EVALUATION OF MEDICINAL PLANTS USED IN THE TREATMENT OF PSORIASIS

A Thesis Presented By

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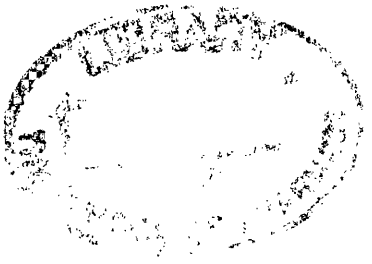

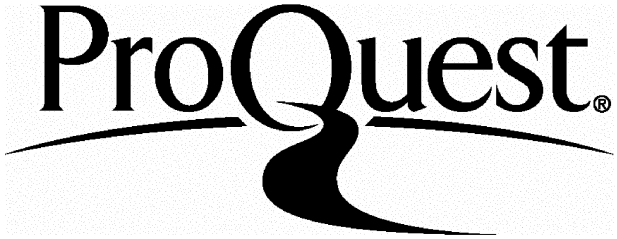

ABSTRACT

Glycyrrhiza glabra, *Galium aparine*, *Trifolium pratense* and *Bellis perennis* have been used traditionally in the treatment of inflammatory skin diseases including *Psoriasis vulgaris*. To verify these past claims the plants were extracted with a range of solvents increasing in polarity and the extracts screened using the anti-inflammatory model, inhibition of chemically induced erythema on the mouse ear (CD1).

Although activity was observed for some extracts of all herbs the ethyl acetate fraction of *G.glabra* and the chloroform fraction of *G.aparine* were most active against chemically induced erythema, with an IC50 of 10 and 5 μg/ear respectively. Inhibition of chemically induced erythema was further employed as a technique for bioactivity guided fractionation of the two extracts, resulting in the isolation of three chalcones, isoliquiritigenin, licochalcone B and echinatin from *G.glabra* and four carotenoids, lutein, anhydrolutein I, 2',3'-anhydroluteion II and 3-hydoxy-3'-methoxy-β-ε-carotene from *G.aparine*.

Isolation of the compounds was achieved through column and preparative thin layer chromatography and identification achieved by spectroscopic techniques including UV analysis, mass spectrometry and one and two dimensional NMR.

The isolated compounds were further examined in a range of biological assay to assess their potential for anti-psoriatic activity and the possible modes of action. The retrochalcone, echinatin, was most active in the inhibition of both normal and TPA induced Swiss 3T3 cell proliferation, at 1μg an 82% and 42% inhibition was exhibited respectively. The retrochalcones, echinatin and licochalcone B also proved most active against chemically induced mouse ear erythema (IC50 1-5μg). Licochalcone B and the carotenoid, lutein, were most active against physically (UV) induced erythema on the dorsal skin of Wistar rats and were able to inhibit the epidermal proliferation by 81% and 77% respectively. Lutein also exhibited most

activity in the inhibition of blood platelet aggregation; at 50μg a 13% and 26% inhibition against TPA and ADP induced aggregation was shown. The chalcones from *G.glabra* showed little activity against blood platelet aggregation.

The results provide some scientific evidence to support the traditional use of the four herbs to treat psoriasis.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
c-AMP	Cyclic adenosine monophosphate
B.	Bellis
br	Broad
^{13}C	Carbon thirteen
Ca^{2+}	Calcium ion
$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
COESY	Two-dimensional ^1H-^1H correlation spectroscopy
^{13}C-NMR	Carbon nuclear magnetic resonance
d	Doublet
dd	Double doublet
ddd	Double double doublet
2D	Two dimensional
D_20	Deuterium oxide
DEPT	Distortionless Enhancement by Polarisation Transfer
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribose nucleic acid
EGF	Epidermal Growth Factor
EtOAc	Ethyl Acetate
EtOH	Ethanol
FABMS	Fast atom bombardment mass spectrometry
FBS	Foetal bovine serum
G.aparine	Galium *aparine*
G.glabra	Glycyrrhiza *glabra*
^1H	Proton
^1H-NMR	Proton nuclear magnetic resonance
HIFCS	Heat Inactivated Foetal Calf Serum
HMQC	Heteronuclear Multiple Quantum Coherence

HPLC	High performance liquid chromatography
H^+	Hydrogen ion
Hz	Hertz
IC_{50}	Inhibition Concentration Fifty
ID_{50}	Dose inducing irritancy in 50% of the test animals
IL	Interleukin
IR	Infrared
J	Coupling constant
l	Litre
μM	MicroMolar
μCi	Microcurie
m	Multiplet
mM	Millimolar
M	Molar
MeOH	Methanol
mg	Milligram
ml	Millilitre
m/z	Mass of the ion in daltons divided by its charge
MHz	Mega Hertz
M^+	Molecular ion
MNOBA	3-Nitrobenzyl alcohol
MS	Mass Spectrometry
nm	Nanometre
NMR	Nuclear magnetic resonance
nM	NanoMolar
NOESY	Two dimensional Nuclear Overhauser Effect Spectroscopy
NaOH	Sodium hydroxide
PKC	Protein kinase C
ppm	Parts per million
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PBS	Phosphate Buffered Solution

PMN	Polymorphonulcear Leukocytes
PTLC	Preparative Thin Layer Chromatography
RNA	Ribonucleic acid
RP-HPLC	Reversed phase high performance liquid chromatography
SD	Standard deviation
s	Singlet
SDS	Sodium dodecyl sulphate
SEM	Standard Error Medium
sh	Shoulder
sp	Species
t	triplet
T.	Trifolium
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TPA	12-O-Tetradecanylphorbol-13-acetate
TMS	Tetramethylsilane
UV	Ultraviolet

CHAPTER ONE

INTRODUCTION

1.1. Psoriasis

Psoriasis, although not a life threatening disease, is a chronic relapsing and remitting skin disease that can be socially debilitating and capable of ruining an individual's life.

The disease, which is a common and complex disorder, is most prevalent among Caucasians affecting about 1-3% of this population and up to 7% of the Japanese population (Mackier, 1997). Clinically, it is identified as a chronic scaly skin disorder which has varying morphological forms, ranging from a finger nail pit, small blemishes on the skin to the total disfigurement of the body when the disease is associated with arthritis. The most common form is *Psoriasis vulgaris*, which is characterised by sharply defined, thickened, scaly and erythematous plaques (Ziboh, 1988). The clinical lesions of psoriasis have been established as the end result of hyperproliferation and the abnormal differentiation in the epidermal layers (Camisa, 1994). The word psoriasis is Greek and means 'the state of having the itch', however texts often say psoriasis does not itch (Livingstone, 1997).

'Psoriasis' may be considered as a single disease that has many morphological variants. The severity of the disease depends on several factors, perhaps the most significant being genetic. People who suffer from psoriasis are often divided into two subgroups, those who develop the disease in their first or second decade having a strong family history with a higher frequency of human leukocyte antigen (HLA) and those who do not develop the disease until their fourth and fifth decade where it is thought not to be genetically inherited. The disease may be influenced by climate or trauma referred to as the Koebner phenomena. This is the tendency of psoriatic lesions to develop at sites of skin trauma, such as mechanical friction, sunburn or lesions of childhood illness e.g. chicken pox. Other diseases, especially

streptococcal infections of the throat are well recognised as a precipitating factor to guttate psoriasis; the immunological status of the host may also contribute to the disease (Camisa, 1994).

Within the USA approximately 150,000-200,000 new cases of psoriasis are reported each year. It is suggested that this could be a significant underestimate as those with mild cases rarely seek medical help and secondly incident figures are derived from hospitals and not family doctors, where most would be treated (Sander *et al.*, 1993).

At present there are no cures for psoriasis and treatments are aimed at controlling the chronic nature of the disease.

1.1.1. Morphology

Psoriasis is characterised by an apparent disruption in a number of regulatory functions (Klem, 1978). The length of the cell cycle and the cell turnover rate are considerably shortened in the epidermis of psoriatic skin. The epidermal cells also fail to differentiate normally and are histologically characterised by para- and hyperkeratosis (Ziboh, 1988) and there is an absence of the granular layer.

The clinical morphology of psoriasis is often summarised by a number of different symptoms; probably the most noticeable are the 'scales', a result of hyper- and parakeratosis. Increased epidermal cell turnover and the abnormal differentiation causes a failure of the granular layer phase of keratinization, resulting in the inflexible horny layer sticking together. Through additional defects in desquamation the individual sheds large squames or 'scales'. Parakeratosis is the production of stratum corneum in which the keratinized cells retain the nuclei. In humans this an abnormal occurrence (Wrench, 1985). Increased epidermal thickness caused by acanthosis ('psoriasiform hyperplasia') and redness of the skin, a result of dilated capillaries and lymphocytes in the dermis, are familiar symptoms. Finally pustules develop as a consequence of the accumulation of lymphocytes in the epidermal layer (figure 1.1.1.).

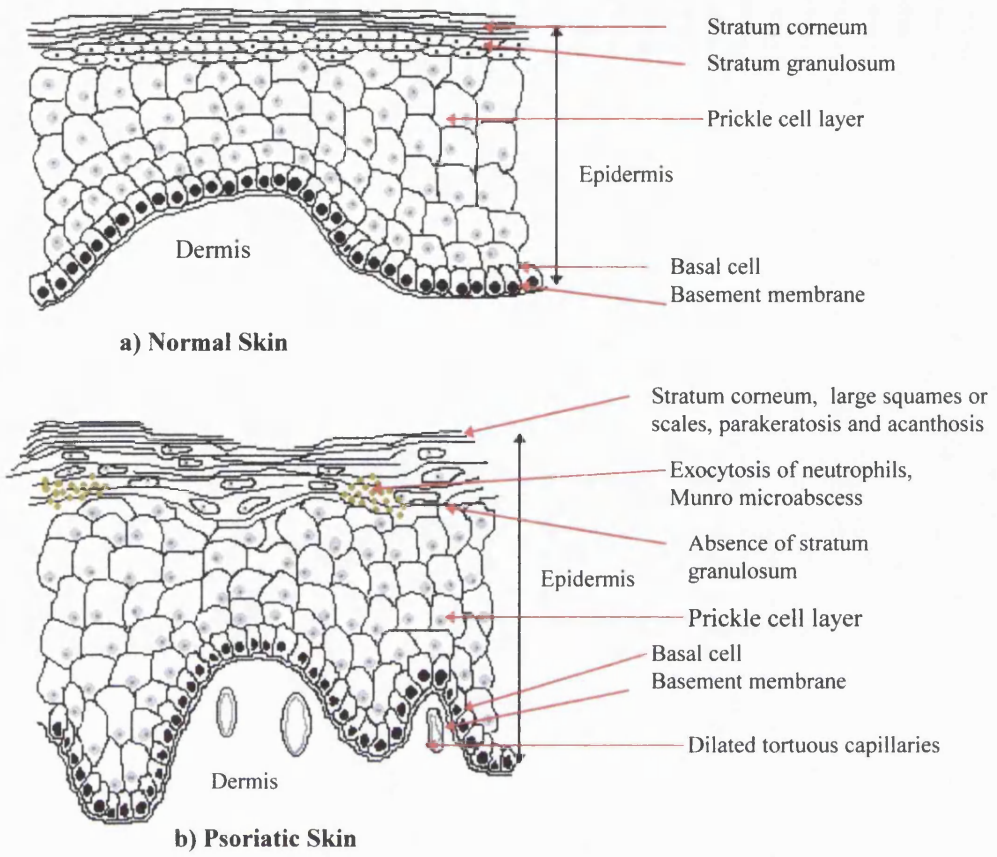


Figure 1.1.1. Comparison of the epidermal structure in normal and psoriatic skin

There are several clinically recognised types of psoriasis They include plaque, guttate, seborrhoeic, erythrodermic and pustular psoriasis. Plaque psoriasis is the most varied and is commonly known as *Psoriasis vulgaris* being characterised by red, round to oval plaques which are surmounted by white silvery scales overlying bone premises (Camisa, 1994). This type of psoriasis involves mainly the knees, elbows, scalp, hands and sacral areas (figure, 1.1.2) but may affect small areas of the skin to almost the whole body surface. The rubbing of lesions will remove the scales to reveal pin-point bleeding from the dilated superficial capillaries; a clinical sign related to the histopathology of the disease.

Guttate psoriasis or 'raindrop' psoriasis, as the name suggests, is characterised by multiple small psoriatic lesions predominantly on the trunk and face of the individual

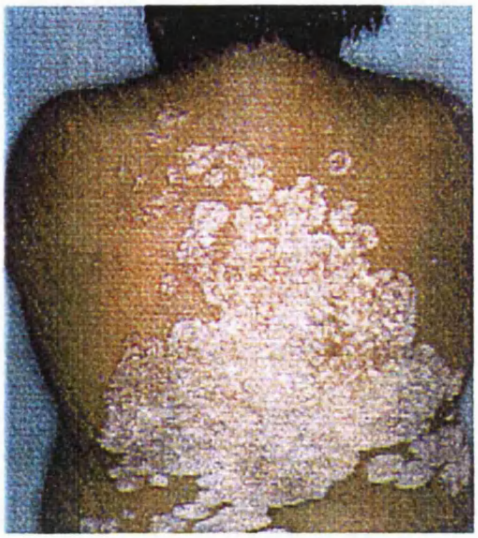


Figure 1.1.2. Photograph of Plaque Psoriasis (*Psoriasis vulgaris)* (Camisa, 1995)

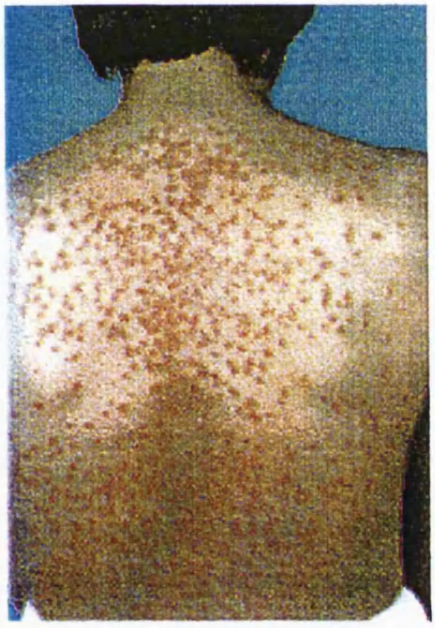


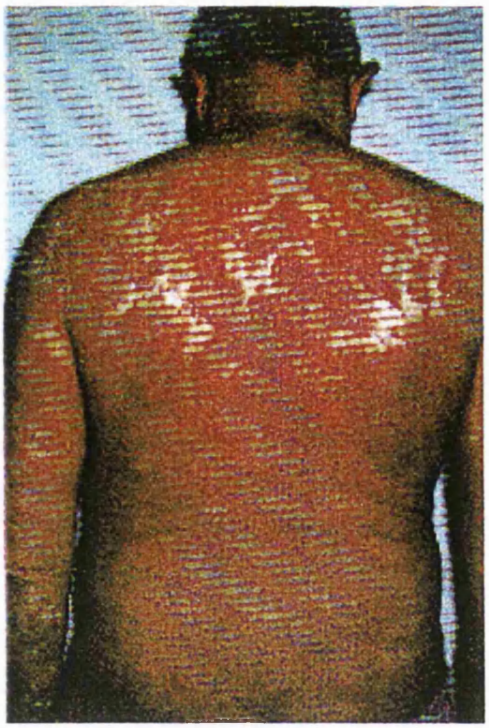
Figure 1.1.3. Photograph of Guttate Psoriasis (Raindrop Psoriasis) (Camisa, 1995)

(figure, 1.1.3). Guttate psoriasis may either be an initial manifestation or an acute flare of a pre-existing condition and tends to be more common in children than adults. Often the individual has a history of respiratory infection, tonsillitis and a streptococcal infection. The streptococcal infection may activate an alternative complement pathway and cross-reactivity between the streptococcal M protein surface antigens and the human epidermis. It is quite common for young individuals to have only one episode of guttate psoriasis (Mackier, 1997).

