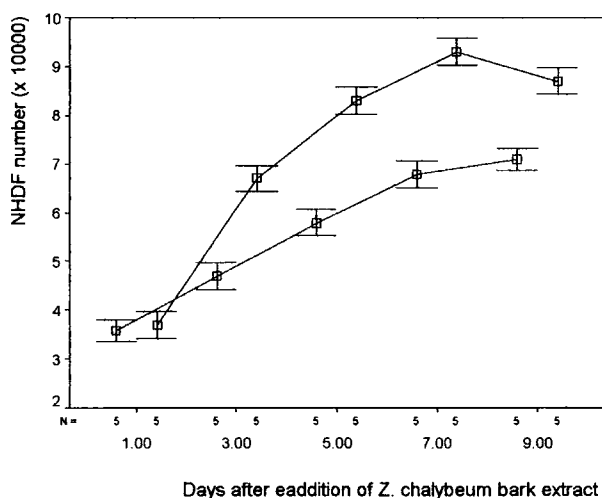


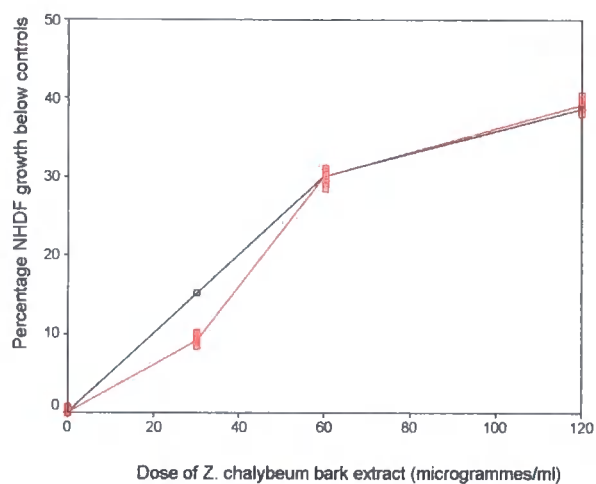
Figure 4.12. Correlation of NHDF number with absorbance and time course for *Z. chalybeum*(B) Upon addition of a single dose of herbal extract, cells were cultured over a period of 9 days in 35 mm culture plates; cell counts and absorbance readings were performed at 1, 3, 5, 7 and 9 days after herbal extract application.

a/ Correlation with absorbance for NHDF treated with 100 $\mu\text{g/ml}$ of *Z. chalybeum*(B)

b/ Time course for NHDF treated with 100 $\mu\text{g/ml}$ of *Z. chalybeum*(B).

(results are the means and standard deviation of five replicate plates).

a/



b/

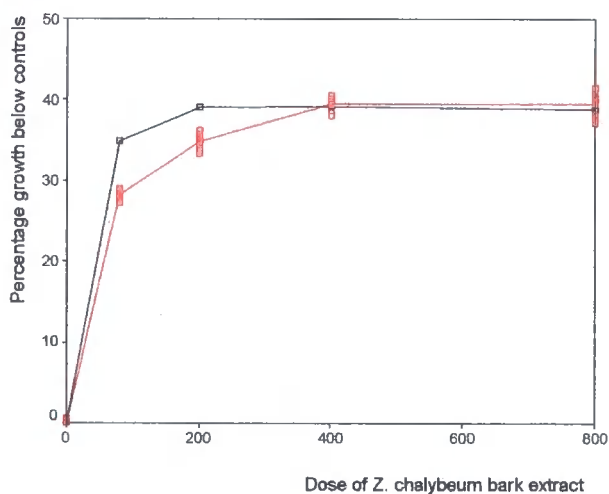


Figure 4.13. Comparison of additive (black curve) and combination (red curve) behaviour for combinations of *C. rotundifolia*(L) and *Z. chalybeum*(B)

a/ Additive and combination behaviour when 60µg/ml of *C. rotundifolia* leaf extract is combined with 30, 60 and 120 µg/ml of *Z. chalybeum* bark extract

b/ Additive and combination behaviour when 400µg/ml of *C. rotundifolia* leaf extract is combined with 80, 200, 400 and 800 µg/ml of *Z. chalybeum* bark extract At *C. rotundifolia*(L) proportion of 2:1, the effects of the combination are less than additive

This trend was also observed upon addition of 70, 80, 90 and 100 µg / ml of *C. rotundifolia*(L) to *Z. chalybeum*(B) in similar fixed mass proportions (data not shown).

Figure 4.13b shows the result of adding 400 µg / ml of *C. rotundifolia*(L) to 80, 200, 400 and 800 µg / ml of *Z. chalybeum*(B). the additive effects attained a maximum value at a *C. rotundifolia*(L) proportion of 2:1; at proportions of 1:1 and 1:2 effects remained unchanged.

At *C. rotundifolia*(L) proportion of 5:1 and 2:1, the effects of the combination were less than additive ($t = 24.1$, $p < 0.001$) and ($t = 12.8$, $p < 0.001$) respectively). As the proportion is reduced to 1:1, and 1:2, additive behaviour is restored.

This trend was observed when doses of 200, 600, 800 and 1000 µg / ml of *C. rotundifolia*(L) were added in similar fixed mass proportions (data not shown)

4.3.4 Discussion

4.3.4.1 Dose response relationships

No effects were observed at doses below 30 µg/ml; this may reflect that the active ingredient(s), due to inhibition by surrounding components, are only able to manifest once a particular concentration has been reached, whereupon effects increase with dose fairly steadily.

The active ingredient(s) of *Z. chalybeum*(B) extract most probably exerts effects by interfering physically or chemically with growth receptor function; this interference reaches a maximum at 100 µg/ml, beyond which further inhibition of growth is not possible, presumably because the sites at which interaction occurs are saturated.

Dose response curves for *C. rotundifolia*(L) and *C. abbreviata*(B), discussed previously, were characterised by effects decreasing steadily once a maximum had been attained, which is in contrast to *Z. chalybeum*(B).

One reason proposed for such decreases was that other components of the herbal extracts displaced the active ingredient(s) from receptors as the herbal extract concentration increased; a possible explanation for the effects remaining at the maximum level in the case of *Z. chalybeum*(B) is that there are lesser amount of these disruptive components.

The reason for the disparity between the curve fitting parameters (E_{max} and IC_{50}) and the actual values recorded are due the software treating the data as sections of larger hyperbolas and hence extrapolating and interpolating the effects.

It is appropriate, however, to apply the model as a dose response relationship up to the maximum.

4.3.4.2 Correlation of NHDF number with absorbance

The assay for cell growth is based on changes in the metabolism of NHDF. The importance of demonstrating that cell number and absorbance are closely correlated is that it removes the possibility that absorbance reductions are due to metabolic suppression by *Z. chalybeum*(B).

The good correlation ($r^2 = 0.97$) established enables not only single, but combined extracts to be assessed unambiguously.

Had there been no such correlation between the MTS assay and NHDF number, the neutral red assay would have been selected for use.

4.3.4.3 Growth rate profile of NHDF cultured in media containing 100 µg/ml of *Z. chalybeum* bark extract

The greatest difference between the growth rates of cells treated with *Z. chalybeum*(B) and controls occurred between days 1 and 3, where the rate of control cells was approximately threefold that of the treatment group.

The growth rate of extract-treated cells continued to increase with each subsequent time point and NHDF numbers in the control group eventually began to fall (most likely due to depletion of nutrients as a result of much larger cell numbers); nevertheless, the early reduction of cell growth in the treatment group was the major determinant of the overall difference in NHDF numbers.

4.3.4.4 Effects of combinations of *Z. chalybeum*(B) and *C. rotundifolia*(L)

Interestingly, despite their ability to enhance the growth of NHDF in both unsupplemented and supplemented media, *C. rotundifolia*(L) and *C. abbreviata*(B) demonstrated no effects on the growth of NHDF in the fibrotic model. One reason for this may be that the amount of growth factors already present in the model (10% FBS+IGF-1) produced a maximum amount of growth which further addition of mitogens was unable to influence.

When evaluating the effects of combinations of *Z. chalybeum*(B) and *C. rotundifolia*(L), it must be realised that due to *C. rotundifolia*(L) having no overt effects, the additive curve was simply the dose response curve for *Z. chalybeum*(B).

When combined with *Z. chalybeum*(B), *C. rotundifolia*(L) reduced the extent to which cell growth was inhibited; because the two extracts are not competing for similar

growth receptors, a negative interaction between them, as opposed to sub-additivity, is believed to be the phenomenon by which effects are reduced.

It seems unlikely that combination with an anti-proliferative herbal extract in some way enabled *C. rotundifolia*(L) to enhance cell growth. This, in part, is supported by the observation that *C. abbreviata*(B), another growth enhancing extract (in basal media and basal media / 2%FBX) had no effect on the performance of *Z. chalybeum*(B). More likely is the premise that *C. rotundifolia*(L), in proportions greater than 1:1, disrupts the inhibitory action of *Z. chalybeum*(B) on growth receptors.

Chapter 5 – Effects of herbal extracts on NHDF production of PICP and KGF

5.1 Effects of herbal extracts on NHDF production of PICP and KGF

5.1.1 Summary of effects

5.1.1.1 Single herbal extracts

Z. chalybeum(L), when applied at a dose range of 0.001-1000 µg/ml, demonstrated no effects on the production by NHDF of PICP and KGF; increased dosage produced no effects up to a dose of 1100, whereupon toxicity was evident.

Z. chalybeum(B), when applied at a dose range of 0.001-1000 µg/ml, demonstrated an apparently inhibitory effect on the production by 'fibrotic' NHDF of PICP and KGF; however, when these effects were indexed to NHDF growth, it was found that there were no intrinsic effects on PICP or KGF production. Toxicity was eventually observed at a dose of 1600 µg/ml

C. rotundifolia(L), when applied at a dose range of 0.001-1000 µg/ml, demonstrated an apparently enhancing effect on the production by NHDF of PICP and KGF; however, when these effects were indexed to NHDF growth, it was found that there were no intrinsic effects on PICP or KGF production. Toxicity was eventually observed at a dose of 1100 µg/ml.

C. abbreviata(B), when applied at a dose range of 0.001-1000 µg/ml, demonstrated an apparently enhancing effect on the production by NHDF of PICP and KGF; however, when these effects were statistically compared to NHDF growth, it was found that there were no intrinsic effects on PICP or KGF. Toxicity was eventually observed at a dose of 1300 µg/ml.

5.1.1.2 Combined herbal extracts

The effect upon NHDF production of PICP and KGF was assessed for the following six paired herbal extract combinations:

Combinations of *Z. chalybeum*(B) / *Z. chalybeum*(L) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 and 1:5 produced no effects.

Addition of *Z. chalybeum*(B) to *C. rotundifolia*(L) in proportions of 5:1, 2:1, 1:1 and 1:2 produced effects which were apparently less than additive when *C. rotundifolia*(L) was present in proportions greater than 1:1; however, when these combination effects were compared statistically to the effects of the same combination on growth-related effects of the NHDF fibrotic model, it was found that there were no significant differences between the two groups.

Addition of *Z. chalybeum*(L) to *C. rotundifolia*(L) in proportions of 5:1, 2:1, 1:1 and 1:2 had no effect upon biomolecule production.

Addition of *Z. chalybeum*(B) to *C. abbreviata*(B) in proportions of 5:1, 2:1, 1:1

and 1:2 had no effect upon biomolecule production.

Addition of *Z. chalybeum*(L) to *C. abbreviata*(B) in proportions of 5:1, 2:1, 1:1 and 1:2 had no effect upon biomolecule production.

Combinations of *C. rotundifolia*(L) / *C. abbreviata*(B) containing *C. rotundifolia*(L) proportions of 5:1, 2:1, 1:1 and 1:2 produced effects which were apparently less than additive when *C. rotundifolia*(L) was present in proportions greater than 1:1 and super-additive when in proportions of 1:1 or less; however, when these combination effects were compared statistically to the effects of the same combination on NHDF growth, it was found that there were no significant differences between the two groups.

Chapter-6 Effects of herbal extracts upon NHDF exposed to oxidants

6.1 Protective effects of herbal extracts

6.1.1 Summary of results

6.1.1.1 Single herbal extracts

Z. chalybeum(B) demonstrated no effects from a dose of 0.001 µg/ml up to 800 µg/ml, whereupon toxicity was evident.

Z. chalybeum(L), when applied at a dose range of 0.001-1000 µg/ml, demonstrated no protective effects; increased dosages produced no effects and toxicity was eventually observed at a dose of 1600 µg/ml.

C. rotundifolia(L), when applied at a dose range of 0.001-1000 µg/ml, demonstrated no protective effects; increased dosages produced no effects and toxicity was eventually observed at a dose of 1400 µg/ml.

C. abbreviata(B), when applied at a dose range of 0.001-1000 µg/ml, demonstrated no protective effects; increased dosages produced no effects and toxicity was eventually observed at a dose of 1600 µg/ml.

6.1.1.2 Combined herbal extracts.

The protective effect upon NHDF was assessed for the following six paired herbal extract combinations:

Combinations of *Z. chalybeum*(B) / *Z. chalybeum*(L) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 and 1:5 produced no protective effects.

Combinations of *Z. chalybeum*(B) / *C. rotundifolia*(L) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 produced no protective effects.

Combinations of *Z. chalybeum*(L) / *C. rotundifolia*(L) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 produced no protective effects.

Combinations of *Z. chalybeum*(B) / *C. abbreviata*(B) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 produced no protective effects.

Combinations of *Z. chalybeum*(L) / *C. abbreviata*(B) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1, 1:2 produced no protective effects

Combinations of *C. rotundifolia*(L) / *C. abbreviata*(B) containing *C. rotundifolia* (L) proportions of 5:1, 2:1, 1:1 and 1:2 produced no protective effects.

6.2 Scavenging effects of herbal extracts

6.2.1 Summary of results

6.2.1.1 Single herbal extracts

C. abbreviata(B), when applied at a dose range of 0.001 – 1000 µg/ml produced no scavenging effects; increased dosages produced no effects and toxicity was eventually observed at a dose of 1300 µg/ml (Appendix 1.3)

C. rotundifolia(L), when applied at a dose range of 0.1-1000 µg/ml, demonstrated an oxidant scavenging effect and toxicity was eventually observed at a dose of 1900 µg/ml.

Z. chalybeum(L), when applied at a dose range of 1.0 -1000 µg/ml, demonstrated an oxidant scavenging effect and toxicity was eventually observed at a dose of 2100 µg/ml.

Z. chalybeum(B), when applied at a dose range of 10 -1000 µg/ml, demonstrated an oxidant scavenging effect and toxicity was eventually observed at a dose of 2000 µg/ml.

6.2.1.2 Combined herbal extracts

Addition of non-scavenging *C. abbreviata*(B) to *C. rotundifolia*(L), *Z. chalybeum*(L)

and *Z. chalybeum*(B) in proportions of 5:1, 2:1, 1:1 and 1:2 did not affect the oxidant scavenging ability of the latter three herbal extracts.

Addition of *Z. chalybeum*(B) to *C. rotundifolia*(L) in proportions of 5:1, 2:1, 1:1 and 1:2 produced purely additive effects.

Addition of *Z. chalybeum*(L) to *C. rotundifolia*(L) in proportions of 5:1, 2:1, 1:1 and 1:2 produced effects which were additive and greater than additive.

Combinations of *Z. chalybeum*(B) / *Z. chalybeum*(L) containing *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1 and 1:2 produced effects which were sub-additive.

6.2.3 Dose response relationships for *C. rotundifolia* leaf extract, *Z. chalybeum* bark extract and *Z. chalybeum* leaf extract.

For *C. rotundifolia*, statistically significant effects, up to a maximum, were observed over the dose range 0.1-30 μ g/ml ($p < 0.001$; $F(12, 117) = 1945.5$).

For *Z. chalybeum* bark extract, statistically significant effects, up to a maximum, were observed over the dose range 10-70 μ g/ml ($p < 0.001$; $F(13, 126) = 1329.3$).

For *Z. chalybeum* leaf extract, statistically significant effects were observed over the dose range 1.0-40 μ g/ml ($p < 0.001$; $F(11, 108) = 1149.4$

Percentage NHDF protection above controls, plotted on a logarithmic scale, is shown in Figure 6.1a. In the case of *C. rotundifolia*(L), there was an initial linear dose response relationship for the first four dose applications; at subsequent dose applications, up to the maximum, effects deviate from linear behaviour. This was not

the case for *Z. chalybeum*(B), which, upon manifesting an effect, exhibits linear behaviour up to the maximum. In the case of *Z. chalybeum*(L), there is a relatively smaller rise in effects between the application of the first and second dose, after which linearity is exhibited up to the maximum.

Generally, all three herbal extracts exhibit common behaviour in that, upon producing a particular maximum effect, further increases in dosage produce only sub-maximal effects which decrease steadily; in particular, upon addition of the maximum dose (1000 µg/ml), *Z. chalybeum*(L) and *Z. chalybeum*(B) both produce effects similar to their initial effect (Figure 6.1a).