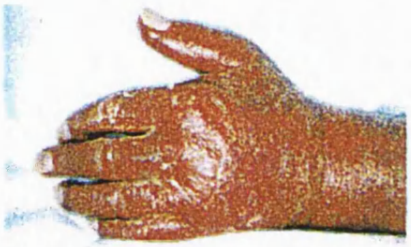
Erythrodermis psoriasis is the least common form and the initial manifestation usually occurs between 48 and 55 years developing during the course of chronic psoriasis (figure, 1.1.4). Erythrodermic psoriasis covers the entire body surface as an extensive erythema which has very few of the classical scaling psoriatic lesions. As the disease develops the distinct outlines of the plaque fade and the body is covered by a bright erythema and a general exfoliation. There is an increased blood flow throughout the body, which consequently leads to a loss of thermoregulation. Precipitating factors are usually alcoholism or stress (Camisa, 1994).

The most serious type of psoriasis is pustular psoriasis, which can require hospitalisation at times. Pustular psoriasis refers to the occurrence of spongiform pustules composed of leukocytes, which may either be localised or generalised (figure, 1.1.5). An example of a localised form is palmoplantar pustulosis, whereby chronic reoccurring eruptions consisting of yellowish pustules occur on the background of red and scaling lesions. A more generalised form often associated with general pustular psoriasis and is onset by the receiving or withdrawal of systemic steroids is referred to as "von Zumbusch", which is serious and life threatening. It consists of fiery red irregular patches with rounded brown borders surmounted by superficial pustules. The pustules can coalesce to form a lake of pus and it is this fluid that can cause an electrolyte imbalance and consequently high output cardiac decompensation. (Mackier, 1997).

Seborrhoeic psoriasis, which is a descriptive term of psoriatic lesions occurring on the scalp and less typically erythematous lesions may also occur in the body folds. It is very similar to seborrohoeic dermatitis, hence diagnosis can be difficult.

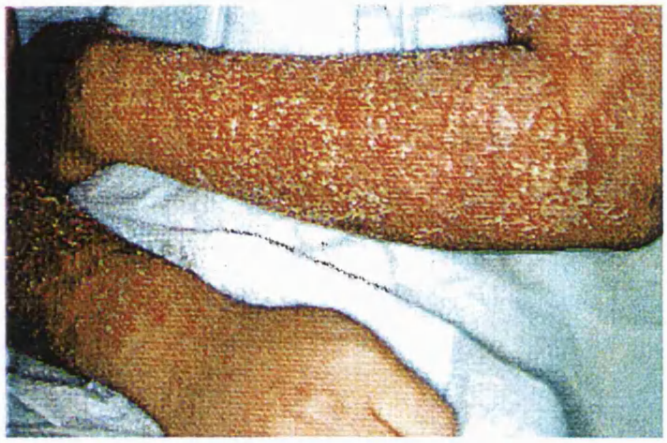


A.



B.

Figure 1.1.4. A. and B.
Photographs of Erythrodermis Psoriasis
(Mackier, 1997)



A.

B.

Figure 1.1.5. A. and B.
Photograph of Pustular Psoriasis
(Mackier, 1997)

1.1.2. Pathology

Cellular

Psoriasis is often described as the hyperproliferation of the epidermis associated with inflammation and vascular changes. The number of basal cells is vastly increased leading to an epidermal turnover rate which is reduced from 27 days to 4 days. An eight fold increase in the germinative cell cycle is experienced with the growth factor increasing from 60% to 100% (Camisa, 1994) resulting in a shortened migration time for the basal cells to reach the surface of the epidermis.

Fibroblasts under normal conditions are the most numerous cells in connective tissue and are responsible for the formation of collagenous and elastic fibres and amorphous ground tissue substances. When fibroblasts have been explanted from both the involved and uninvolved skin of a psoriatic patient they are in a state of persistent hyperproliferation with increased glycosaminoglycan and collagen production (Boss, 1988; Priestely, 1983). Fibrosis, the thickening and scarring of fibrous connective tissue, is not apparent in the psoriatic skin.

In contrast to proliferation, the keratinocytes suffer from an abnormal differentiation. The granular layer is reduced or absent, and hyperkeratosis (excessive production of keratinocytes, above the normal) or parakeratosis (the production of stratum corneum in which the keratinized cells retain nuclei) develops (Wrench, 1985). There is a 12-fold increase in the basal and suprabasal keratinocytes in cell cycling, and clinically apparent is the thickening and scaling of the skin. In the uninvolved skin there is an increase of 2.5 fold in the proliferating keratinocytes (Boss, 1988).

Endothelial cells are those which line the blood vessels and lymphatic vessels. The most superficial endothelial cells of psoriatic skin develop abnormally. For example they have an increased permeability, are dilated and tortuous and show enhanced proliferation (Ascheim, & Farber, 1996). Angiogenic factors (e.g. interleukin 1) have been found in the keratinocytes and blood mononuclear cells of psoriatic patients.

Polymorphonuclear leukocytes (PMNL's) are often associated with developing localised/generalised pustulation, which has evolved from the plaque disease. The pustules are a visual collection of pus beneath the epidermis (Camisa, 1994). It is suggested that the PMNL's of psoriatics are more sensitive to usual chemoattractants and may be passively trapped or produced in the psoriatic epidermis. T-lymphocytes have been identified as a major infiltrating cell type in the psoriatic lesions as identified by the use of monoclonal antibodies.

Dendritic cells accumulate in the dermis of psoriatic patients at a higher percentage than those suffering from other dermatoses such as pityriasis rosea, nummular dermatitis and lichen planus. Dendritic cells have the capacity to function as accessory or antigen presenting cells as part of an immune response.

The main characteristics of psoriasis remain as the epidermal hyperproliferation and dyskeratinization, due to the eight fold increase in basal and suprabasal keratinocytes in the cell cycle. There is a shortened migration time for the basal cells to the surface of the epidermis, which means a disappearance in the granular layer and an absence in the maturation and keratinization of the keratinocytes; consequently abnormal differentiation results.

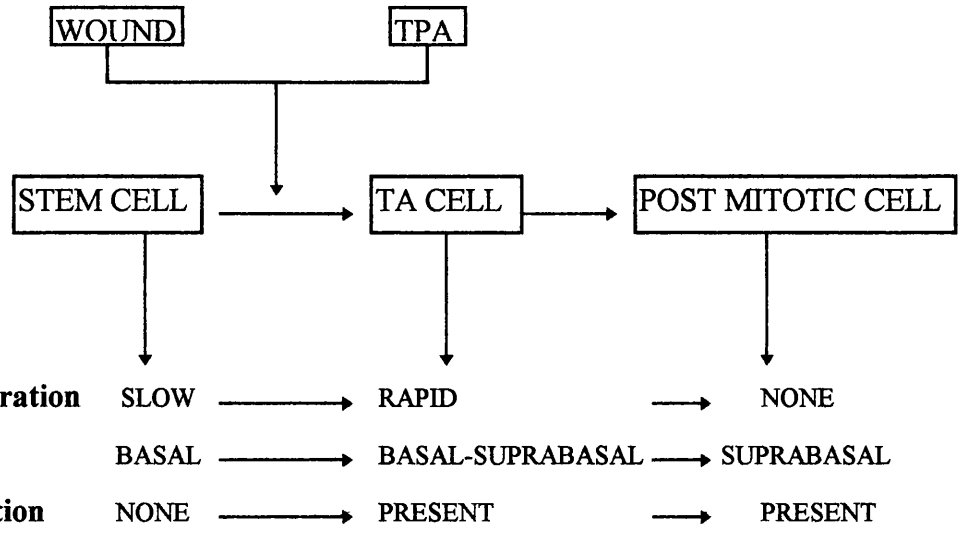
The stem-cell transient amplifying concept was originally defined by Potten (1986) and by Lavker and Sun (1983) (figure 1.1.6), according to this view there are two types of cell that proliferate in the epidermis (Ilzuka and Takahashi, 1993). One is the stem cell which has a low mitotic activity, slow cycling and little differentiation; on division it produces a transient amplifying cell (TA cell) and the daughter stem cell. The TA cell has a high proliferation activity to amplify the number of keratinocytes. Normally the division of one stem cell produces one stem cell and eight TA cells. The stem cell transient amplifying concept thus explains the clustering of mitoses of the epidermis. In a normal epidermis there is a low stem cell to TA cells transition. This depends on the stem cells having a long cell cycle time, because the number of stem cells exceed that of sporadically clustering TA cells. When there is abnormal activity, such as psoriasis, the increase in stem cell to TA

cell transition results in the predominance of TA cells resulting in the apparent decrease in the epidermal cell cycle time. The stem cells are induced to proliferate by tissue demand or specific stimuli e.g. wound healing. Epidermal hyperproliferation is accompanied by an increased epidermal turnover, therefore the keratinocytes in a hyperproliferation condition should complete their keratinocytes process in a much shorter time than usual resulting in a deranged keratinization, a feature shared with the psoriatic epidermis. The psoriatic epidermis is comparable to wound healing, with an increased transition from stem cells to TA cells. The tumour promoter TPA also induces this transition. The process of keratinization in psoriasis or TPA induced is described as defective, incomplete or delayed (Ilzuka & Takahashi, 1993).

Figure 1.1.6.

Stem Cell Transient Amplifying Concept (after Isuka and Takahashi,1993)

Illustration of the two types of cells that proliferate in the epidermis, the stem cell and the TA cell.



Intracellular

Studies at the biochemical level in psoriatic skin have revealed a number of abnormalities in the transmembranous signal transducing system, including a reduced cAMP cascade activity, high lesional guanosine monophosphate (cGMP)

levels (Vorhees, 1973), increased calmodulin levels (Tucker, 1985), increased phospholipase C (PLC) activity, increased tyrosine kinase activity and increased epidermal growth factor (EGF). The phospholipase C (PLC) activity is greatly increased in the psoriatic skin, PLC forms diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3) from the plasma membrane and these products act as secondary messengers in cell activation and replication. Activated PLC may induce guanylate cyclase which by the formation of cGMP additionally initiates cellular responses. Antigens, growth factors, neurotransmitters, hormones and platelet activating factors can all induce inositol PLC.

Adenylate cylcase, guanylate cylcase, phospholipase C and tyrosine kinase all regulate cell proliferation and differentiation by their effects on intracellular protein phosphorylation, thus in the involved psoriatic skin they are all altered, all associated with cell proliferation, protein activation and abnormal differentiation, (Bouclier *et al.* 1990).

Intercellular

It has been demonstrated that the supernatant of the mononuclear cells has both an enhancing effect and inhibitory effect on Hela cell cultures, (Kruger and Jederberg 1981). Supernatants obtained from psoriasis patients had more of an enhancing effect suggesting that the epidermal hyperplasia in psoriasis is the result of an imbalance of mediator release. It is now understood that the psoriatic lesions are composed of mononuclear cells, predominantly T-lymphocytes, and the putative soluble mediators termed interleukins which originate from them (Bjerke *et al.* 1979) are the possible mediators of the inflammatory characteristic of the psoriatic disease. The suggested cellular mediators are eicosanoids, platelet activating factor, interleukins and interferons.

Arachidonic acid (AA), is the precursor of the family of the inflammatory and mitogenic mediators, the eicosanoids, and is found in abnormally high levels in psoriatic skin (Ziboh, 1988) (figure 1.1.7.). It is suggested that psoriatic skin might contain an endogenous inhibitor to cycloxygenase resulting in the diversion of

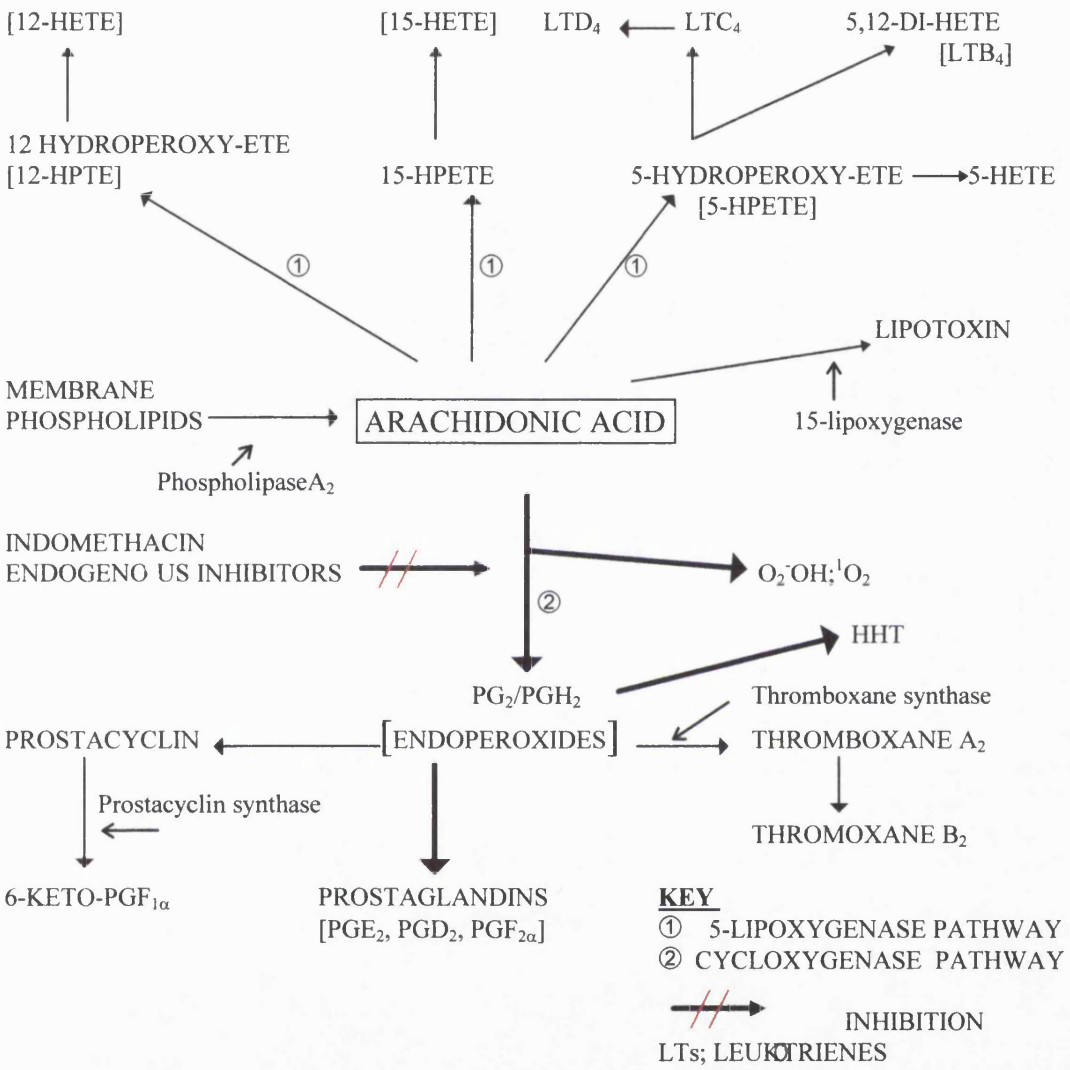
arachidonic acid to the lipoxygenase pathway and the generation of proinflammatory products 12-hydroxyeicosatetraenoic acid (12-HETE), leukotriene B_4 (LTB_4) and prostaglandins PGE_2 and PGF_2. All are found to be elevated in the psoriatic lesions. PGE_2 and PGF_2 are partly responsible for vasodilatation and keratinocyte hyperproliferation. LTB_4, a potent chemoattractant for PMNL and inducer of proliferating keratinocytes is found increased in allergic contact dermatitis and atopic dermatitis. Initially it was thought that the increased AA levels were primarily the result of increased phospholipase A_2(PLA_2) (Boss, 1988) in the psoriatic lesion, however PLA_2 is also elevated in uninvolved skin. Evidence now suggests that diacyl glycerol (DG) is an alternative source of increased AA. The secondary messenger DG produced by phospholipase C from the plasma membrane after receptor-ligand interaction, is subsequently metabolised into AA.