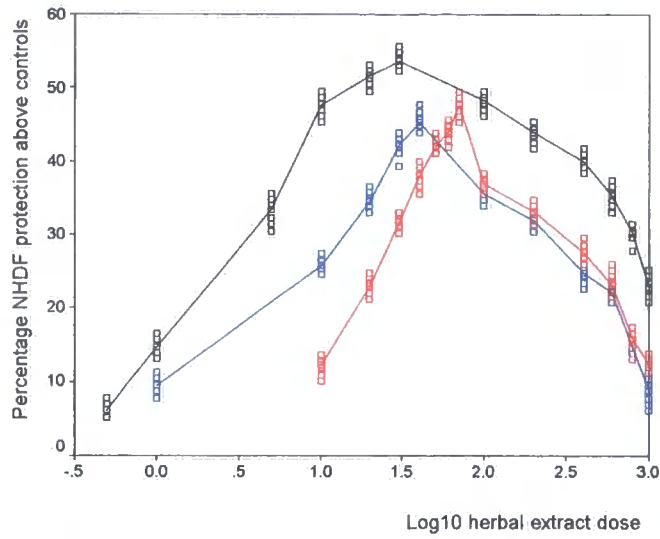
C. rotundifolia(L) is the more potent herbal extract and also attains a higher maximum effect (53.74%) than either of the other herbal extracts; additionally the decrease in effects once the maximum has been attained is less pronounced when compared with the other extracts.

Z. chalybeum(L) is more potent than *Z. chalybeum*(B), up to the maximum effect; the maximum for both are comparable (47.65 and 45.38% respectively) and the eventual decrease in effects is very similar for both these herbal extracts.

The ascending portion of the dose response curves were also plotted on a linear dose scale and fitted to hyperbolic models which are briefly described below. These plots are shown in Figure 6.1b.

For *C. rotundifolia*(L), the value of Emax (60.3%) compares favourably with the actual maximum effect (53.74%) and the EC50 (3.3 µg/ml) appears to be a reasonable approximation for the dose which results in half the actual maximum

a/



b/

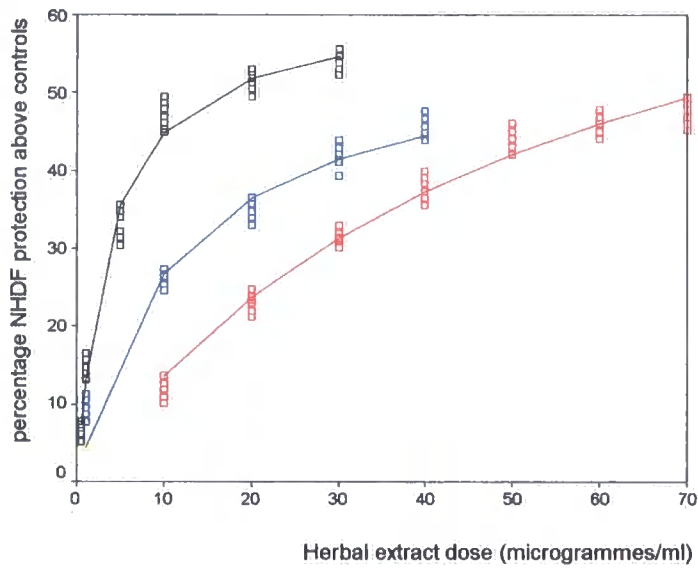


Figure 6.1 Dose response relationships for *C. rotundifolia* leaf extract (black curves), *Z. chalybeum* leaf extract (blue curves), *Z. chalybeum* bark extract (red curves).

a/ effects versus dose plotted on a logarithmic scale.

b/ effects versus dose (up to maximum effect) plotted on a linear scale and fitted to hyperbolic model.

For *Z. chalybeum* (L), the comparison of Emax (57.0) with the actual value (47.65%) is less favourable; however, the EC50 (11.3 µg/ml) appears to be a reasonable approximation of the dose resulting in half the actual maximum.

For *Z. chalybeum* (B), the actual maximum effect (45.38%) does not approximate well to the curve fitting parameter Emax (87.1%) and the EC50 (53.2 µg/ml) appears to be more than double that which produces half the actual maximum.

6.2.4 Effects due to paired combinations of *C. rotundifolia*(L), *Z. chalybeum*(B) and *Z. chalybeum*(L)

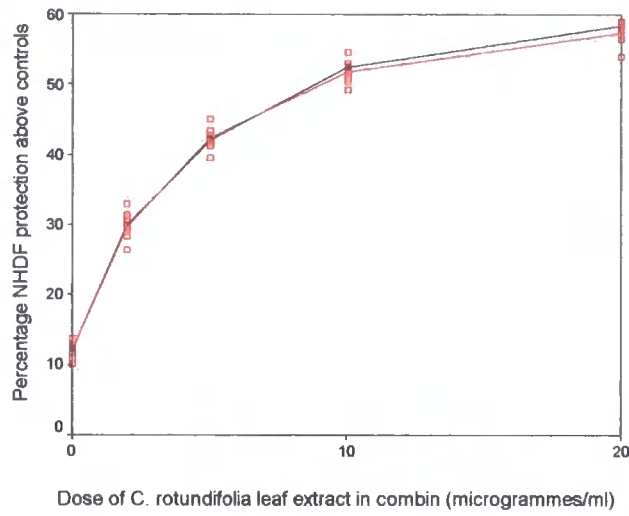
6.2.4.1 *C. rotundifolia*(L) and *Z. chalybeum*(B)

Figure 6.2a shows the effect of adding 10 µg/ml of *Z. chalybeum*(B) to 2, 5, 10 and 20 µg/ml of *C. rotundifolia*(L). At all proportions of *Z. chalybeum*(B), there were no differences in additive and combination behaviour; effects increased smoothly with doses of both extracts.

The addition of 20 µg/ml of *Z. chalybeum*(B) to 4, 10, 20 and 40 µg/ml of *C. rotundifolia*(L) is shown in Figure 6.2b. There was no difference, between additive and combination behaviour; however, in contrast to Figure 6.2a, a maximum is produced at a 1:2 proportion of *Z. chalybeum*(B) and remains unchanged as *Z. chalybeum*(B) is added to larger amounts of *C. rotundifolia*(L).

The behaviour shown in Figure 6.2a continued until the addition of a dose of 70 µg/ml of *Z. chalybeum*(B) to 14, 35, 70 and 140 µg/ml of *C. rotundifolia*(L), which is shown in Figure 6.3a. Effects began to decline at *Z. chalybeum*(B) proportions of 1:1 and 1:2

a/



b/

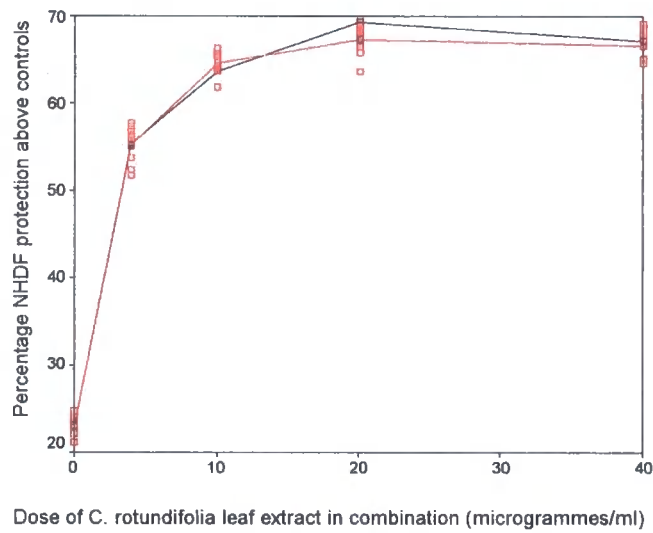
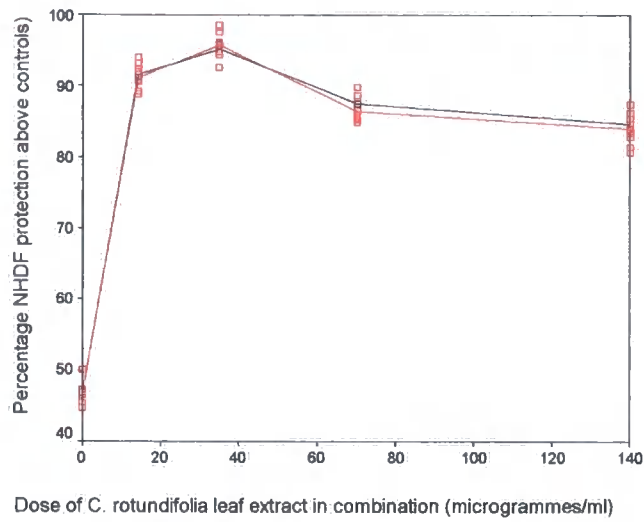