Platelet activating factor (PAF) is also found elevated in the psoriatic lesions and like $_{12}$-HETE and LTB_4 is a cell membrane derived product of the increased PLA_2 activity demonstrated in uninvolved and lesional psoriatic activity. It has been considered that PAF may activate phospholipase activity with the subsequent activation of a particular cell function (Boss, 1988).

Interleukin is the generic term for a group of protein factors produced by macrophages and T-cells; fifteen forms have been identified. Interleukin$_1$ (IL-$_1$) is a mediator which stimulates thymocyte proliferation, as well as the stimulation of a wide array of cell types including lymphocytes and keratinocytes. Interleukin$_1$ (IL-$_1$) is present in psoriatic plaques and scales and it is suggested that it may induce keratinocyte growth and induce lymphocyte activation in the epidermis as well as fibroblasts and endothelial cells, (Boss, 1988).

Both gamma-interferon and alpha-interferon have been found in the blister fluid of psoriatic lesions. Activated T-cells may be responsible for the production of gamma-interferon and the monocytes and macrophages the alpha-interferon. Both may stimulate the IL-$_1$ production (Boss, 1988).

Figure 1.1.7. **Metabolic Pathway Of Arachidonic Acid In Tissues And Cells (Ziboh, 1988; Williamson *et al.*, 1996)**



Psoriasis And Protein Kinase C

Protein kinase C (PKC) was first discovered in 1977 as a proteolytically activated protein kinase found in many tissues and it did not appear to have any obvious role in signal transduction (Inoue, 1977). Later PKC was shown to be a Ca^{2+} activated phospholipid-dependent enzyme, linked to signal transduction (Takai *et al.*, 1979:A; Nishizuka, 1984). PKC is an isozyme family with at least eleven mammalian

members that play important roles in cell growth regulation and differentiation (O'Brian, 1998). Independent of calmodulin, PKC can be inhibited by a large number of phospholipid interacting drugs e.g. chlorpromazine and trifluroperazine; an example of a plant derived PKC inhibitor is chelerythrine (Herber *et al.* 1990). The anti-oestrogen tamoxifen inhibits PKC, indicating a link between PKC signal transduction pathway and the steroid receptor family (Hahnel and Gschwendt, 1995).

The protein serine/threonine kinases of PKC are important components of the major signalling pathways regulating cell proliferation and differentiation. There are two sites within the cell cycle where protein kinase C is implicated, during the G1 and S progression and at G2 to M transition. PKC activation at the G1 progression modulates the specific cyclin-dependent kinases (CDKs) activity. CDKs phosphorylate the retinoblastoma susceptibility gene product (RE) which is a pivotal event in cell cycle progression leading to the G1/S transition. The inhibition or enhancement of CDK is mediated by the PKC enzyme and the phosphorylation of RE is dependent on the precise timing of PKC activation during G1 and the particular cell type (Fishman *et al.*, 1998).

PKC was originally assumed to be activated in psoriasis (Ilzuka and Takahashi 1993). However it is known that PKC activity is decreased following its activation and the decreased PKC activity in psoriasis probably represents this down regulation. Jun and Fos are immediate early genes, expressed following cell activation. In psoriasis there is a decrease in Jun/Fos expression, probably as a result of the decrease in PKC activity (Denning *et al.* 1995). Evidence suggests a heterogeneity of PKC and there are at least eight subspecies, of which their specific functions remain largely unknown. It is thought that perhaps one may trigger cell proliferation whilst another triggers differentiation. Wevers *et al.*, (1992) suggest that the PKC enzyme plays a regulatory role in epidermal proliferation and differentiation. Studies have shown a difference in the activity and distribution of PKC in psoriatic lesions and healthy skin (Wevers *et al,*1992).

PKC does appear to be associated with the pathophysiology of psoriasis. PKC activation explains the various psoriatic phenotypes including increased involucrin expression, increased keratinocyte differentiation, and neutrophilic exocytosis. However the PKC subspecies does make it difficult to interpret the PKC-dependent inflammatory processes. Inhibitors of PKC are therefore seen as an obvious way of treating inflammatory skin diseases (Wevers, *et al.* 1992).

1.1.3. Treatments of Psoriasis

The prognosis of a patient suffering from psoriasis depends on the age of the individual as well as the extent and severity of the initial outbreak, for example the younger they are the greater the severity of the disease. Permanent remissions are rare and present therapy focuses on the management of the disease because at present no cures exist. It is for this reason and due to the nature of the disease i.e. ranges of severity and the varying forms, that there are so many treatments available which correspondingly also have a wide range in effectiveness and safety (table, 1.1.3.).

1.1.4. Synthetic drugs

Corticosteroids penetrate the stratum corneum and bind to steroid receptors in the cytosol of living keratinocytes and function by altering the DNA synthesis and gene transcription. Their potency is dependent on the affinity of the receptor for the corticosteroid. Corticosteroids are most useful in treating those with limited psoriasis (<10% body surface), and are most effective when used under occlusive polyethylene covering and applied at night. The toxicity of corticosteroids when most potent includes severe pituarity-adrenal-axis suppression and Cushing's syndrome. Local side effects include cutaneous atrophy, rupturing of connective tissue and stretching of the epidermis (Camisa, 1994; BNF, 1997).

Retinoids are naturally or synthetically derived from vitamin A (retinol) and the effects are similar to those of vitamin A (retinol). They are anti-inflammatory, anti-keratinizing and anti-proliferating. Acitretin is a metabolite of etretinate and has

Treatments	Mechanism Of Action	Type of Psoriasis	Adverse Effects
Acitretin (Metabolite of etretinate)	Anti-inflammatory, anti-proliferative by decreasing levels of epidermal IL-1 & increasing polyamine synthesis	Severe or resistant psoriasis	Teratogenicity, mild pruritus, nose bleeds.
Cyclosporin	Powerful immunosuppressant, interfering with T-cell function, inhibit cytokine cascade	Severe and persistent psoriasis, requires specialist hospital care.	Nephrotoxicity, hypertension, CNS disturbances, carcinogenicity
Methotrexate	Anti-metabolite, folic acid antagonist. Inhibition of (s) phase cell cycle.	Resistant psoriasis	Bone marrow depression, liver damage, renal failure & tubular necrosis
Methoxsalen	Used with UVA (PUVA), induces cross-linking of DNA strands leading to inhibition of DNA synthesis in the epidermal basal cells	General psoriasis, first form of systemic therapy that will be used	Skin cancer, nausea, severe burning, eye damage and mental effects
Calcipotriol	Suppress proliferation of keratinocytes & induce differentiation	Mild to moderate plaque psoriasis	Local irritation of skin
Dithranol	Inhibit metabolism of cells by free oxygen radical	Mild to moderate psoriasis	Severe irritation , staining of skin and clothes
Coal Tar	Suppress epidermal DNA synthesis	Mild psoriasis	Irritation of skin, recognised carcinogen, unpleasant smell
Corticosteroids	Bind to steroid receptors in cytosol altering DNA synthesis and gene transcription	Mild to moderate psoriasis	Pituarity-adrenal-axis suppression, Cushing's syndrome, cutaneous atrophy, stretching of epidermis
UVB Radiation	Phototoxicity for inflammatory cells	Mild- moderate psoriasis, used in combination with methoxsalen & coal tar	Skin cancer

(Martindale, 1996; B.N.F, 1996; Camisa, 1994; Mackier, 1997)

Table 1.1.3. Treatments for Psoriasis

generally replaced all other retinoids as it is eight hundred times more potent than the latter. Acitretin and other retinoids are used for the treatment of severe and resistant or complicated psoriasis. Most patients suffer from dryness and cracking of the lips, other side effects include mild pruritus, paronychia and nose bleeds. It is also a known teratogen (BNF, 1997).

Methotrexate (MTX) was first used in 1958, and has become the most commonly prescribed systemic anti-metabolite. MTX is a folic acid antagonist and acts by competing for the binding sites on the intracellular enzyme dihydrofolate reductase (DHFR), converting dihydrofolate to tetrahydrofolate. The mechanism of MTX is the inhibition of the (s) phase of the cell cycle and in psoriasis a greater fraction of the cells are in the s-phase. MTX is used in the treatment of acute lymphoblastic leukaemia, meningeal leukaemia, choriocarcinoma, rheumatoid arthritis as well as resistant psoriasis. Most common side effects include bone-marrow depression, ulceration of the mouth and gastro-intestinal disturbances. Other side effects include liver damage, renal failure, tubular necrosis, skin reactions, alopecia, osteoporosis fertility impairment and teratogenicity (Martindale, 1996). MTX is an effective treatment, but potent side effects can lead to death, therefore it is only used in severe or disabling psoriasis (Mackier, 1997).

Salicylic acid can be used in the treatment of all hyperkeratotic and scaling conditions to enhance the rate of loss of surface scale . Side effects include irritation and occasionally salicylate toxicity.

1.1.5. Natural Products

A considerable number of the pharmacologically active compounds used for treating psoriasis are derived from natural sources.

Dithranol (Anthralin, USA) was first described by Balmano Squire in 1878 as being effective in the treatment of psoriasis. Dithranol, once called Goa powder, is derived from the bark of the Brazilian tree *Andira araroba* Aguiar (Fabaceae). The active compound was the hydroxanthracene 'chrysabin' which on the loss of a methyl

becomes dithranol. Dithranol inhibits cell growth and thymidine incorporation into human fibroblasts and is applied topically to the skin in either the form of an ointment or cream. Treatment schedules often involve coal tar and UV irradiation. Dithranol, however can cause severe irritation in the form of a burning sensation and, for this reason, it is usual to start with low concentrations and build up. Patients with fair skin are more sensitive than those with dark skin. It is an irritant to the eyes and mucous membranes and should avoid being used on skin flexures, genitals and face (Farber, 1992; BNF, 1997).

Cyclosporin, a cyclic polypeptide consisting of eleven amino acids, is obtained from the soil fungus *Tolypocladium inflatum* Gams. Cyclosporin A is a unique immunosuppressant because it interferes directly with T-cell function by inhibiting the cytokine cascade (interleukins-I (IL-1) and -2(IL-2) and IL-2 receptor (IL-2R)) without myelosuppresive effects. Cyclosporin may be used in association with PUVA treatment (Camisa, 1994) but only in the treatment of severe and persistent psoriasis which requires specialist care in hospital. Approximately one third of all patients suffer from nephrotoxicity; other side effects include gastro-intestinal disturbances, hepatoxicity, acne, oedema, neurotoxicity and alopecia. Like many immunosuppresants cylcosporin is associated with an increased incidence of malignancy, lymphoma and skin cancers, as well as the increased risk of infections.

Psoralens are natural compounds from *Psoralea coryfolia* L. (Fabaceae) which interact with sunlight to produce biological effects (Psoralens and high-intensity ultraviolet A = PUVA). Xanthotoxin (8-methoxypsoralen, 8-MOP) and the synthetic trioxalen (3,5,8-trimethylpsoralen, TMP) are the most commonly used and are given by mouth to sensitise the skin to the effects of irradiation. With activation by light, psoralens produce the cross-linking of DNA strands leading to the inhibition of DNA synthesis in the epidermal basal cells. Although this treatment is clean and may produce remissions for several months, short term effects include severe burning and long term hazards include cataract formation, accelerated ageing of the skin and the development of skin cancer (Livingstone, 1997; BNF, 1997).

Coal tar is a by-product of the processing of coke and gas from bituminous coal. It is a chemical complex of tens of thousands of compounds, described as aromatic hydrocarbons. Coal tar and UVB phototherapy (Goeckerman Regime) suppress the epidermal DNA synthesis; generally coal tar is suitable for most cases but is limited by the unpleasant smell. They may also cause irritation and acne-like eruptions of the skin and should not be applied to inflamed and broken skin; coal tar is also a recognised carcinogen.

Fish oil can be used by individuals with psoriasis as a dietary supplement. Fish oil contains ω-3 polyunsaturated fatty acids which in theory can substitute for AA as a substrate for the lipoxygenase and cycloxygenase enzymes. Fish oil is also a source of eicosapentaenoic acid (EPA), which helps to decrease the amount of polymorphonuclear (PMNLs) (50%) produced by LTB_4. Relatively high doses of fish oil are required to attain mild effects. It is suggested, therefore, that fish oil may be used as an adjunct therapy to retinoids or cydcosporin, mainly due to its low incidence of side effects (Camisa, 1994).

Identification of environmental factors may have important implications for the prevention and treatment of the disease. A low prevalence of the disease is experienced among Lapps and Innuits who have a high consumption of fish. A study done in Italy reported on the relationship on selected foods and psoriasis (Naldi *et al.* 1996). It was found that the risk of psoriasis increased with increasing body mass index and was inversely related to the consumption of carrots, tomatoes and fresh fruit, and to the index of beta-carotene intake.

1.2. The Medicinal Use Of Plants

The plant kingdom is full of a wealth of chemical information, which can be used to assist medicinal chemistry and act as diagnostic tools in pharmacological studies.

There are approximately 250,000 species of angiosperms on earth, hence in natural product research it is essential that selection and screening of plants is highly effective (Heywood,1993). Selection techniques for biological screening can be

described as either random or targeted. A random based technique involves the mass screening of plants which is an expensive operation. This was used by the United States National Cancer Institute (NCI) between the 1950's and 1980's, as it considered cancer to be a disease where the present range of drugs was seriously inadequate or inefficient, therefore justifying the high expense of the chosen selection technique. Targeted techniques include information based, ethnomedical and taxonomic based strategies (Cordell, 1995). The information based strategies rely on the availability of ethnomedical, biological, pharmacological and chemical data already collected. Ethnomedical strategy involves the collection of data from traditional cultures, which have a sound ethnomedical tradition, an establishment in the residing area for many generations and the presence of a diverse flora, all of these enhance the likelihood of discovering novel compounds. An ethnomedical strategy focuses on the establishment of a long-term relationship with the indigenous communities and the provision of short and long term benefits to them (Cox, 1990). The latter point at present is receiving much attention with regards to intellectual property rights (IPR). Targeted strategies include taxonomic and phytochemical studies and focuses on the plants or isolation of compounds which are close relatives or of similar groupings to those already known to possess some biological activity (Cordell, 1995). The aim of targeted strategies is to isolate compounds of greater activity.

The understanding of biological processes related to specific disease with the availability of receptors, enzymes and genetic switches has led to the development of automated high-throughput screening enabling hundreds of thousands of specimens to be screened in a few days. An effective screening procedure should be sufficiently selective to limit the number of leads for follow up evaluation but be highly sensitive enough to detect low concentrations of active compounds (Evans, 1996).

Bioassay guided fractionation is a popular technique used to isolate biologically active compounds. It involves the fractionation of extracts and consequently isolation of compounds as guided by a particular activity (e.g. anti-inflammatory etc.) displayed at each step of the fractionation process. However, it is sometimes the case that a fraction containing significant biological activity possesses no single active

constituent. The biological activity displayed is the result of synergism or antagonism due to the complex nature of the extract (Evans, 1996) and it is not uncommon that the active compound is already a well-known structure with known biological activity (Bruhn & Bohlin, 1997). Bioassay guided fractionation has been used in this thesis to isolate anti-psoriatic compounds from various medicinal plants chosen on the basis of their reported use in treating psoriasis. The plants were chosen because they were most frequently mentioned in treating psoriasis from a wide selection of past and present herbal books.

1.3. *Glycyrrhiza glabra* L. (Liquorice)

G.glabra is a perennial angiosperm which is used extensively in homoeopathic medicine and especially in Chinese medicine (figure, 1.3). Although all parts of the plant can be utilised in medicine (Werbach & Murray, 1994), it is generally the stolon which contains most of the biological activity and is consequently used. Liquorice is documented as being taken as a drug in the oldest medical recipe dating from the 3rd century BC found in the ancient tumulus, Ma-Wang-Dui, at Changsa, China (Shibata, 1994:A). *G.glabra* is not only an important commodity for the pharmaceutical industry, but is also important for the tobacco and confectionery industries (Fenwick *et al.*, 1990) and therefore one of the most widely investigated of the economically important plants.

1.3.1. Botany

G.glabra is a member of a large family the Leguminosae, consisting of 700 genera and 15,000 species. It is separated into three subfamilies, Papilionoideae, Mimosoideae and Caesalpinoideae (Heywood, 1993). The family is quite distinct by the characteristic papilionate flower which has one adaxial petal (standard), 2 lateral petals (wings) and 2 lower petals joined together by their lower margins (Clapham *et al*, 1987). One other distinctive feature is the presence of nodules, which contain bacteria (*Rhizobium* bacteria), on the roots. The Leguminosae are described as cosmopolitan in distribution with major economic uses as food crops (peas, beans,

Figure 1.3. **Photograph of *Glycyrrhiza glabra* L.**

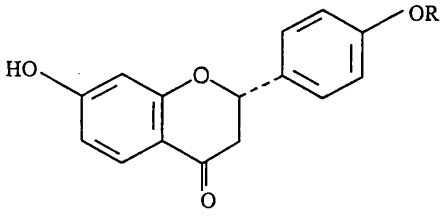
groundnut, soybean) forage crops (clover, lucerne) and other uses such as dyes and timber (Heywood, 1993).

G.glabra is a member of the Papilionoideae which are temperate, tropical and sub-tropical in distribution and are mostly herbs, trees and shrubs. *Glycyrrhiza* is the name of the genus and includes the following species *G.glabra*, *G.echinata*, *G.glandulifera*, *G.hirusta*, *G.brachycarpa*, *G.uranlesis*, *G.pallidiflora*, *G.korshinski*, *G.inflata*, and *G.lepidota* (Fenwick *et al.*, 1990). *G.glabra* is a shrub growing to a height of 11-12 metres, with leaves consisting of 9-17 ellipitic leaflets covered with sticky glandular trichomes It has bluish/violet flowers arranged in spike like clusters, flowering in June and July. *G.glabra* grows on stony places and dry woods and is often cultivated. It is native to the Mediterranean region, central Russia and Asia minor as far as Iran (Fenwick *et al.*, 1990).

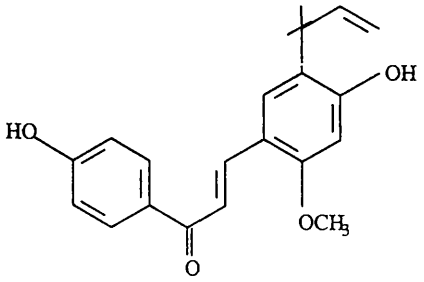
Liquorice is the drug, and consists of the dried unpeeled roots and stolons. When cut the stolons appear roughly fibrous, due to the very abundant fibres which occur in groups. Also noticeable are the cork fragments which are orange brown in colour. The stolons appear lemon-yellow in colour, have a faint but characteristic odour and are very sweet but have a mildly aromatic taste (Ody, 1993).

1.3.2. Phytochemistry

G.glabra has been extensively investigated for its constituents. A vast number of flavonoids have been identified, such as flavanols and isoflavanones including formononetin, glabrin, glabrol, glabrone, glyzarin, glycyrol, glabridin,kumatakenin, licoflavanol, licoisoflavanones A and B, licoisoflavanone, licorcone, liquiritin and phaseollinisoflavan (Newall *et al.*,1996). Chalcones present in *G.glabra* include neoliquiritin, neoisoliquiritin, isoliquritigenin, licuraside, echinatin, neolicuroside, isoliquiritigenin and licochalcones A and B (Shibata *et al.*, 1994:B). Due to the active anti-tumorigenic properties of the chalcones many other synthesised compounds have been made (Garecki *et al.*, 1991)(figure 1.3.2.)



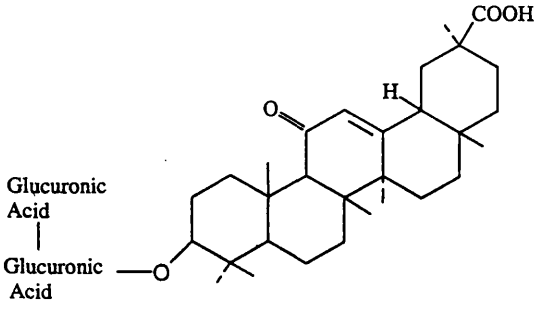
Liquiritigenin R=H
Liquiritin R=Glu = flavonoid



Licochalcone A

Figure 1.3.2. Molecular Structure of Liquiritigenin and Licochalcone A

The terpenoids of *G.glabra* are probably the best known as they include the glycoside glycyrrhizin. Glycyrrhizin accounts for 2-9% of the dried stolon and is also known as glycyrrhizinic acid yielding glycyrrhetinic (or glycyrrhetic) acid (as the aglycone) and glucuronic acid following hydrolysis (Takino et al., 1979). Other terpenoids include glycyrrhetic acid-3-0-glucuronide, glycyrrhetol, glabrolide, liquiritic acid, β-amyrin and licoric acid (Tsubone *et al.*,1982; Yamamura *et al*, 1991; Elgamal & El-Tawil, 1975; Killacky *et al.*, 1976; Takeda *et al.*, 1990). Other saponins present in *G.glabra* include glabranin A and glabranin B, which are both glycosides of glycyrrhetinic acid (Varshney *et al.*, 1983), also betulinic acid and the soyasaponins (Hayashi *et al.*, 1990) (figure 1.3.2.1.).



Glycyrrhizinic acid

Figure 1.3.2.1. Molecular Structure of Glycyrrhizinic Acid

Coumarins present in liquorice include glycyrin, liqcoumarin, umbelliferone and 3-arylcoumarin derivatives. The volatile oils make up 0.047% of which 80 components have been identified, including anethole, benzaldehyde, butryolactone, cumic alcohol,

eugenol, fenchone, furfuryl alchohol, hexanol, indole, linalool, γ-nonalactone, oestragole, propionic acid, α-terpineol and thujone (Newall *et al.*, 1996).

Other constituents of liquorice include amino acids, amines, gums, starch, sterols, (β-sitosterol and stigmasterol), sugars and waxes and those found in other regions of the plant except the stolon include kaempferol, isoquercetin, phytoalexins and apigenin, coumarins, phytoestrogens, β-sitosterol and saponeretin (Yahara and Nishioka, 1984).

1.3.3. Pharmacological and Biological Activities

The flavonoids are one of the most numerous and widespread groups of natural constituents. They are 15-carbon skeleton compounds in which one or more hydrogen atoms are replaced either by hydroxyl groups or methoxyl groups (Harborne and Mabry, 1982). The parent compounds are the chalcones which are classified as minor flavonoids and appear as a dominant class of compound within the *Glycyrrhiza* species exerting a wide range of biological activities.

Licochalcone A from *Glycyrrhiza inflata* shows strong anti-inflammatory action towards mouse oedema induced by TPA and arachidonic acid by topical application (Shibata *et al.*, 1991), acts as an anti-tumour agent against 7,12-dimethyl-benz[*a*]anthracene (DMBA) promoted by TPA and inhibits *in vitro* ^{32}P-incorporation to phospholipids in Hela cells promoted by TPA (Shibata, 1994:B). It was suggested that there maybe a competitive interaction of licochalcone A with the TPA receptors in the cell membrane. Shibata (1994:B) synthesised a further 40 compounds and found chalcone 3'and 4'-methyl-3-hydroxychalcone showed the highest potency in inhibiting tumorigenesis and also showed inhibitory effects on the proliferation of HGC-27 cells derived from human gastric cancer. Isoliquiritigenin inhibits epidermal ornithine decarboxylase (ODC) induction and ear oedema formation by TPA and inhibits DMBA initiated and TPA promoted papilloma formation in CD1 mice. Epidermal ODC induction and skin tumour promotion caused by 7-bromoethylbenz[*a*]anthracene (BrMBA), a non TPA type of tumour promoting agent, was inhibited by isoliquiritigenin as was TPA stimulated prostaglandin E_2 (PGE2)

production in intact epidermal cells. 12-Lipoxygenase, 5-lipoxygenase and phospholipase A2 activity of platelet sonicates was not inhibited, whereas the platelet 12-lipoxygenase and 5-lipoxygenase in polymorphonuclear leukocyte activity was. This led to the suggestion that isoliquiritigenin was exerting anti-tumour activity through the lipoxygenase inhibition by acting on cells other that the target epidermal cells (Yamamoto *et al.*, 1991).

Hsieh *et al.* (1998) reported that chalcones showed a strong inhibitory effect on the release of β-glucuronidase and histamine from rat peritoneal mast cells. Chalcones and 4'hydroxyflavone exhibited potent inhibitory effects on the release of β-glucuronidase and lysozymes from rat neutrophils stimulated with formyl-methionyl-leucyl-phenylalamine(fMLP). Some chalcones showed potent inhibitory effects on superoxide formation of rat neutrophils stimulated with fMLP/cytochalasin B (CB) or tetradecanoyl phorbol acetate (TPA). The 2',3-dihydroxy-, 2',5'-dihydroxy-4-chloro, and 2',5'-dihydroxychalcone showed inhibitory activity effects on hind-paw oedema induced by polymyxin B in normal as well as in adrenalectomized mice (Hsieh *et al.*, 1998). The conclusion from this study was that the anti-inflammatory effects of the chalcone compounds were mediated through the suppression of chemicals released from mast cells and neutrophils.

The chalcones and flavanones from *G.inflata* were investigated for effects on human polymorphonuclear neutrophils (PMNs). Licochalcones A and B at concentrations between 10^{-6}M to 10^{-3}M inhibited calcium ionophore A23187, leukotriene B4 (LTB4), cytochalasin B(cyto B), platelet activating factor (PAF), cyto B- and N-formyl-methionyl-leucyl-phenylalamine (fMLP) plus cyto-B-induced enzyme release from human PMNs (Kimura *et al.*, 1993:A). Isoliquiritigenin, isoliquiritin, liquiritigenin and liquiritin had no effect on PMN degranulation induced by various stimuli. Licochalcones A and B at concentrations of 10^{-4}M to 10^{-3}M increased cyclic AMP in human PMNs. At similar concentrations they reduced the elevation of cytosolic free calcium concentration induced by calcium ionophore ionomycine, fMLP, PAF and LTB_4 (Kimura *et al.*, 1993:A).

Flavonoids and chalcones exert a wide variety of biological actions on mammalian systems and the anti-tumorigenic and anti-carcinogenic properties have been studied. Iwata *et al.* (1995) examined forty chalcone derivatives for structure-activity relationships against tumorigenesis by testing for inhibitory activities of chalcones against ^{32}Pi-incorporation into phospholipids of HeLa cells enhanced by TPA. It was established that 3-hydroxy-chalcone derivatives possessing a methyl group in 3'-, 4'-, or 2'-position and isoliquiritigenin homologs showed potent inhibitory activities in the phosphorylation test. De Vincenzo *et al.* (1995) examined fifteen different naturally occurring chalcones on the proliferation of both established and primary cancer cells expressing type II oestrogen binding sites (type II EBS). At concentrations from 0.1 to 10μm chalcones inhibited ovarian cancer cell proliferation and [^{3}H] oestradiol ([^{3}H]E2) binding to type II EBS (De Vincenzo *et al.* 1995).

Leishmaniases are a broad spectrum of diseases caused by species belonging to the genus *Leishmania* and are prevalent in Africa, Asia and Latin America. Numerous studies have been conducted on the antiparasitic activity of the chalcones, Licochalcone A in particular. Chen *et al.* (1993) studied the inhibitory activity of licochalcone A on the growth of *Leishmania major* and *Leishmania donovani* promastigotes and amastigotes. The growth of *L.major* whilst in the stationary phase was completely inhibited by Licochalcone A at 5μg/ml, as measured by the uptake of [^{3}H]-thymidine. At 0.5μg/ml licochalcone A markedly reduced the infection rate of human peripheral blood monocyte-derived macrophages and U937 cells with *L.major* promastigotes and was able to kill the parasite intracellularly. From this study it was concluded that licochalcone A exhibited antileishmanial activity whilst being non-toxic to the host cells. It was hypothesised that the target organelle was the parasite mitochondria (Chen *et al.*, 1993). Bioassay guided fractionation was used to extract the chalcone (E)-1-[2,4-dihydroxy-3-(3-methyl-2-butenyl)phenyl]-3-[4-hydroxy-3-(3-methyl-2-butenyl]phenyl-2-propen-1-one from Chinese liquorice and showed strong inhibitory activity against the uptake of thymidine in proliferating promastigotes of *L.donovani* (Christensen, 1994). Further studies have been done on synthesised liquorice chalcones whereby it was concluded that echinatin and licochalcone A inhibited the growth of Leishmanias parasites in concentrations which only slightly effected the lymphocytes (Nielsen *et al.*, 1995).