Figure 6.2 Additive behaviour (black curve) compared with combination behaviour (red curve) for *Z. chalybeum* bark extract / *C. rotundifolia* leaf extract combination.

a/ Additive and combination behaviour when a dose of 10 $\mu\text{g/ml}$ of *Z. chalybeum* bark extract is added to the indicated doses of *C. rotundifolia* leaf extract.

b/ Additive and combination behaviour when a dose of 20 $\mu\text{g/ml}$ of *Z. chalybeum* bark extract is added to the indicated doses of *C. rotundifolia* leaf extract

a/



b/

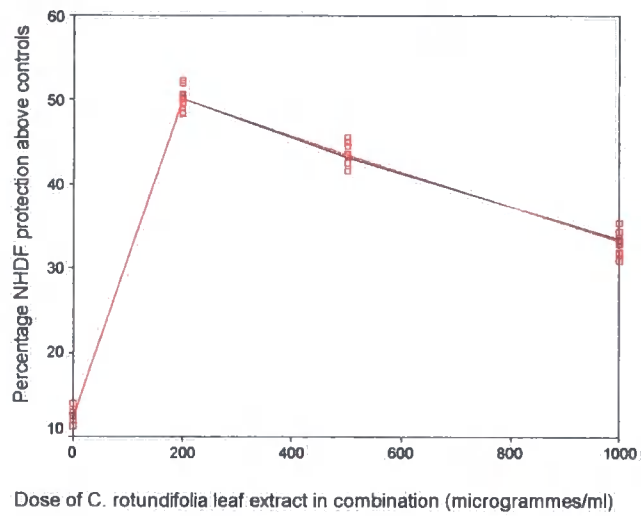


Figure 6.3 Additive behaviour (black curve) compared with combination behaviour (red curve) for *X. chalybeum* bark extract / *C. rotundifolia* leaf extract combination.

a/ Additive and combination behaviour when a dose of 70 $\mu\text{g/ml}$ of *Z. chalybeum* bark extract is added to the indicated doses of *C. rotundifolia* leaf extract

b/ Additive and combination behaviour when a dose of 1000 $\mu\text{g/ml}$ of *Z. chalybeum* bark extract is added to the indicated doses of *C. rotundifolia* leaf extract.

In these combinations, there was no difference between additive and combination behaviour at any relative herbal extract proportion.

Different behaviour was evidenced at additions of 100, 200, 300, 400, 600 and 800 and 1000 µg/ml of *Z. chalybeum*(B), the latter of which is shown in figure 6.3b. Upon attaining a maximum effect at a *Z. chalybeum*(B) proportion of 5:1, addition of *X. chalybeum*(B) to larger amounts of *C. rotundifolia*(L) resulted in a steady decline in effects. There were no differences between additive and combination behaviour for any of the proportions for this combination.

The attainment of the greatest maximum effect (100.63% protection) corresponds to the addition of 60 µg/ml of *Z. chalybeum*(B) to 30 µg/ml *C. rotundifolia*(L).

6.2.2 *C. rotundifolia* leaf extract and *Z. chalybeum* leaf extract

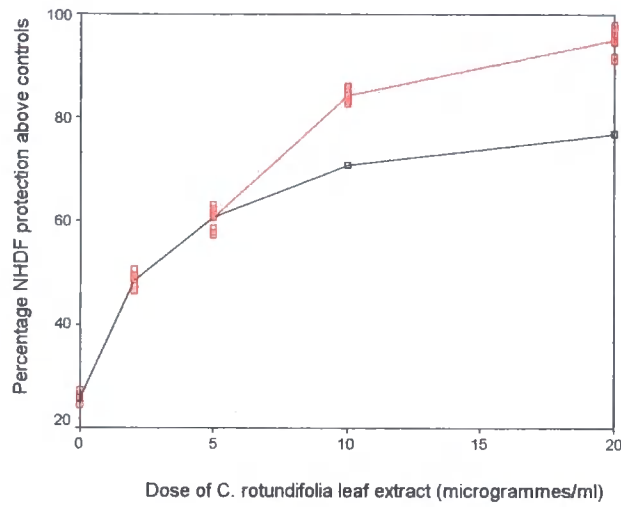
Comparison plots for additive and combination effects.

Figure 6.4a shows the addition of 10 µg/ml of *Z. chalybeum*(L) extract to 2, 5, 10 and 20 µg/ml of *C. rotundifolia*(L). There was no difference between additive and combination behaviour until the proportion of *Z. chalybeum*(L) was reduced to 1:1, at which point the effects of combinations exceeded additive effects ($t = 22.7$, $p < 0.001$); the divergence of the curves increased when the proportion was further reduced to 1:2 ($t = 20.7$, $p < 0.001$).

This behaviour was also evidenced due to the addition of 20 and 30 µg/ml *Z. chalybeum*(L) in similar fixed proportions to above (data not shown).

An addition of 40 µg/ml, to *C. rotundifolia*(L) doses of 8, 20, 40 and 80 µg/ml is shown in Figure 6.4b. There was no difference between additive and combination

a/



b/

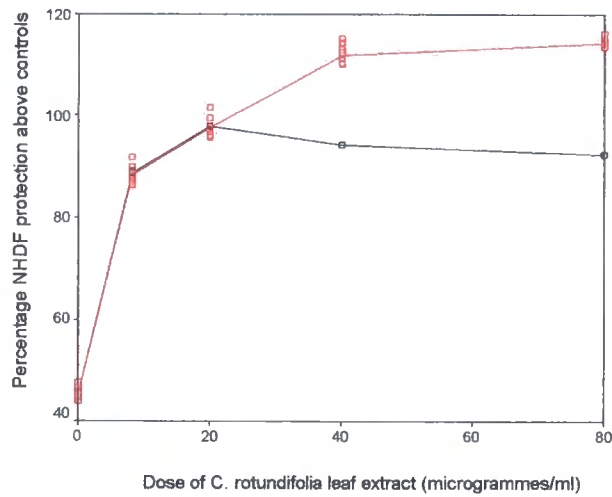


Figure 6.4 Additive behaviour (black curve) compared with combination behaviour (red curve) for *Z. chalybeum* leaf extract / *C. rotundifolia* leaf extract combination.

a/ Additive and combination behaviour when a dose of 10 µg/ml of *Z. chalybeum* leaf extract is added to the indicated doses of *C. rotundifolia* leaf extract.

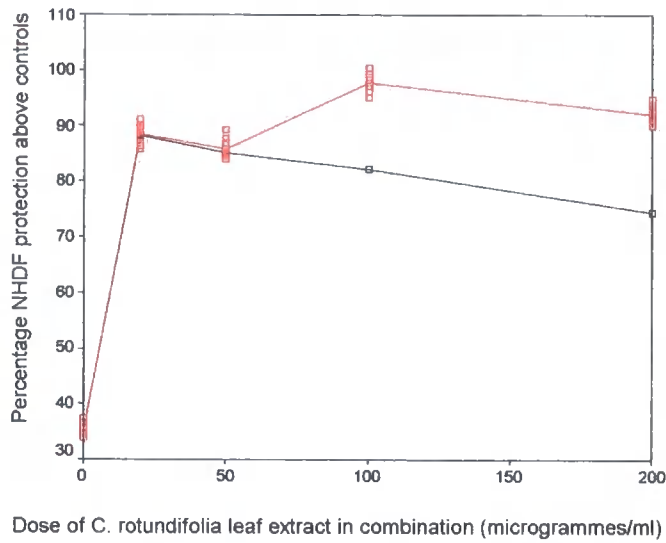
b/ Additive and combination behaviour when a dose of 40 µg/ml of *Z. chalybeum* leaf extract is added to the indicated doses of *C. rotundifolia* leaf extract.

behaviour until the proportion of *Z. chalybeum*(L) was reduced to 1:1 and 1:2, at which point the effects of combinations exceeded additive effects ($t = 24.5$, $p < 0.001$) and ($t = 73.8$, $p < 0.001$) respectively. In the case of additive effects, at a *Z. chalybeum*(L) proportion of 2:1, the effects reach a maximum; subsequently, effects began to decline as the proportion of *Z. chalybeum*(L) lessened. This was not the case for combination behaviour, where at a *Z. chalybeum*(L) proportion of 1:1, effects reached a maximum which was maintained at the lesser proportion of 1:2. Thus, when the proportion of *Z. chalybeum*(L) decreased from 1:1 to 1:2, there were differences in terms of both the magnitude of effects and the direction in which effects varied with dose for additive and combination behaviour.