Licochalcones A-D and echinatin from *Glycyrrhiza inflata* have been reported as showing antibacterial activity against some Gram-positive bacteria. Licochalcones A and C were most potent. The chalcones inhibited the oxygen consumption in susceptible bacterial cells, oxidation of NADH in bacterial membrane preparations and NADH-cytochrome c reductase, however cytochrome c oxidase, NADH-CoQ reductase and NADH-FMN were not inhibited. It was hypothesised that the site of respiratory inhibition of licochalcones was between CoQ and cytochrome c in the bacterial respiratory electron transport chain (Haraguchi *et al.*, 1998). The compounds pinocembrin, licoflavone and cycloflavone obtained from *G.glabra* were not very active against *Bacillus subtilis*, *Staphylococcus aureus* and *Candida albicans* and showed no activity against *Escherichia coli* (Fukui *et al.*, 1988).

Isoliquiritigenin, glabridin, licoarylcoumarin and licoricidin were identified as strong inhibitors of adenosine 3',5'-cyclic monophosphate (cAMP) phosphodiester. Some of the cAMP phosphodiesterase inhibitors also have strong inhibitory activity on platelet aggregation (Kusano *et al*, 1991). Licochalcones A and B, isoliquiritigenin, isoliquiritin, liquiritigenin and liquiritin have been studied for their effects on arachidonate metabolism and aggregation in human platelets (Kimura, 1993:B). Licochalcones A and B at concentration of 10^{-5}M to 10^{-3}M inhibited the formation of 12-hydroxy-5,8,10-heptacatrienoic acid (HHT) and thromboxane (TXB_2) dose dependently. At higher concentration isoliquiritigenin and liquiritigenin inhibited the formation of HHT and TXB_2 while increasing the formation of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). They did not effect arachidonate metabolism or prevent thrombin induced platelet aggregation . Licochalcones A and B inhibited thrombin induced platelet aggregation ($2x10^{-5}$ M to $2x10^{-4}$ M) and inhibited the elevation of $[Ca^{2+}]$ induced by thrombin. Contradicting these results Tawata *et al.* (1992) reported that isoliquiritigenin isolated from *G.radix* inhibited thrombin induced platelet aggregation, comparable to that of aspirin, by inhibiting cyclo-oxygenase, lipoxygenase and peroxidase activity and inhibited platelet aggregation *in vivo*. Isoliquiritigenin was the only aldose reductase inhibitor with significant anti-platelet action (Tawata *et al.*, 1992) In previous reports isoliquiritigenin inhibited rat lens aldose reductase activity with an IC_{50} of 3.2 $x10^{-7}$M, inhibited sorbitol accumulation in human red blood cells *in vitro* with an IC_{50} of

2 x 10^{-6}M and suppressed sorbitol accumulation in the red blood cells, the sciatic nerve and the lens (Aida *et al.*, 1990). It was therefore suggested that isoliquiritigenin could be potentially useful in treating diabetic complications.

Isoliquiritigenin has been investigated for its potential cardiac effects (Wegner and Nawarth, 1997:A; Wegner and Nawarth, 1997:B) and was found to induce a concentration-dependent relaxation (EC50 9.4μM) on the tension in endothelial free rat aortic rings pre-contracted with phenylephrine (3μM). However at 10μM the effects were potentiated, Wegner and Nawarth ((B)1997) suggested that the effects maybe due to the inhibition of phosphodiesterase activity. Further work on the effects of isoliquiritigenin on the force contraction (F_c), L-type Ca^{2+}(I_{Ca}) current and intracellular Ca^{2+} concentration([Ca^{2+}]i) in rat ventricular heart muscle, resulted in an increase in F_c and I_{Ca} in the myocardial preparations , with the conclusion being that it was again a direct inhibitory response on the phosphodiesterase activity and the subsequent accumulation of cyclic AMP (Wegner and Nawarth 1997:B).

The saponin glycyrrhizin and its derivatives have been recognised as effective anti-inflammatory agents for many years, Finney and Somers in 1958 reported on the anti-inflammatory activities in the use of four different animal models; the cotton pellet method of Meir, Schuler and Desaulles, inhibition of tuberculin reaction in B.C.G. sensitised guinea pigs, the rat foot test (formaldehyde) and the granuloma pouch method. The inhibitory activity of glycyrrhetinic acid, as its dihemiphthalate derivatives have been examined on arachidonic acid (AA)-induced ear oedema in mice (Dehpour *et al.*, 1994). Glycyrrhetinic acid and deoxyglycyrrhetol were ineffective at the inhibition of AA (4mg/ear) induced oedema at doses of 1mg/ear, although three derivatives showed strong inhibition on both topical (ID50 1.7, 1.9 and 2.8 mg/ear) and oral (ID50 88, 90 and 130 mg kg^{-1}) administration (Inoue *et al.* 1988). They also inhibited lipoxygenase and cyclooxygenase activities and prevented the formation of gastric ulcers. Similar tests were carried out to examine the anti-inflammatory action on TPA-induced (2μg/ear) oedema. Again the parent compounds glycyrrhetinic acid and deoxyglycyrrhetol produced little inhibition by oral administration (less than 200mg kg^{-1}), although glycyrrhetinic acid and its analogues were very effective when topically applied (1mg/ear) inhibiting oedema

by 80% (Inoue *et al.* 1989). Both AA and TPA induced oedema were more effectively inhibited when the anti-inflammatory agents were applied 30 minutes prior to the application of the irritant.

Glycyrrhetinic acid expresses a dose and time dependent inhibitory response on TPA stimulated 3-*O*-methyl-glucose transport in mouse Swiss 3T3 fibroblasts (Kitagawa *et al.*, 1984). From the kinetic studies it was suggested that TPA-induced the stimulation of hexose transport through the enhancement of the number of hexose carriers and this could be completely inhibited by glycyrrhetinic acid. Kitagawa *et al.* (1984) concluded that glycyrrhetinic acid could be applicable in cancer prevention because of the non-mutagenic and non-cytotoxic but strong anti-tumour promoting activity *in vitro*. They also reported on the unpublished results of the suppressing action of glycyrrhetinic acid on teleocidin skin tumour formation in mice initiated with 7,12-dimethylbenz[*a*]anthracene. Glycyrrhetinic acid has been investigated for its action on histamine metabolism in cultured mast cells cocultured with Swiss 3T3 fibroblasts; 50μM of glycyrrhetinic acid strongly inhibited histamine synthesis which was believed to be derived from the histidine decarboxylase activity which was inhibited by 80%. Glycyrrhetinic acid significantly reduced the mRNA expression of novel protein kinase C δ (nPKCδ), which led to the suggestion that perhaps glycyrrhetinic acid inhibited histamine synthesis through the regulatory effect of nPKCδ (Lee *et al.*, 1996).

Glycyrrhizin when fed orally to Sencor mice, gives substantial protection against skin tumorigenesis by 7,12-dimethylbenz(a)anthracene (DMBA) initiation and 12-tetradecanoylphorbol-13-acetate (TPA) promotion. The chronic oral feeding inhibits the binding of topically applied [^{3}H]Benz(a)pyrene to epidermal DNA (Agarwell *et al,* 1991).

Neither liquorice nor glycyrrhizin promoted the growth of plaque formation of the cariogenic bacteria *Streptococcus mutans*. At 0.5-1% glycyrrhizin, inhibition of the plaque was almost complete (Segal *et al.*, 1985).

The polysaccharide fractions of *Glycyrrhiza uralensis* have been examined for *in vitro* immunostimulating activity. The most potent fraction contained about 60% galacturonic acid with arabinose, galactose and glucose as the major neutral sugars and a small quantity of protein. When the extract was further separated the fraction containing rhamnose, fucose, arabinose, mannose, glucose, galactose, glucuronic acid and galacturonic acid with a small quantity of protein had the most potent anti-complementary and mitogenic activities, thus leading to the suggestion that polysaccharides containing a polygalacturonan moiety were the most effective immunostimulants (Zhao *et al.*, 1991).

1.3.4. Medicinal Uses

The drug liquorice obtained from *G.glabra* has been used for over 1200 years (Shibata, 1994:A). A Dutch doctor in the late 1940's noticed that patients with gastric ulcers were being cured by high doses of liquorice extract dispensed by a local pharmacist. An investigation into this led to the identification of glycyrrhizin and glycyrrhetinic acid as the main active constituents. Due to the relatively poor water solubility of these two compounds a semi-synthesised derivative was developed called carbenoxolone. Carbenoxolone became the major anti-ulcer drug of the 1960's until the first acid inhibitors were developed. Unfortunately both carbenoxolone and glycyrrhetinic acid cause significant side effects, mainly fluid retention and hypertension.

The British Herbal Pharmacopoeia (1990 vol.1) quotes that *G.glabra* can be used as an expectorant, demulcent or as a spasmolytic. The tincture of *G.glabra* is used as an anti-inflammatory for arthritic or allergic conditions and as a digestive stimulant for lung disorders. A decoction is used to reduce stomach acidity in ulceration and the syrup made from the decoction is used as a soothing expectorant for asthma and bronchitis (Ody, 1993). *G.glabra* has been used traditionally as a laxative and against bronchial catarrh, colic and primary adrenocortical insufficiency (Newall *et al.*, 1996). *Glycyrrhiza* is used as part of the Japanese traditional herbal medicine, Kampo, in treating diabetic neuropathy (Aida, 1990).

Although liquorice is beneficial medicinally, side effects can occur. The excessive ingestion of liquorice may result in sodium and water retention, hypertension, hypokalaemia and suppression of the renin-aldosterone system (Farese *et al.*, 1991; Heikens *et al.*, 1995). It was thought that liquorice produced these effects by binding its active component glycyrrhizic acid and its hydrolytic metabolite glycyrrhetinic acid to mineralocorticoid receptors. Farese *et al.* (1991) report on several findings which argue against this theory. The affinity of glycyrrhetinic acid for mineral corticoid receptors is 0.01% of aldosterone and glycyrrhetinic acid does not have mineralocorticoid effects in patients with Addison's disease or adrenalectomized rats unless cortisone or hydrocortisone is administered concomitantly. Heikens *et al.* (1995) report that the hydrolytic metabolite of glycyrrhetinic acid, glycyrrhizic acid, is the active component of liquorice causing the inhibition of the peripheral metabolism of cortisol. Cortisol binds with the same affinity as aldosterone to the mineralocorticoid receptor resulting in hypermineralocorticoid condition resulting in the retention of sodium and water, hypertension, hypokalaemia and the suppression of the renin-aldosterone system.

1.4. *Galium aparine* L. (Goosegrass or Cleavers)

G.aparine is a common annual herb, commonly known as goose grass or cleavers and has been a popular herb in folk medicine throughout the centuries (Mabry, 1996) (figure, 1.4.). The young shoots of *G.aparine* are the first of many weeds which appear in the spring and it is these shoots that are used in the herbal remedies. Culpepper once said ' it is also an inhabitant in gardens that it ramps upon and is ready to choke what ever grows near it'. Indeed this is still true, *G.aparine* is most certainly a prolific weed (Williamson and Evans, 1988). *G.aparine* is used today as fodder for geese and chickens and the round fruits were once used to make tops for lace pins.

1.4.1. Botany

G.aparine belongs to one of the largest flowering plant families, the Rubiaceae, containing about 500 genera and 7,000 species. Three subfamilies exist within the

Figure 1.4. **Photograph of *Galium aparine* L.**

Rubiaceae, the Rubiodeae (11 tribes), Cinchonoideae (17 tribes) and the Guettardoideae (1 tribe) (Heywood, 1993). The diagnostic features of the family are the opposite whorled leaves and the presence of stipules, which in *Galium* and *Asperula* are leaf like; flowers are borne in panicles, cymes or congested heads which are bisexual and the fruits are either as capsules or drupes. The Rubiaceae is mainly tropical and subtropical in distribution with a few species in the temperate and cold regions. The tropical species are mainly trees and shrubs whilst all temperate species are herbaceous (Clapham *et al.*, 1987). The family is important economically as it contains both *Coffea* and *Cinchona* genera, producing coffee and quinine respectively (Heywood, 1993).

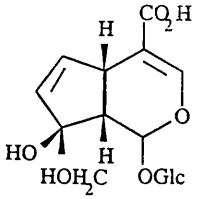
The British representatives of this family, including *Galium*, all belong to the sub family Rubiodeae and the tribe Galieae. There are about 150 species of *Galium* in Europe which are classified into ten morphologically well-defined sections. Within each of these sections the classification is less clear, species often represent polyploid complexes for example *G.mollugo* group and the *G.lucidum* group within the section Leiogalium (Kuiper and Labadie, 1981).

G.aparine is an annual herb, with prostrate scrambling branched stems 15-20cm in length which are quadrangular in shape and rough to the touch because of the downwardly-directed prickles. The leaves are found in whorls of about 6-9, about 12-60mm in length and 3-8mm in width. They are described as being narrowly to broadly oblanceolate or elliptical. The flowers are 1.5mm in diameter and are found in 2-5 axillary cymes with the peduncles topped by a whorl of 4-8 bracts, olive/purplish in colour covered by white hooked bristles with tuberculate base and are hermaphrodite and protandrous. *G.aparine* is a native of the British Isles, occurring in hedges and waste places and is abundant throughout the UK, Europe and North and West Asia (Clapham *et al.*, 1987).

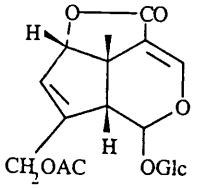
1.4.2. Phytochemistry

G.aparine possess a number of iridoids, which are cylopentan-[c]-pyran monoterpenoids. Iridoids frequently occur combined with a sugar molecule as the

glucoside. There are many seco-iridoids in which the pyran ring is left open and less common when the pyran ring oxygen is replaced by a nitrogen. Iridoids present in *G.aparine* include asperuloside, monotropein (Figure1.4.2.) (Newall *et al.*, 1996), deacetylasperuloside (Williamson and Evans, 1988) scandoside and geniposidic acid. HPLC determination of the iridoids in six *Galium* species led to the identification of monotropein, aucubin and asperulside (figure 1.4.2.) in all six (Ergun and Sener, 1986) and a non-glycosidic iridoid form has been identified from *G.macedonicum* (Mitova *et al.*, 1996). Iridoids occur in plants both in the free state and in the glycosidic form, from which the free toxin is liberated after enzyme hydrolysis. They are present in about fifty plant families belonging to the dicotyledon section.