An addition of 100 $\mu\text{g/ml}$ of *Z. chalybeum*(L) to 20, 50, 100 and 200 $\mu\text{g/ml}$ of *C. rotundifolia*(L) is shown in Figure 6.5a. Difference between additive and combination behaviour only became significant when the proportion of *Z. chalybeum*(L) was reduced to 1:1 and 1:2 ($t = 24.5$, $p < 0.001$) and ($t = 73.8$, $p < 0.001$) respectively. The additive curve reached a maximum at a *Z. chalybeum*(L) proportion of 5:1 and as the proportion decreased to 2:1, the additive and combination behaviour was identical. Between *Z. chalybeum*(L) proportions of 2:1 and 1:1, the combination effects altered direction and increased to a level which declined slightly when the proportion was finally reduced to 1:2. In contrast, the additive curve continued to decrease in a linear fashion as *Z. chalybeum*(L) proportions were reduced to 1:2. This trend also resulted from the addition of 200 and 400 $\mu\text{g/ml}$ of *Z. chalybeum*(L) in similar fixed proportions (data not shown).

Commencing at an addition of 600 $\mu\text{g/ml}$ of *Z. chalybeum*(L), differences between combination and additive behaviour became less pronounced, a trend which increased upon addition of 800 $\mu\text{g/ml}$ (data not shown).

a/



b

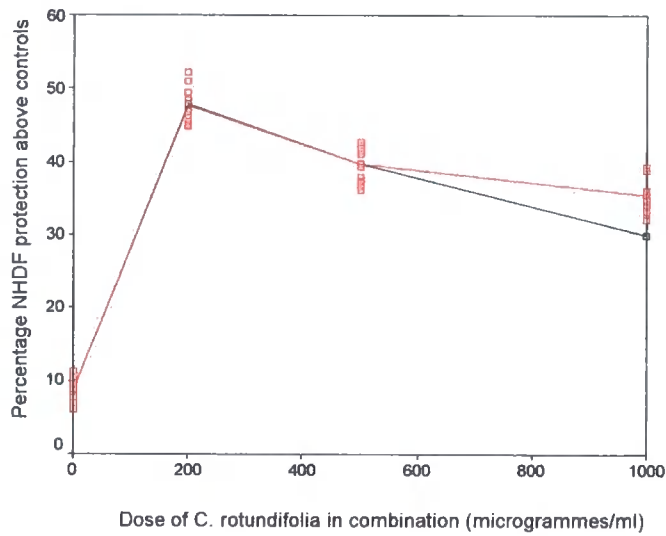


Figure 6.5 Additive behaviour (black curve) compared with combination behaviour

(red curve) for *Z. chalybeum* leaf extract / *C. rotundifolia* leaf extract combination

a/ Additive and combination behaviour when a dose of 100 µg/ml of *Z. chalybeum*

leaf extract is added to the indicated doses of *C. rotundifolia* leaf extract

b/ Additive and combination behaviour when a dose of 1000 µg/ml of *Z. chalybeum*

bark extract is added to the indicated doses of *C. rotundifolia* leaf extract.

The case of an 1000 µg/ml addition to 200, 500, and 1000 µg/ml of *Z. chalybeum*(L) is shown in Figure 6.5b, where additive and combination behaviour was identical between *Z. chalybeum*(L) proportions of 5:1 and 2:1; as the proportion is reduced to 1:1 and 1:2 there was a slight upward divergence of combination behaviour from additive behaviour ($t = 4.9$, $p < 0.001$).

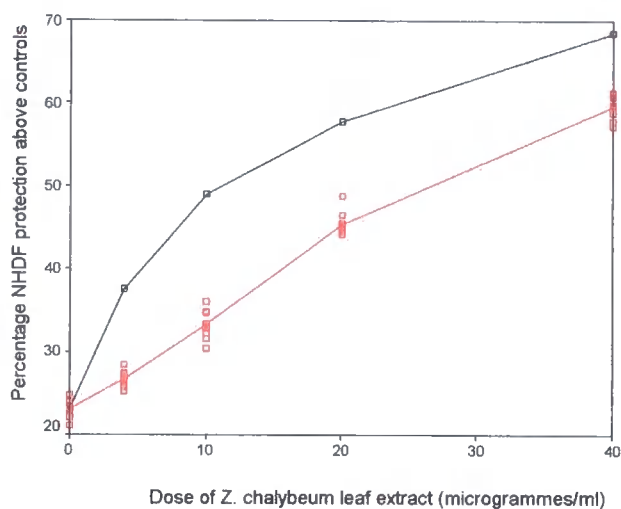
The highest maximum value(114.6% NHDF protection above controls) was attained when 30 µg/ml of *Z. chalybeum*(L) was added to 30 µg/ml of *C. rotundifolia*(L).

6.2.3 *Z. chalybeum* leaf extract and *Z. chalybeum* bark extract

An addition of 20 µg/ml of *Z. chalybeum*(B) to 4, 10, 20 and 40 µg/ml *Z. chalybeum*(L) is depicted in Figure 6.6a. At all proportions of *Z. chalybeum*(B), 5:1, 2:1, 1:1 and 1:2, the effects produced by the combinations were less than additive ($t = 21.4$, $p < 0.001$), ($t = 20.2$, $p < 0.001$), ($t = 18.0$, $p < 0.001$) and ($t = 12.4$, $p < 0.001$) respectively. This was most pronounced at a *Z. chalybeum*(B) proportion of 2:1; as the proportion was reduced to 1:1 and 1:2, there appeared to be a constant difference between additive and combination effects.

When 30 µg/ml of *Z. chalybeum*(B) are added to 6, 15, 30 and 60 30 µg/ml of *Z. chalybeum*(L), shown in Figure 6.6b, combination effects were again less than additive for all *Z. chalybeum*(B) proportions of 5:1, 2:1, 1:1 and 1:2 ($t = 18.8$, $p < 0.001$), ($t = 22.6$, $p < 0.001$), ($t = 19.7$, $p < 0.001$) and ($t = 7.0$, $p < 0.001$) respectively. Both curves rise steadily and attain maximum effects at a *Z. chalybeum*(B) proportion of 1:1. From this point, a slight decline in additive effects coupled with a steady increase in combination effects results in approximately additive behaviour by the

a/



b/

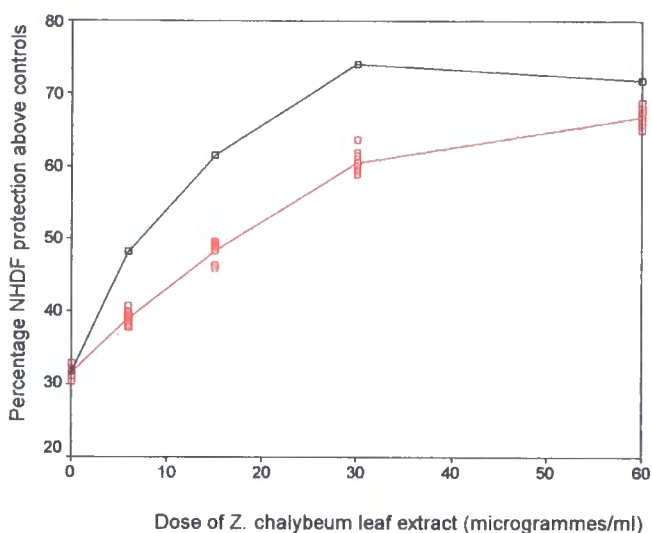


Figure 6.6 Additive behaviour (black curve) compared with combination behaviour (red curve) for *Z. chalybeum* bark extract / *Z. chalybeum* leaf extract

a/ Comparison of additive and combination behaviour when a dose of 20 $\mu\text{g/ml}$ of *X. chalybeum* bark extract is added to the indicated doses of *Z. chalybeum* leaf extract.

b/ Comparison of additive and combination behaviour when a dose of 30 $\mu\text{g/ml}$ of *X. chalybeum* bark extract is added to the indicated doses of *Z. chalybeum* leaf extract.

combination at a *Z. chalybeum*(B) proportion of 1:2.

The addition of 40, 50 and 60 µg/ml of *Z. chalybeum*(B) resulted in a common trend and Figure 6.7a depicts the results of adding 60 µg/ml of *Z. chalybeum*(B) to 12, 30, 60 and 120 µg/ml of *Z. chalybeum*(L). All *Z. chalybeum*(B) proportions, 5:1, 2:1, 1:1 and 1:2 resulted in less than additive effects ($t =$, $p < 0.001$), ($t =$, $p < 0.001$), ($t =$, $p < 0.001$) and ($t =$, $p < 0.001$) respectively. Both curves attained a maximum at a *Z. chalybeum*(B) proportion of 1:1; after this point, additive effects began to decrease whereas combination effects remained constant until the proportion was decreased to 1:2, at which point they also began to decline.

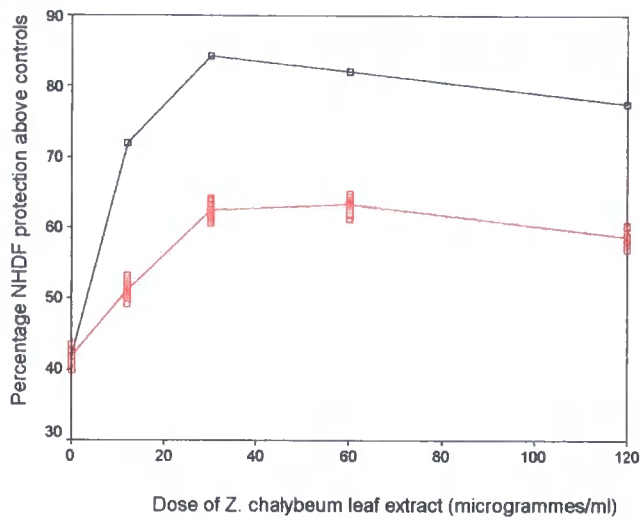
This trend continued upon addition of 70, 100 and 200 µg/ml of *Z. chalybeum*(B) in similar fixed mass ratios (data not shown).

The addition of 400, 600 and 1000 µg/ml of *Z. chalybeum*(B) to *Z. chalybeum*(L) in similar fixed mass ratios also resulted in a common trend and the addition of the latter amount is shown in Figure 6.9b. All *Z. chalybeum*(B) proportions, 5:1, 2:1 and 1:1 resulted in less than additive effects ($t = 19.5$, $p < 0.001$), ($t = 28.6$, $p < 0.001$) and ($t = 18.0$, $p < 0.001$) respectively.

Both the additive and combination curves attained a maximum at a *Z. chalybeum*(B) proportion of 5:1 after which point there is a steady decline in effects for both types of behaviour.

The highest maximum value (67.14% NHDF protection above controls) was attained when 40 µg/ml of *Z. chalybeum*(B) was added to 40 µg/ml of *Z. chalybeum*(L).

a/



b/

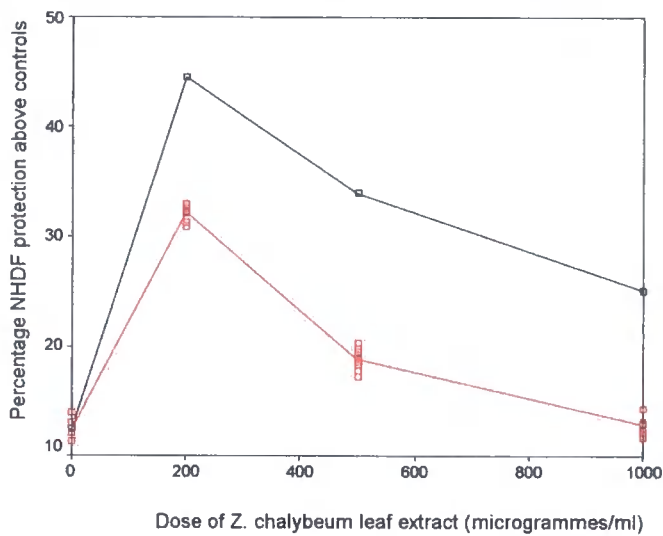


Figure 6.9 Additive behaviour (black curve) compared with combination behaviour (red curve) for *Z. chalybeum* bark extract / *Z. chalybeum* leaf extract

a/ Additive and combination behaviour when a dose of 60 µg/ml of *Z. chalybeum* bark extract is added to the indicated doses of *Z. chalybeum* leaf extract.

b/ Additive and combination behaviour when a dose of 1000 µg/ml of *Z. chalybeum* bark extract is added to the indicated doses of *Z. chalybeum* leaf extract.

6.3 Discussion

6.3.1 Individual dose response relationships for *C. rotundifolia*(L), *Z. chalybeum*(B) and *Z. chalybeum*(B)

Strictly speaking, the ability of drugs to 'scavenge' free radicals is not a physiological effect. Any action on the free radicals by the herbal extract components takes place extracellularly and is a purely chemical phenomenon. However, the scavenging ability of herbal extracts is often dependent on the binding of components to specific sites on the free radical molecule prior to a chemical reaction between them; this situation is analogous to drug-receptor binding and the use of a similar means of visual representation is therefore appropriate.

Generally, all three herbal extracts exhibited common behaviour in that, upon producing a particular maximum effect, further increases in dosage produce only sub-maximal effects which decrease steadily (Figure 6.1a). This behaviour deviates from classic pharmacological behaviour, where, as effects approach a maximum level, further increases in dosage produce negligible changes in effect. One explanation for this difference is the fact that herbal drugs contain several components which may, in sufficient quantities, displace bound active ingredients from receptors or, in this particular case, free radicals.

In the case of *Z. chalybeum*(L) and *Z. chalybeum*(B), the maximum effects for both were comparable (47.65 and 45.38% respectively) and the decrease in effects after attainment of the maximum followed a very similar profile for both these herbal extracts. These observations suggest that *Z. chalybeum*(L) and *Z. chalybeum*(B) may possibly exert their scavenging effects by similar mechanisms.

In contrast to *C. rotundifolia*(L) and *Z. chalybeum*(B), for *X. chalybeum*(B), the actual maximum effect (45.38%) does not approximate well to the curve fitting parameter (87.1%) and the EC50 (53.2 µg/ml) appears to be more than double that which produces half the actual maximum (Figure 6.1b). The reason for this discrepancy is that, in the case of *Z. chalybeum*(B) in particular, the data is treated as being a portion of a larger curve and the higher values of Emax and EC50 are the result of interpolation.

Nevertheless, in all three cases, up to the maximum effect, the hyperbolic models are good mathematical descriptions of the relationship between effects and dose.

6.3.2 Effects of paired combinations of herbal extracts.

6.3.2.1 *C. rotundifolia*(L) and *Z. chalybeum*(B)

Overall, there was no deviation from additive behaviour when the effects of combinations of these herbal extracts were compared with theoretical additive effects; this was the case when effects were increasing and declining (Figures 6.2 and 6.3) and suggests that the mechanisms of free radical scavenging for *C. rotundifolia*(L) and *Z. chalybeum*(B) are independent. Effects produced by the herbal extracts are consistent with their individual dose response curves and it would appear that there was no restriction or interference between the active ingredient(s) of each.

6.3.2.2 *C. rotundifolia*(L) and *Z. chalybeum*(L)

Generally, when the proportion of *C. rotundifolia*(L) is greater than or equal to 1:1, it

appears that there is interaction between the two herbal extracts, resulting in combination behaviour which is greater than additive.

Considering the proportions in which the herbal extracts were combined, 1:1 appears to be the critical proportion at which an interaction becomes manifest.

The existence of additive behaviour at proportions less than 1:1 suggests that the herbal extracts scavenge free radicals via independent mechanisms until the point at which *C. rotundifolia*(L) is present in sufficient quantities to initiate an interaction.

6.3.2.3 *Z. chalybeum*(B) and *Z. chalybeum*(L)

Overall, the addition of *Z. chalybeum*(B) to *Z. chalybeum* in any proportion consistently resulted in combinations which produced less than additive effects. There are two possible reasons for this.

1. The two herbal extracts may exert their scavenging effects by the same mechanism, binding to a similar site on the free radical; in this case, the herbal extracts compete for the binding site and the active ingredient with the more compatible structure is preferentially bound. In such a scenario, the less compatible herbal extract will produce an inferior effect in comparison to when it is applied alone.
2. The two herbal extracts may or may not act on the same receptor but nevertheless interact or influence one another in a way which disrupts the scavenging activity of one or both of them (antagonism). Given the multitude of components present in herbal extracts, it is possible that such interference may be between components other than the active ingredients.

Chapter 7 – General discussion and further work

7.1 General discussion

Only in one case were effects manifest at a dose which was less than 1 µg/ml (the free radical scavenging effects of *C. rotundifolia*(L) became apparent at 0.1 µg/ml) . Herbal extracts contain a myriad of chemical components and, unlike the case of pharmaceuticals based on single chemical entities, there may be more than one active ingredient present; extending such rationale, the effects of a given herbal drug may actually represent the resultant effects of interactions between the components and said interaction may only manifest at a particular overall dose.

With the exception of the anti-proliferative effects of *Z. chalybeum*(B), all effective herbal extracts followed the trend of attaining a maximum effect prior to a steady decline in effects when the dose was further increased. It has been proposed that this is due to displacement of active ingredients from receptors and is probably related to the likelihood of physical interaction (due, in turn, to the abundance of chemical components in any herbal extract). Previous work on wound healing herbal medicines also evidenced this decline in post maximal effect but a reason was not proposed.^{27,48}

An indication was given as to the different dynamics of herbal extracts as they are introduced into differing wound environments. In the case of *C. rotundifolia*(L) and *C. abbreviata*(B), applied in basal media, the aforementioned decline in post maximal effects was more pronounced in the case of the latter herb; application of extracts in basal media / 2% FBS produced the opposite result.