Monotropein



Asperuloside

Figure 1.4.2. Molecular Structure of Monotropein and Asperuloside

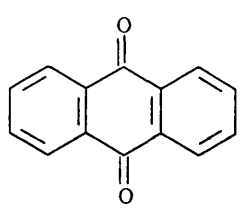
Coumarins present in *G.aparine* are unspecified, however scopoletin and umbelliferone are reported for the related species *Galium cruciata* and *Galium tauricum* (Newall *et al.*, 1996). Coumarins are derivatives of benzo-α-pyrone and are common in plants both in the free state or as the glycosides. Coumarins give the characteristic odour of new-mown hay and occur in many species of the Leguminosae. *G.aparine* also possesses a number of polyphenolic acids, for example caffeic, p-coumaric, gallic, p-hydroxybenzoic, salicylic and citric acids (Newall *et al.*, 1996).

Also present in *G.aparine* are a number of sterols and fatty acids which vary in quantity according to the season in which they are harvested. Unsaponifiable sterol

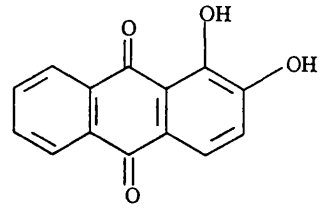
constituents include campesterol, stigmasterol, Δ^5-avenasterol, Δ^7-stigmasterol and Δ^7-avenasterol. Lanosterol, cycloartenol and β-amyrin are only found in the spring samples (Tzakou *et al.*, 1990). Fatty acids present include lauric, myristic, palmitic, palmitoleic, , stearic, oleic, linoleic and linolenic. The content of linolenic acid is slightly higher in the winter sample (Tzakou *et al.*, 1990).

Flavonoids are the largest group of naturally occurring phenols and appear both in the free state and as glycosides. Flavonoids are biosynthesised from three acetate units and a phenylpropane unit. Within *Galium verum* flavonoids represent 2% of the dry weight and include quercetin glycosides such as isorutin, palustroside and cynaroside and the flavone luteolin (Williamson and Evans, 1988).

Anthraquinones are formed by the union of two anthrone molecules and are derived from the shikimate and mevalonate pathway or the acetate-malonate pathway and occur in the roots of *Galium* species. The genus *Galium* is comprised of about 150 species in Europe classified into ten morphologically defined sections, the species representing polyploid complexes. The anthraquinones of *Galium* have been examined to assist in the determination of these polyploid complexes leading to a classification which involves both morphological and chemical features. Approximately 25 anthraquinones (aglycones) have been identified within the genus including alizarin (figure1.4.2.1.), purpurin and rubiadin (figure 1.4.21.)(Kuiper and Labadie , 1982). From *G.album* the A-ring prenylated anthraquinone, galiprenylin, has been identified (figure 1.4.2.1.) (Kuiper and Labadie, 1982). Within *G.aparine* the anthraquinone derivatives including alizarin and derivatives, xanthopurpurin and its esters and galiosin have been identified (Williamson and Evans, 1988). El-Gamal *et al.* (1995 and 1997) have extensively studied the anthraquinones from *G.sinaicum* and have isolated twenty six novel anthraquinones. Undifferentiated cell cultures of *G.verum* have been used specifically to produce anthraquinones (Banthorpe and White, 1995).



Anthraquinone



Alizarin

Figure 1.4.2.1. Molecular Structure of Anthraquinone and Alizarin

Other constituents present include the N-alkanes with the C_{29} alkanes being the most abundant (49%) followed by C_{27} (11%), C_{31} (8%) and C_{25}, C_{23} and C_{30} (2%) (Corrigan *et al.*, 1978). The isoquinoline alkaloid protopine has been found in *G.aparine* of Turkish origins (Sener and Ergun, 1991). A lignan glycoside (lariciresinol-based lignan) has been found in *Galium sinaicum* which are the first reports of this compound in the family Rubiaceae (El-Gamal *et al.*, 1997).

1.4.3. Pharmacology and biological activities

The anthraquinones from *G.sinaicum* were isolated using cytotoxicity testing on P388 cell lines (Mouse P388 leukaemia cells) (El-Gamal *et al.*, 1995). The n-hexane extract has an IC_{50} of 3.8μgml^{-1}, however the most active compounds were not as active as the crude extract. The most toxic was quinizarin which had an IC_{50} of 5.8μgml^{-1} and copareolatin 6-methyl ether which was 5.5 μgml^{-1}(El-Gamal *et al.*,, 1995). Anthraquinones from the most polar fractions of *G.sinaicum* were also studied for cytotoxicity activity on the mouse P388 leukemic cell line and the CH_2Cl_2 and n-BuOH extracts had IC50s of 16 and 70μgml^{-1} respectively. The eight anthraquinones included five free anthraquinones, which were the most potent and had IC50s between 1 and 10.4μgml^{-1} and three anthraquinone glycosides (IC50s, 48-100μgml^{-1}). The lignan glycoside isolated from *G.aparine* exerts a weak cytotoxic activity against the P388 cell lines (El-Gamal, 1997).

The iridoids asperuloside and monotropein have been reported to exert a mild laxative affect in mice and were established as being fifteen times less potent than senna (*Cassia senna*) (Newall *et al.*, 1996). Iridoids have been tested for anti-inflammatory actions on carrageenan-induced mouse paw oedema and TPA-induced

mouse ear oedema. The iridoid loganic acid was most active on mouse paw oedema (44.4% inhibition, loganic acid 100mg/kg per, carageenan 0.05ml). However aucubin which is found in *G.aparine* showed an 80% inhibition against mouse ear oedema (aucubin 1mg/ear, TPA 2.5µg/ear), (Recio et al., 1993) Other activities shown by the iridoids include antimicrobial, antitumoral, haemodynamic, choleretic and hepatoprotective (Recio *et al.*, 1993).

Coumarins reportedly show oral anti-coagulant activity. They inhibit the clotting mechanism of blood whilst having no effect on platelet aggregation and are of value in arterial thrombosis. The 4-hydroxy-coumarins reportedly found in *G.aparine* act by antagonising the effects of vitamin k. Vitamin k acts by indirectly activating those substances necessary for the conversion of prothrombin to thrombin (Evans, 1996).

1.4.4. Medicinal Uses

Aerial parts of *G.aparine* are used as a potent diuretic and lymphatic cleanser and have been reported as being effective in many cases involving swollen or enlarged lymph glands (Newall *et al.*, 1996). The British Herbal Pharmacopoeia (vol.1, 1990) has described its medicinal use as a diuretic and mild astringent. It is described as a blood purifier and is used for skin problems and other conditions where the body is failing to rid itself of toxins (Ody, 1993).

The juice of *G.aparine* is used to make an effective diuretic and lymphatic cleanser for a range of conditions including glandular fever, tonsillitis and prostate disorders. The infusion is used for urinary problems such as cystitis and gravel and is also taken as a cooling drink in fevers. The cream is regularly used to relieve the symptoms of psoriasis and a hair rinse is used to treat dandruff or scaling scalp problems (Ody, 1993).

1.5. *Bellis perennis* L. (Daisy)

Bellis perennis is commonly known as the English or Lawn daisy (figure, 1.5.). It is a perennial often found in the short turf of paddocks and lawns where it can grow in constellations so dense that there is no space between the flowers.

1.5.1. Botany

Bellis perennis L. belongs to the Compositae (Asteraceae), the sunflower family, which has around 1,100 accepted genera and about 25,000 species. The family is separated into two subfamilies the Lactucoideae and the Asteroideae. Most of the members are evergreen shrubs or subshrubs or perennial rhizomatous herbs and are described as having a cosmopolitan distribution being absent only from the Antartic regions. They are particularly well represented in the semi arid regions of the tropics. The daisies, thistles and dandelions exemplify one of the most characteristic features of the Compositae, the head-like inflorescence known as the capitulum (Heywood, 1993). The capitulum is made up of numerous small individual flowers called florets, which are surrounded by an involucre of protective bracts resembling a single flower and consequently functions biologically as a single flower. The Compositae is of an economic importance mainly because of its contribution to the stability of grasslands, also its use as food plants, for example *Lactuca sativa* (lettuce) or *Cichorium tybus* (chicory); other uses include medicinal and drug plants, ornamentals and succulents.

Bellis perennis is a perennial herb belonging to the sub-family Asteroideae, tribe Astereae. Within Europe and the Mediterranean region there are fifteen species of *Bellis*, however in the UK *B.perennis* is the only species. *B.perennis* has a short erect stout stem with fibrous roots The leaves are 2-4 cm in length and are confined to a basal rosette. They are obovate, broad and rounded at the end. The flower head is 16-25mm in diameter and the bracts are oblong, with green tips. The ray florets are numerous and narrow, either pink or white in colour and the achenes are bright yellow. It is a native plant of short grasslands throughout the British Isles, Europe and West Asia, (Clapham *et al.*,1987).

Figure 1.5. **Photograph of *Bellis perennis* L.**

1.5.2. Phytochemistry

Schopke *et al.* (1992) have extensively studied the saponins present in *B.perennis* isolating and elucidating the structures of the two acylated triterpenoid saponins bellisaponins BA_1 and BA_2. Previously four triterpenoid saponins of the rarely occurring sapogenin polygalacic acid (Schopke *et al.*, 1986) were isolated from the rhizomes. They included bellissaponin BS1 and BS2 and the two acylated bellissaponins BA_1 and BA_2. The bellisaponins BS4, BS5, BS6 and BS7 (figure 1.5.2.) were isolated from the more polar fractions of the alkaline hydrolysate of the ester saponin mixture from the rhizome, as were asterogenic acid, bayogenin, hederagenin and oleanic acid (Schopke *et al.*, 1991; Schopke *et al.*, 1992). *B.perennis* contains saponins of nearly all sapogenins from those plants belonging to the Asteraceae. Schopke *et al.* (1996) have continued their studies on triterpenoid saponins within the other species of *Bellis*. Recent studies have included the use of positive-ion electrospray ionisation mass spectrometry to analyse the saponin mixture of *B.annua*, concluding that saponins accounted for 4% of the dry weight.

The sugars rhamnose, xylose and glucose have been isolated from the ethanol extract. Two other minor compounds were also isolated and identified as the genin of polygalsin D and the peracetate of polygalacic acid (Deseaeueday *et al.*, 1989).

	R^1	R^2	R^3	R^4
BS4	OH	H	Glc	Rha
BS5	H	OH	Glc	Xyl
BS6	H	OH	GLC	Rha
BS7	H	OH	Rha	Glc

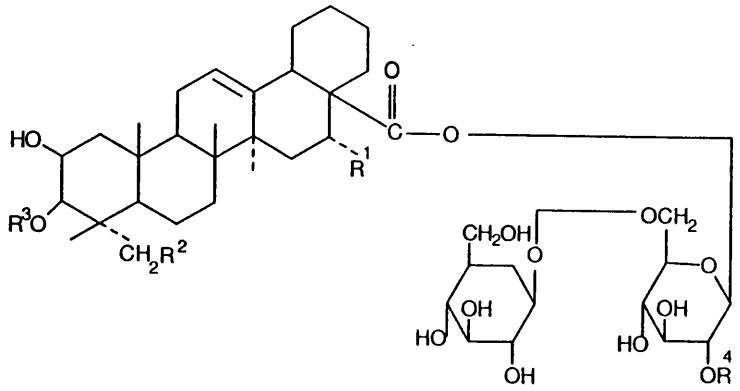


Figure 1.5.2. Molecular Structure of Bellis Saponins

The chemical constituents of the essential oils from the leaves and flowers of *B.perennis* plants collected in Italy have been analysed (Pinarosa & Tawa, 1985). The monoterpenes were present in the largest quantities (47% and 62% from the leaves and flowers respectively) with β-myrcene (15% leaves; 28% flowers) and

geranylacetate (12% leaves; 6% flowers) as the main volatiles. There was found to be little variation in the composition profile of the two organs apart from *cis*-3-hexenol (15% leaves; 0.4% flowers). Sesquiterpenes found in the essential oils of *B.perennis* include β-caryophyllene and α-cubebene. The C_6 leaf alcohols have been suggested as being responsible for the typical 'green leaf odour'(Avato and Tava, 1995).

1.5.3. Pharmacology and biological activities

The saponin rich fraction of *Bellis perennis* exhibits a strong activity *in vivo* and *in vitro* experiments on the fungus *Ceratocystis ulmi*, the fungus responsible for Dutch Elm disease (Deseaueday *et al.*, 1989). *In vitro* anti-fungal activity was tested on petri dishes inoculated in the centre with young mycelium and the substances to be tested deposited into a hole bored into the dish. *In vivo* tests were carried out on diseased elms whereby the extract was injected into four cylindrical holes bored into the bottom of the trees. The greatest activity was obtained from an *n*-BuOH extract obtained from the EtOH extract. Analysis of this extract revealed three sugars and three genins.

Very little else has been written on the pharmacological/biological activity and constituents of *B.perennis.* However substantial work has been done on other members of the Compositae family. Erythro-alkane-6,8-diols from *Carthamus tinctorius* has a 50% inhibitory dose of 0.5-0.7mg/ear against TPA induced inflammation at 1μg/ear. It furthermore suppressed the effect of TPA on skin tumour formation on mice following the initiation with 7-12-dimethylben[α]anthracene (DMBA) (Yasukawa *et al.*, 1996; Akihisa *et al.*, 1996:A).

The ten dihydroxy- and trihydroxy triterpenes, four taraxastanes (faradiol, heliantrial Bo, heliantriol C and arnidial), two lupanes (calenduladiol and heliantrial B_2), two oleananes (moniladiol and longispinogenin), two ursanes (brein and uvaol) isolated from the non-saponifable lipids of *Carthamus tinctorius, Chrysanthemum morifolium and Helianthus annuus* all have a 50% inhibition at 0.03-0.2mg/ear over TPA inflammation (1μg)(Akhisa *et al.*, 1996:B). Taxasterol from *Cynara xalynus* and

faradiol from *Chrysanthemum marifolium* inhibited initiation at 2.0μmol/mouse, TPA (1μg/mouse) induced skin tumour formation following initiation with DMBA (50μg/mouse) (Yasukawa *et al.,* 1996). The sterols stigmasterol and scholterol from *Carthamus tintorius* also inhibited TPA inflammation (Kasahara *et al*, 1994).

1.5.4. Medicinal Uses

Traditionally *B.perennis* has been used as a poultice of the fresh plant or as compress soaked in an infusion to relieve bruises and sprains (Ody, 1993), treating wounds (Schauenberg and Paris, 1977) and chest ailments (Bunney, 1994). *B.perennis* is used as skin wash for eczema, a douche for thrush, and as an expectorant for coughs. It has also been suggested that *B.perennis* may slow the growth of breast tumours from evidence of research (Bremness, 1994). An infusion of *B.perennis* is added to baths to help revive sallow skins. The stem sap is applied to spots and the flower tea is given to listless children, (Bremness, 1994). Traditionally *B.perennis* has been used in the treatment of rheumatism and as an expectorant (Avato and Tavo, 1995). The usefulness of *B.perennis* for pain and swelling occurring from injury or trauma to deep seated tissue (Hammen, 1996), has given rise to its reputation in the treatment of arthritis and rheumatism as well as in liver and kidney problems. Within veterinary medicine it has been used as a vulnerary and against ecchymoses. It has also been shown to have antifungal activities.