Although a causal relationship may be claimed between the application of *C. rotundifolia*(L), *C. abbreviata*(B) and *Z. chalybeum*(B) and altered production of PICP and KGF, it is important to establish whether effects are intrinsic or growth related; referencing apparent effects to growth effects removes the possibility of resources being designated to further work based on an ambiguous or false conclusion.

Regarding anti-oxidant properties, in NHDF, the hydroxyl radical is believed to exert cytotoxic effects by oxidation of proteins and DNA.^{78.79}

The herbal extracts, either singly or in combination, when incubated with cells prior to exposure to oxidants, were unable to afford protection to NHDF; this may reflect either an inability to interact in any way with sensitive biomolecules and / or organelles, or more fundamentally, herbal extracts may be unable to prevent oxidants from permeating the cell membrane.

From a bio-prospecting perspective, the philosophy behind the general approach of this study is emphatically targeted screening, whereby herbal medicines are examined in the context of a specific purported effect in man.

Strictly speaking, the cell culture-based screening of herbal medicines for the possible development of new drugs is an expedient, rudimentary procedure and would not include herbal combinations; indeed, none of the herbal medicines discussed in the current work are used in combination

This work has investigated combination behaviour (as compared to a theoretical additive model) for the following reasons:

1. For a given wound healing effect, either in the event of one effective and one inert drug or two inert drugs, their combination presents the potential to create an entirely new pharmacological species
2. Combinations consolidate the study of effective single herbal extracts as a means of assessing advantages due to combination and gaining an insight into mechanism.

Only one published study exists pertaining to the investigation of interactions between herbal medicines; although, the study did not involve wound healing, it is felt that a brief discussion of methodology is of merit. The effects on cultured PC 3 human prostate cancer cells of PC SPES (an eight-component herbal product marketed for the treatment of prostate cancer) was investigated; the manufacturers of PC SPES claimed that synergy existed between the herbal components. The MTT assay was used to establish the relative cytotoxicity of added herbs and paired combinations, expressed in the form of linear dose response curves.

The most cytotoxic extract, *Panax notoginseng*, was deemed antagonistic to the other seven herbal extracts, although no attempt was made to distinguish between antagonism and sub-additivity. The authors concluded that the methodology for the assessment of pharmaceutical combinations may be applied to herbal combinations.

From a purely pharmacological perspective, the use of linear dose response curves to determine interactions is not inferior to the method employed in this work; the maximum effect and hence optimal relative herbal extract doses may be determined in either case. However, it is felt that greater insight was gained from a consideration of all the data, in terms of trends and the extent to which increments altered additive and combination behaviour.

In the case of herbal extract combinations which affected growth and antioxidant properties, there were instances when either the combination curve was ascending as additive effects declined or vice versa; such instances give a greater indication of the extent to which combination and additive behaviour diverge, a perspective which would be lost were only the linear portions of the dose response curves considered.

When effects are sub-maximal, superior effects of combinations can be explained in terms of an interaction producing a greater than additive effect; however, super-additivity in the case of declining effects is less easily accounted for. Clearly, the combination of the herbal extracts reduced the extent to which post maximal effects decline.

Two reasons are proposed for this:

1. There may be components in the combined herbal extract inhibitory to the action of species which displace agonists from receptors.
2. The herbal extracts may contribute components which ultimately combine to comprise a completely new active ingredient which acts on a different receptor.

Without a detailed analysis of receptors, and indeed, knowledge of the nature of the composite components of the herbal extracts, it is generally impossible to offer more than speculation when discussing the nature of receptor-agonist dynamics. However, to some degree the additive model is able to provide the basis for emphatic determinations. For example, in the case of the scavenging effects of *C. rotundifolia*(L) / *X. chalybeum*(B) combinations, the observation that effects were not statistically different from the additive model was strongly suggestive of the extracts exerting their scavenging effects by independent means.

Interestingly, in cases where the behaviour of combinations was both super-additive and less than additive, super-additivity was manifest when the more potent individual extract was present in a proportion of at least 1:1; thus, in these cases, the proportion of the more potent herbal drug determined the extent of super-additivity.

In the case of *C. rotundifolia*(L) and *C. abbreviata*(B), the presence of 2% FBS completely altered the relative behaviour of dose response profiles of herbal interaction. When extracts were applied in basal media only, effects of combinations were less than additive when *C. abbreviata*(B) was present in proportions less than 1:1; on the other hand, combinations applied in supplemented media were super-additive at all *C. abbreviata*(B) proportions. The implication is that 2% FBS (a realistic representation of the chronic wound milieu) would be a favourable environment in which to introduce combinations of these herbs.

Generally, the situation whereby two non-effective herbal extracts are combined to produce effects was not evident in any of the investigations; it can only be concluded that there were no physical or chemical interactions which produced a required agonist or antagonist to the corresponding receptor.

The study of herbal extract combinations is fairly straightforward on a cell culture basis, due to the fact that cells are phenotypically identical and large quantities may be used with good reproducibility. Such an undertaking would present a serious challenge if attempted in human studies. Sample numbers would be difficult to reproduce and the construction of additive curves would present problems due to the fact that there is greater variation between two human subjects than there is between two identical fibroblasts. The behaviour of one herbal extract in a group of subjects may differ significantly from those of another; hence, the summation of the effects of two different drugs may not have the same pharmacological or statistical validity as

for the case of different groups of cells.

For these reasons, it may be the case that cell culture based studies of drug combinations are the only practical indication of the efficacy of drug combinations.

7.2 Further work

Each herbal extract exhibited at least one effect on the wound healing parameters considered and the results presented in this work are a platform for the further study of all the herbal extracts.

Although NHDF have been shown to be crucial to the onset of wound healing disorders, it would be remiss not to turn attention to the other main reparative cells, namely endothelial cells and keratinocytes.

Insight would be gained from the application of the extracts to more complex dermal and epidermal models, used for the assessment of drug effects on simulated wounds; notable examples are dermal equivalents such as collagen gels to determine the effects of drugs on fibroblast contraction and keratinocyte monolayers for the simulation of wounded epidermis and assessment of the extent of re-epidermalisation.

To a lesser extent, the use of whole skin organ culture has been used for the investigation of the influence of drugs on several reparative cells simultaneously. knowledge of toxicity. This may be extended in human studies by the analysis of tissue samples (biopsies) and the altered growth of their constituent cells due to the action of a particular drug; such samples may be taken on a regular basis and hence provide a time course for particular *in vivo* cellular effects.

The study of wound contraction may also be extended to human studies; the reduction

of wound area is a common means of gauging the efficacy of drugs and has been used in the clinical study of *Chromolaena odorata*.²⁷

The study of herbal extracts in animals is not felt to be appropriate, as the very nature of such study involves the infliction of wounds upon subjects.

Overall, with regard to a suitable paradigm for the comprehensive assessment of the herbal extracts, a progression of experimental complexity is required; this would be represented by cultured cell monolayers, three dimensional skin representations, measurement of biopsied cells and tissues to application in living human systems.

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Appendix 1 – Toxicity data

Appendix 1.1 Visual representation of toxicity for proliferation experiments

In these cases, toxicity is the minimum effect level deemed to be representative of viable, non-proliferating cells. Proliferative effects due to herbal extracts have been expressed as percentage absorbance increases or decreases above or below untreated, growing cells; the absorbance of viable, non-growing cells may also be expressed in these terms and when plotted on dose response axes, give a horizontal line below which effect levels are representative of non-viable cells.

This is exemplified in Figures A1.1, which shows the eventual toxicities in basal media for non-effective herbal extracts *Z. chalybeum*(B) and *Z. chalybeum*(L) and A1.2, which shows data for the same extracts in basal media/2% FBS.

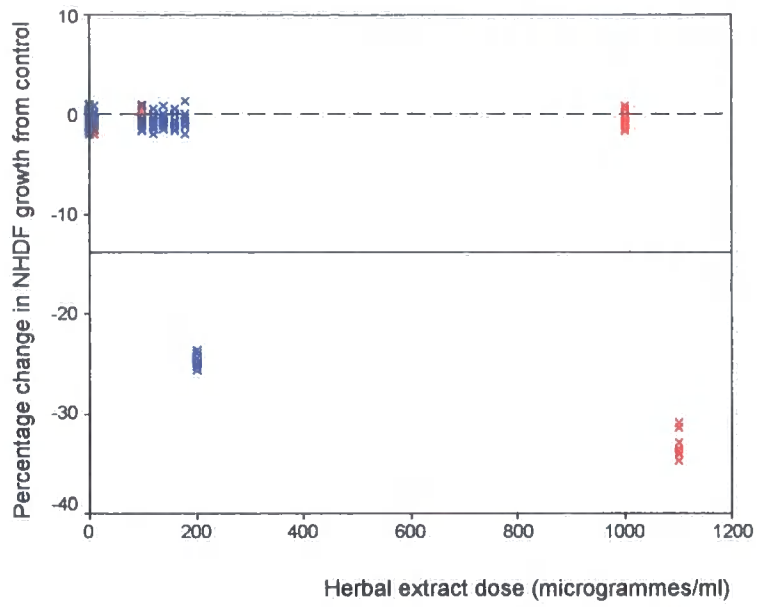
Appendix 1.2 Visual distinction between growth inhibition and toxicity

In addition to determining limiting cytotoxicity for proliferative herbal extracts, the principles described in Appendix 1.1 may be used to gauge the transition between growth inhibition and cell death / damage.

Figure A1.3 shows the eventual toxicity due to herbal extracts *C. rotundifolia*(L), *C. abbreviata*(B) and *Z. chalybeum*(L) when these non-effective herbs were added to basal media /10% FBS + IGF-1.

Figure A1.4 illustrates how the line of toxicity acts as a partition between areas of reduced growth and reduced cell viability (toxicity).

a/



b/

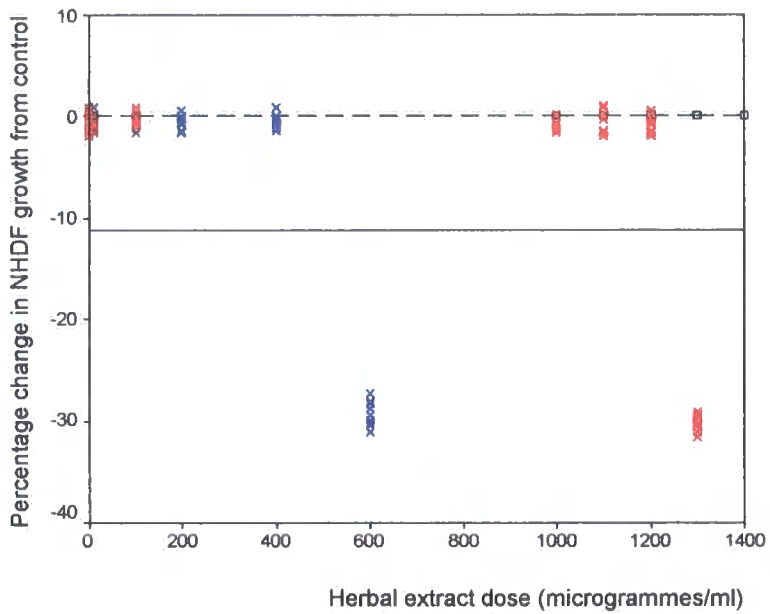
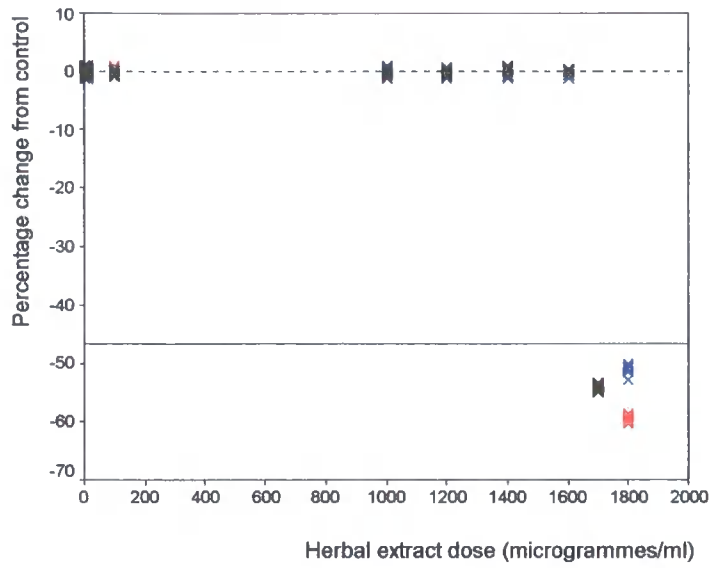


Figure A1.1. Eventual toxicity of non-effective herbal extracts *Z. chalybeum*(B) (blue crosses) and *Z. chalybeum*(L) (red crosses) in proliferation experiments

a/ Herbal extracts applied in basal media

b/ Herbal extracts applied in basal media / 2 % FBS

a/



b/

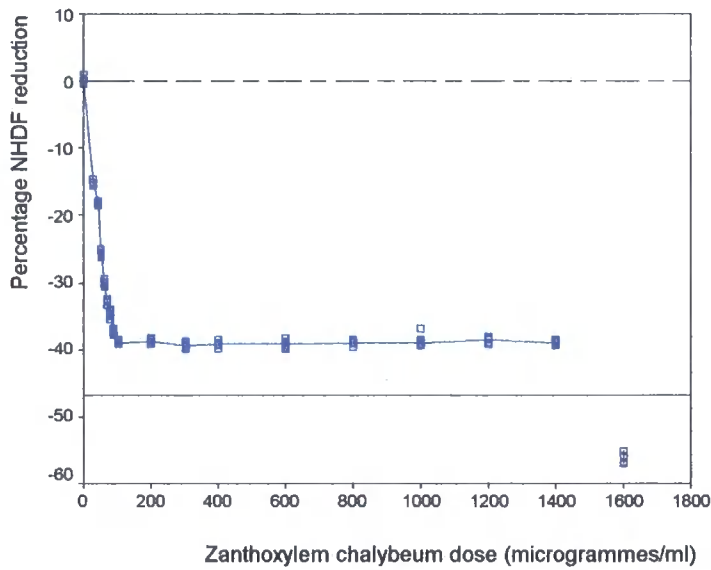


Figure A1.2. Eventual toxicities of herbal extracts when applied in basal media / 10% FBS + 70µg/ml IGF-1.

a/ Non effective herbal extracts *C. rotundifolia*(L) (black crosses), *C. abbreviata*(B) (blue crosses) and *Z. chalybeum*(L) (red crosses)

b/ Anti-proliferative herbal extract *Z. chalybeum*(B)

Appendix 1.3 Distinction between oxidant cytotoxicity and herbal extract-induced toxicity

This distinction is more straightforward than for the previous cases. For all experiments using oxidant solutions, effects due to herbal extracts were defined as the percentage absorbance above that of oxidant-treated cells. The zero line on the horizontal axis thus represents the toxicity due to oxidant alone; effect levels below this represent the additional toxicity due to herbal extracts.

This is illustrated in Figure A1.3, which shows the eventual toxicity of the non-effective scavenging herbal extract *C. abbreviata*(B).

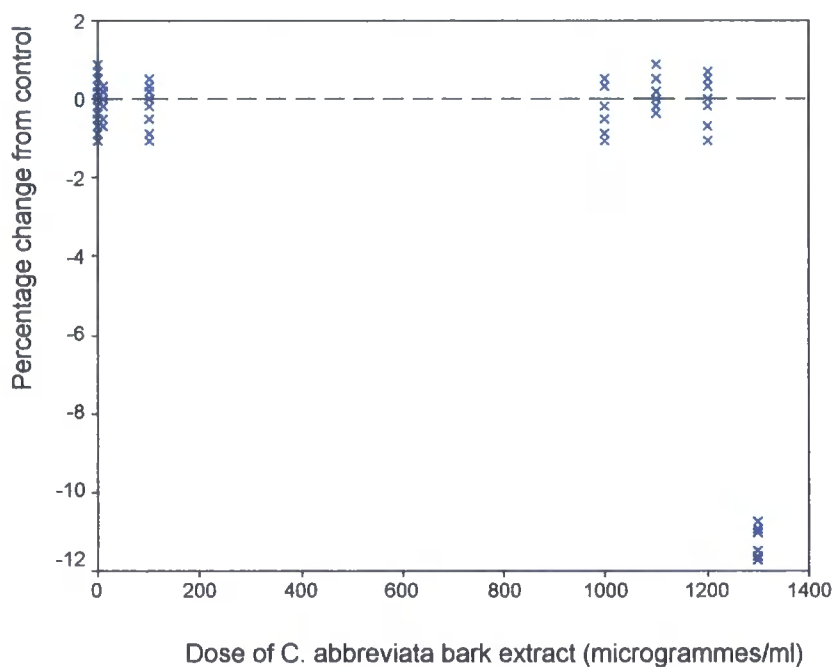


Figure A1.3. Eventual toxicity due to herbal extract *C. abbreviata*(B) when applied in oxidant scavenging experiments