1.6. *Trifolium pratense* L. (Red clover)

Trifolium pratense, commonly known as red clover or meadow trefoil, is a perennial common throughout the British Isles in its native form and a more robust form sown as fodder crops (figure, 1.6.). Meadow trefoil meaning 'three-leaved grass' because of its familiar three-lobed leaves was once associated by medieval Christians with the Trinity.

Figure 1.6. **Photograph of *Trifolium pratense* L.**

1.6.1. Botany

T.pratense belongs to the sub-family Papilionoideae, genus *Trifolium* of which there are a total of twenty-two species in the UK. *T.pratense* is an erect pubescent perennial 5-100cm in length, possessing leaflets (-50mm) which are obovate, hairy beneath, glabrescent above, with a white crescentric spot towards the base. The flower heads are terminal, 20-40mm in length globose in shape and subtended by a pair of short-petiolate leaves (Clapham *et al.*, 1987). This plant is a native of the British Isles.

1.6.2. Phytochemistry

The main constituents of *T.pratense* are the isoflavanones. Isoflavanones present in *T.pratense* include biochanin A (figure 1.6.2.), daidzein, formononetin, genistein , pratensein, trifoside, calycosine and galactoside (figure 1.6.2.)(Saxena and Jain, 1987), 4',7;-dimethoxyisoflavanone, narigenin and galangin (Chae *et al.*, 1991; Nicollier and Thompson, 1982), conjugated isoflavonoids including formonetin-7-O-glucoside-6-11-malonate (FGM), maackein 3-O-glucoside-6"-O-malonate (MKGM) and an unknown 5,7-dihydroxy containing isoflavonoid metabolite (Edwards *et al.*, 1997). Flavones present include 4',7-dihydroxyflavanone (DHF) and pectolinarin (Orgamide *et al.*, 1994)

Phytoalexins are anti-microbial metabolites that are absent in healthy plants but accumulate in high concentrations in damaged and around damaged cells. Medicarpin and maackian (figure 1.6.2.) have been isolated from *T.pratense* (figure 1.6.2.) (Dewick, 1975). These two phytoalexins are produced when leaves were inoculated with *Helminthosporium turicum*. Further work has led to the isolation of medicarpin and maackiain from *T.pratense* seedlings on treatment with heavy metal ions (Dewick, 1977).

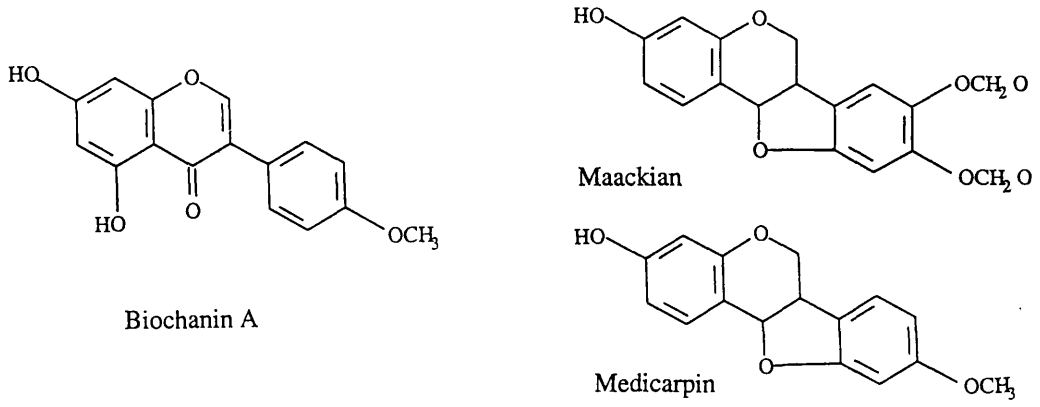


Figure 1.6.2. Molecular Structure of Biochanin A, Maackian and Medicarpin

T.pratense contains the flavonoids isorhamnetin, kaempferol, quercetin and their glycosides (Jain and Saxena, 1986). Over fifty essential oil constituents have been identified from clover flowers using flame ionisation and gas chromatography-mass spectrometry. The following compounds were isolated maltol (8%), linalool (4%), I-phenylethyal (3%), phenol (3%), phenyl ethyl acetate (3%), acetophene (2.5%) and (2)-3-hexanyl acetate (2%) (Buchbauer *et al.,* 1998).

Soyasapogenols B-F and the carbohydrates arabinose, glucose, glucuronic acid, rhamnose and xylose have been derived by acid hydrolysis (Olesek and Jurzysta, 1986). Medicagenic acid, medicagenic acid 3-O-glycopyranoside and soyasaponin 1 are all present in *T.pratense* (Czeczot *et al.*, 1994).

Other constituents of *T.pratense* include coumaric acid, phaseolic acid, salicyclic acid, trans- and cis- clovamide (L-dopa conjugated with trans- and cis- caffeic acids), resin, volatile oils (furfural), fats, vitamins, minerals (Newall *et al.,* 1996) and the coumarins, coumarin and medicagol.

1.6.3. Pharmacology and Biological Activities

The isoflavone biochanin A has been reported to inhibit carcinogen activity in cell culture, which has led to the suggestion that this compound may be used as a potential chemopreventative agent. Cassady *et al.*, (1988) measured the effects on the metabolism of [^{3}H]benzo(*a*)pyrene (B(*a*)P) in hamster embryo cell cultures. A 95% ethyl alcohol extract of *T.pratense* significantly inhibited the metabolism of B(*a*)P and decreased the level of binding of B(*a*)P to DNA by 30-40%. Using

bioassay guided fractionation biochanin A was isolated and shown to inhibit B(*a*)P binding by 54% at a dose of 25 μg/ml (Cassady *et al.*, 1988).

Further work led to the effects of twenty-three flavonoids, structurally related to biochanin A on the metabolism of B(*a*)P using hepatic microsomes prepared from rats treated with various cytochrome P-450 inducers. Flavones that contain free 5- and 7- hydroxyls were identified as potent inhibitors of cytochrome P-450 induced by β-naphthoflavone (P-4501A1 and/or P-4501A2) and therefore were concluded as potentially useful chemopreventative agents against hydrocarbon induced carcinogenesis (Chae *et al.*, 1991). Biochanin A has demonstrated anti-tumourogenic activity against a number of carcinogens requiring metabolic activation such as aflatoxin B, 2-amino-anthracene and against one direct acting carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (Chae *et al.*, 1991).

Biochanin A, formononetin, pratensein, genistein, daizein and the coumarin derivative coumestrol have been examined for their effects on the reproductive systems of animals especially sheep (Sachse, 1974). Biochanin A, formononetin and genistein have been identified as possessing oestrogenic properties (Newall *et al.*, 1996).

The mutagenic activities of medicagenic acid, medicagenic acid 3-O-glucopyranoside and soyasaponin 1 were tested by the Ames method with *S.typhinimurium* strains TA97, TA98, TA100 and TA102 in the absence and presence of metabolic activation. They were all found to be non-toxic and non-mutagenic for the testing doses (Czeczot *et al.*,1994). The saponin constituents are also reported to lack any haemolytic or fungistatic activity (Newall *et al.*, 1996).

1.6.4. Medicinal Uses

T.pratense has been used as a dermatological agent treating skin complaints such as psoriasis and eczema, where it is suggested that a tincture should be taken internally (Ody, 1993). Within the British Herbal Pharmacopoeia (vol.1, 1990) it is quoted as being used as a dermatological agent. The herb may be used fresh or as an ointment

to treat lymphatic swellings, skin sores, ulcers, burns, insect bites and stings and as an eye wash for conjunctivitis and sore eyes (Ody, 1993). The tannins are known to possess astringent properties (Newall, *et al.*, 1996).

The syrup which is made from the infusion is used for treating stubborn dry coughs and bronchial conditions or as a mild antispasmodic and expectorant (Williamson and Evans, 1988). A compress is used for arthritic pains and gout (Ody, 1993).

Adverse side effects of *T.pratense* have been documented, generally due to the oestrogenic isoflavanone constituents, especially formononetin, whereby infertility and growth disorders have been reported. For this reason large doses should be avoided to prevent possible anticoagulant (coumarin) and hormonal effects especially during pregnancy and lactation.

1.7. Biological tests for psoriasis

The diseased skin of psoriatic patients shows a benign epidermal hyperplasia and abnormal differentiation plus dermal inflammation. A variety of simulated psoriatic models have been produced, but they are few and inadequate. Models therefore tend to focus on different characteristics of the disease, for example epidermal hyperplasia. These models have been used to study either the kinetics and biochemical abnormalities of the disease or screen potentially effective and anti-psoriatic drugs (Lowe, 1988).

The biological tests for psoriasis are easily separated into *in vivo* and *in vitro*. In choosing a screening test, it is essential that both efficacy and efficiency are considered carefully especially if an animal model is to be used.

1.7.1. *In vivo* Tests

Rat ultraviolet-ray-B (UVB) photodermatitis is a useful model to investigate antipathogenic mechanisms in psoriasis as well as testing drugs for antipsoriatic activity. Rat UVB-dermatitis is characterised by a sharply demarcated red lesion with scale formation. Histologically there is microvascular dilation, intraepidermal accumulation of PMNL with microabscess, mononuclear cell infiltration at the papillary dermis and hyperproliferation of the epidermal cells. The test involves exposure of the dorsal skin to UVB (302nm) for a set period of time depending on the intensity of the light. Observations of the inhibition of UVB induced dermatitis by anti-psoriatic agents may be done in several ways. Initial examination can easily be seen macroscopically by the reddening and scaling of the skin. A biphasic erythema is usually observed whereby a faint erythema will be seen immediately after irradiation. A second phase starts at six hours peaking between 24 and 48 hours. Histological examinations are best taken five days post-irradiation, at this time thickening of the epidermis, microabscess formation and PMNL infiltration are clearly visible in the rat species, but within the guinea-pig and mouse only microvascular dilation is visible. To evaluate the intensity of the epidermal proliferation and identify the proliferating sites DNA labelling can be done whereby intra-nuclear 5-bromo-2-deoxyuridine (BrdU) is incorporated into the keratinocytes (Nakaguma *et al.*, 1995). Epidermal PMNL infiltration can be examined by the morphological polarisation assay (Cianonciolo and Snyderman, 1981). Peters *et al.* (1977) showed that UV-erythema was partially the result of local release of prostaglandins by studying the effects of topically applied agents to the skin of guinea pigs and found that prostaglandin synthetase inhibitors, for example indomethacin and pirprofen, to be highly effective suppressors of UV erythema. Snyder (1975) established that indomethacin could decrease skin temperatures and hyperplasia to near normal levels in both guinea-pigs and humans. This was done using a grading system with the following parameters: intensity of erythema, skin temperature, pain perception, increased pigmentation and scaling. *In vivo* ^{3}HTdR incorporation by irradiated sites on the guinea-pigs was used as a measure of

epidermal proliferation, and keratinocyte cell death measured by counting the abnormally stained cells.

Parakeratosis refers to the production of a stratum corneum in which the keratinized cells retain nuclei. This is a natural occurrence in mouse tail scales, mammalian external nares (nostrils) and whale epidermis. In human body skin parakeratosis is an abnormal condition and occurs in psoriasis as the lesional epidermal cells or keratinocytes undergo the accelerated and disturbed differentiation. The majority of human skin differentiates in an 'orthokeratotic' fashion, where late in the keratinocyte life cellular organelles are degraded by hydrolytic enzymes which are released or activated in the granular region. The nucleus is therefore destroyed, the plasma membrane becomes toughened through keratin synthesis and the final product of differentiation is the horny cell. The horny cell is devoid of most of the original internal structure which allows it to have a considerable amount of flexibility, obviously required by the skin. The mouse tail test has been used to evaluate drugs for anti-psoriatic activity (Wrench and Britten, 1975; Wrench, 1985). There are two forms of tests. Parakeratotic to orthokeratotic conversion in adult mice studies the ability of anti-psoriatic drug to change the regions of normal parakeratotic differentiation in the adult mouse tail to an orthokeratotic type. The pattern of keratinization in adult mice is established by two weeks of age but neonate mice have completely orthokeratotic tails and one can therefore study the inhibition of parakeratotic development in infant mice. Evaluation of the test can be done by measuring the epidermal thickness and observing the changes in the fluorescence of the stratum corneum. After fixation the sections are fluorochromed in a congo red and tian yellow mixture previous to immersion in a solution of thioflavine T. Visualisation with UV reveals that normal human body skin and perifollicular tail skin (both have an orthokeratotic differentiation) fluoresce red, whilst psoriatic epidermal layer and untreated mouse skin fluoresce blue or green (Wrench, 1985).

In mouse skin a single application of the tumour promoter phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) will induce skin inflammation and epidermal hyperproliferation followed by hyperplasia, which on repeated administration would become permanent. The induction of epidermal hyperplasia in mouse skin is

mediated by a short pulse of prostaglandin E synthesis accompanied by certain biochemical events such as an increase of orthine decarboxylase (ODC), the rate-limiting step of polyamine biosynthesis (Bourin *et al.* 1982). This is a relatively simple test referred to as chemically induced erythema. TPA is not the only reagent used to induce proliferation, other reagents include croton oil, 12-deoxyphorbolphenylacetate (12-DOPP), arachidonic acid and ethyl phenyl proprionate (Williamson *et al*, 1996). Erythema of the mouse ear is very easy to perform as mice have translucent ears and reddening is clearly visible, also mice can be reused and the course of inflammation can be studied over a period of time. However quantification of the redness can be subjective. Oedema of the ear is easier to quantify and is estimated by punching a disc from the ear and weighing, or measuring the thickness with a pair of callipers. DNA synthesis, orthine decarboxylase activity (ODC) and prostaglandin synthesis can all be examined in chemically induced erythema (Bourin *et al*, 1982). The rat or mouse paw oedema test is used as a test for inflammation. Inflammatory agents are injected into the hind paw of the animal and the oedema measured by a suitable volume displacement method (Bouclier *et al*, 1990). All of these assays, erythema of the ear and oedema of the ear or paw, can be altered to create models as tests for inhibition of inflammation. For example by applying topically an anti-inflammatory agent to the ear of a mouse twenty minutes prior to the application of TPA, the normal inflammatory response will either be inhibited or not (Williamson and Evans, 1981).

Sellotape ® stripping on the human epidermis has been used to study the level of orthine decarboxylase (ODC) inhibition by antipsoriatic drugs. Sellotape ® stripping is known to induce ODC production, PMN infiltration into the epidermal layers and thickening of the epidermis; just a few of the symptoms expressed in psoriasis. ODC activity can be assessed by the measurement of CO_2 production and soluble protein determined in the clear supernatant by direct fluoresence (280nm and 340nm); biopsies can be taken at a depth of 0.2mm by using a Castroviejo keratome set to study the histology of the skin (Arnold *et al.*, 1993).

The athymic nude mouse provides a model for studying skin diseases that may be grafted onto immunologically defective mice. Skin grafts can be maintained for long

periods of time because of the deficient lymphocyte-mediated response in the animal. Flaky skin (fsn) is an autosomal recessive mouse mutation with papulosquamous disease features similar to psoriasis. The fsn gene was discovered by the Jackson Laboratory research group (Bar Harbor, Maine U.S.A.) in 1985 (Sundberg *et al*, 1994). In fsn skin there is marked acanthosis, hyperkeratosis with focal parakeratosis, subcorneal pustules, dermal capillary dilation and a marked diffuse dermal infiltration of mixed inflammatory cells such as lymphocytes. The histological phenotype of the donor was maintained in the skin graft for up to ten weeks, the symptoms primarily being epidermal proliferation and dermal inflammation. The flaky skin mouse mutation is a potentially useful animal model that will serve as an important tool to unravel the mechanisms behind the diseases such as psoriasis and in testing for anti-psoriatic compounds. As yet, there have been no published studies of the value of this model and the testing of anti-psoriatic compounds (Sundberg *et al*, 1994; Lowe, 1988).

1.7.2. *In vitro* Tests

Klem (1978) was the first to publish a study on the effects of anti-psoriasis drugs on the growth of epidermal cells. Klem used GP18 cells, a clonal strain of epithelial-like cells derived from guinea pig epidermis selected for rapid growth and epithelial morphology. Anti-psoriasis drugs were tested for the effect on the ability of cells to incorporate thymidine into DNA, the ability of cells to incorporate amino acids into protein and the number of cells attached to the growing surface (population density) in each culture. It was established that the antipsoriasis drugs methotrexate, anthralin, mycophenolic acid, azaribine and hydroxyurea all inhibited thymidine incorporation into DNA, but not amino acid incorporation into protein. At concentrations of 0.5-50μg/ml the anti-psoriasis drugs inhibited cell proliferation. Anthralin was most potent against cell growth inhibiting the growth irreversibly and inhibited the utilisation of thymidine and deoxyuridine equally.

Recent studies have led to the development of human keratinocyte cell cultures. The bark extract from *Mahonia aquifolium* L. has been used in the past as a treatment for various skin diseases including psoriasis however its mechanism of activity has not

yet been established. Muller *et al.* (1995) have evaluated the anti-proliferative activity of the extract of the bark and its main constituent alkaloids against the growth of HaCaT cells, a rapidly multiplying human keratinocyte cell line. The proliferation of the keratinocytes was determined directly by counting the dispersed cells under a phase-contrast microscope after a 48 hour treatment. It was determined that the bark extract was an inhibitor of keratinocyte growth with an IC50 of 35μM. However the benzylisoquinoline alkaloids berbamine and oxyacanthine were more potent inhibitors by a factor of three (Muller *et al.* 1995).

Swiss 3T3 fibroblasts have been extensively used to analyse the mechanisms of mitogenic stimulation by neuropeptides and polypeptide growth factors and they provide a useful model system for the investigation of cell proliferation control. Potent mitogenic factors such as platelet-derived growth factor (PDGF) stimulate DNA synthesis in the absence of any other growth factors. The effects of these factors are mediated by multiple synergistic signalling pathways including arachidonic acid release and production of PGE_2. Castano *et al.* (1997) examined the inhibition of DNA synthesis by aspirin induced by these factors to discover that aspirin inhibits DNA synthesis and that the effect maybe mediated through the inhibition of prostaglandin synthetase (PGHS). *In vitro* cultured cell systems therefore can be useful in identifying new topically active drugs which are potentially useful as antipsoriasis agents or in the treatment of other proliferative skin disorders.

Enzymes used in bioassays to isolate active anti-psoriatic compounds include the cyclo-oxygenase and 5-lipoxygenase enzymes. 5-Lipoxygenase is an enzyme complex which catalyses the oxygenation of higher unsaturated fatty acids in position C-5 followed by dehydration. Products of this reaction are 5-HETE, 12-HETE, lipoxins, leukotrienes and LTB_4 and they promote inflammatory processes including allergic reactions such as asthma (Williamson *et al* 1996) (figure 1.1.7.). Psoriasis is characterised by the infiltration of macrophages and neutrophils mediated by the arachidonic acid metabolites e.g. 5-lipoxygenase. The recognition of lipoxygenase products as mediators of inflammation has led to a better understanding of the pathogenesis of psoriasis and has provided a new target for therapeutic intervention. Lipoxygenase inhibitors are categorised as antioxidants or radical scavengers and

inhibit the lipid peroxidation. They may also remove oxygen radicals which mediate the cell damage in skin diseases such as psoriasis (Muller and Ziereis, 1993) Lipoxygenases are prone to inactivation by active oxygen species, therefore lipoxygenases may be activated or suppressed and this is in line with antipsoriatic drugs which may either generate or scavenge active oxygen species. Two studies have been carried out on *Mahonia aquifolium,* with respect to lipoxygenase activity. Muller and Ziereis (1993) examined the main constituents berberine, berbamine and oxyacanthine testing them against 5-lipoxygenase peroxidation in phospholipid liposomes induced by 2,2'-azo-(bis-2-amidinopropane), deoxyribose degradation and for their activities against free radical 2,2-diphenyl-1-picrylhydrazyl. The bark extract of *M.aquifolium* was able to inhibit lipid peroxidation (IC_{50}=5μM), whilst its alkaloid constituents berberine, berbamine and oxyacanthin did not. Again a similar pattern was shown in the inhibition of 5-lipoxygenase (IC_{50}=50μM, *M.aquifolium* bark extract). Misik *et al* (1995) studied the four protoberberine alkaloids berberine, oxyberberine, jatrorrhizine, columbamine and the two aporphine alkaloids magnoflorine and corytuberine. Oxyberbarine, corytuberine and columamine were the most potent lipoxygenase inhibitors tested and berberine and magnoflorine exhibited only low potencies. A strong linear correlation between the lipoxygenase inhibition and lipid antioxidant properties of these compounds was found. From this data Misik concluded that the mechanism of lipoxygenase contributed the therapeutice effect of *M.aquifolium* in the treatment of psoriasis, whereas Muller and Ziereis concluded that there was no appreciable effects on lipoxygenase inhibition by the alkaloids. The 5-lipoxygenase enzyme can be obtained from homogenates of intact pig or rabbit leukocytes, rat PMNs or human neutrophils.

Cyclo-oxygenase catalyses the metabolism of arachidonic acid to prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$ and PGI_2) and may be obtained from sheep and pig seminal vesicles, rabbit and rat medulla from the kidneys, or the polymorphonuclear leukocytes (PMNs) or blood platelets maybe used as a whole system (Williamson *et al.,* 1996). The inhibition of blood platelet aggregation is often used as a test for anti-inflammatory compounds as blood platelets release inflammatory mediators in response to an external stimulus, which affect the proliferation of cells, an important part of the disease psoriasis.

Inflammatory responses, the release of reactive oxygen species and in the regulation of cell proliferation and tumour promotion, protein kinase C (PKC) has been identified as a key regulatory enzyme in the cellular signal transduction via the inositide cascade (Hegemann *et al.* 1992). PKC has also been identified as the cellular receptor for phorbol esters and there is evidence for the involvement of PKC in the pathophysiology of psoriasis. Hegemann *et al* (1992) tested the antipsoriatic drug anthralin on protein kinase C. Anthralin is known to attenuate lesional inflammation and often generates perilesional dermatitis. This phenomenon is reflected by its action on human leukocytes. Anthralin inhibits reactive oxygen species in phorbol ester-activated leukocytes, however it will also directly induce this response in unstimulated cells. Anthralin was found to inhibit the enzyme PKC in a dose-dependent manner, but did not display any stimulatory effects when investigated in a cell-free system (Hegeman *et al*, 1992).

AIMS AND OBJECTIVES OF THE RESEARCH

Psoriasis is a complex disease with ranges of severity from finger nail pits, small blemishes on the skin to the total disfigurement of the body which can be associated with arthritis.

Plant products are increasing in popularity amongst the general public for the treatment of a variety of conditions and in Europe plant remedies have always formed part of medicinal practise. Using this past experience four traditional plants were chosen which could prove useful in the treatment of psoriasis.

The objective of the research was to verify these past claims and investigate the mechanisms of action and constituents responsible for them. The aim was therefore to isolate the active compounds using the technique of bioassay guided fractionation, identify these compounds using modern spectroscopic techniques and finally evaluate their potential anti-psoriatic activity using a range of biological assays.

CHAPTER TWO

METHODS

2.1. Plant Material

G.glabra, G.aparine, B.perennis and *T.pratense*

Plant materials were obtained from the Herbal Apothecary, Syston, Leicestershire based in the United Kingdom. The stolons of *Glycyrrhiza glabra* originated from China, *Bellis perennis* flowers from France, *Galium aparine* herb from North America and the flower tops of *Trifolium pratense* came from Albania. All herbs were delivered as the dried plant material and stored in a dark dry environment. Voucher specimens have been retained for each plant species in the herbarium at the School of Pharmacy.

2.2. Extraction of Plant Material

G.glabra, G.aparine, B.perennis and *T.pratense*

100g of plant material was finely chopped using an electronic mill and exhaustively extracted using a soxhlet apparatus. As a screening process of anti-psoriatic activity was to be carried out with each plant, a range of solvents was used differing in polarity so that the compounds were already partially fractionated at the screening stage. The non-polar solvent hexane was used first to extract the plant material followed by chloroform, ethyl acetate, ethanol (all solvents supplied by BDH) and distilled water. Approximately 1.75 litres was used in each solvent extraction and each extraction took about twenty-one hours. Anti-bumping granules were used with all solvents.

The extracts, with the exception of the water extract, were evaporated under vacuum using a Buchi rotavaporator at 35°C. The crude extracts were further dried using a vacuum desiccator, weighed and stored under nitrogen at -20 °C. The water extract

was dried down overnight using an Edwards freeze drier and stored in the same manner as the other extracts.

Each extract was tested separately at 10 and 100μg for inhibition against chemically induced erythema on the mouse ear (assay one).

2.3. Chromatographic Separations

Materials

Silica gel 60 (70-230 microns) used for column chromatography was obtained from Merck (Germany), LH20 from Pharmacia Biotech (Sweden), sorbisil C60 (40-60 microns) from Rhone-Poulenc (UK) and Polyamide (>200 microns) from Schleicher and Schull (Germany).

Analytical T.L.C. plates used were aluminium silica G/UV254 from Whatman and micro-polyamide plates (type F1700) from Shleicher and Schull (Germany). Silica gel GF254 was obtained from Fluka Chemika (Switzerland).

The H.P.L.C system used consisted of a Waters 991 photodiode-array detector obtained from Millipore (UK), two pumps (Altex 11A, USA) and a controller (Altex 421, USA). A stainless steel C_{18} reversed-phase column, 25cm in length with a 0.5cm internal diameter and packed with 3-10 micron particles (MicroBond, UK) and a C_{18} waters pre-column (Millipore, UK) was used.

All solvents used were obtained from BDH, the acetone and chloroform was redistilled when used in final purifications.

2.4. *G.glabra* - Silica column

Silica gel 60 was used in a column (size 30x1.5cm) to fractionate the crude ethyl acetate extract from *G.glabra* which proved to be most active in the screening test, inhibition of chemically (TPA) induced erythema on the mouse ear.

1.2g of the ethyl acetate fraction was dissolved in acetone and adsorbed onto silica gel and dried. The whole procedure was carried out in a fume cupboard. Once the extract was adsorbed onto the silica gel it was applied to the top of the column. A layer of acid washed sand was placed on top followed by a piece of cotton wool to prevent any disturbance of the column. Elution of the column commenced with chloroform. The polarity of the solvent was then gradually increased using the following percentages of methanol 2, 5, 10, 20, 50, 80, 100%. The fractionation process was traced using analytical silica T.L.C. (solvent system 80%$CHCl_3$:20%MeOH and 70%$CHCl_3$:30%MeOH) and the running solvent of the column changed according to the elution of compounds. Initially 150 fractions, 15ml each, were collected and grouped into 12 fractions based on their similarities on analytical T.L.C.. The fractions were tested separately for inhibition of chemically induced erythema on the mouse ear. Further extraction and fractionation of the ethyl acetate fraction of *G.glabra* was done to obtain more material to work with but as the active fractions had been identified 100ml fractions were collected from the column instead of 15ml until the point at which the required fractions were eluted.

2.4.1. *G.glabra* - Polyamide column

Two active fractions had been identified from the screening assay. A range of solvents and adsorbents (e.g. silica, paper, cellulose and polyamide) were used in analytical T.L.C. to establish a suitable method of further fractionation. Silica, cellulose and paper did not result in successful separations. A sorbisil C60 (Rhone-Poulenc) and an LH20 (Pharmacia) column were also used without success in the separation of compounds. Polyamide was finally chosen as the most suitable adsorbent for further fractionation.

A polyamide (>200 microns) column was used to further fractionate active fractions five and six (0.367g) obtained from the ethyl acetate extract of *G.glabra* separated using the silica column. A column (5x1cm) was loaded with polyamide gel. The column commenced with a mixture of solvents, chloroform 20ml: methanol 10 ml: propan-2-ol 5ml : acetone (redistilled) 1ml. All solvents used were HPLC grade.

The quantity of methanol increased from 10,15, 20 to 25 ml, whilst the volumes of chloroform, propan-2-ol and acetone remained the same. Five millilitre fractions were collected and grouped together based on their similarities according to analytical T.L.C. (silica), the solvents used to elute the column increased in polarity according to the T.L.C. Visualisation of the compounds were assisted by observing the plate under U.V.light at 366nm and 254nm. Further analysis was done by spraying the plate with 60% H_2SO_4 and heating at 110°C for ten minutes. A total of thirty fractions were collected and were grouped together to form six, at this stage each fraction now contained between one and four compounds. The fractions were again tested separately for anti-inflammatory activity on the mouse ear.

2.4.2. *G.glabra* - Thin Layer Chromatography

Preparative T.L.C. using silica plates were used in further separation of fraction three from the silica column. Preparative T.L.C. using polyamide plates was used in the final purification of the active compounds isolated from *G.glabra.*

The preparative plates were made from silica gel GF254 and distilled water, one part silica to one part water. The adsorbent and water were mixed thoroughly and then spread on to the plates at a thickness of 0.2cm. Once dried the plates were activated in an oven at 100-110°C for thirty minutes and then cleaned in a tank using the proposed running solvent for the separation of compounds (90% chloroform and 10% methanol). This was done to clean the silica prior to the separation of the compounds. The plates were then reactivated by heating as before in the oven. Once cooled fraction three from the silica column was loaded on to the plate as a straight line 0.5cm from the base line and developed accordingly. The different bands, as identified using UV (365nm) and the spray reagent 60%H_2SO_4 (only a 0.5cm strip of the plate was sprayed), were then scraped off and eluted into solvent (90% chloroform and 10% methanol). Six fractions were obtained from the preparative T.L.C. and each one was tested separately using the screening assay. The active fraction from the six tested was purified using prep. polyamide T.L.C. as described below to give a single isolated compound.

Micro-polyamide plates were used to purify the two compounds isolated from the polyamide column and the single compound isolated from the preparative silica T.L.C. Plates were pre-run using the appropriate running solvent to clean the plates. The compounds were then loaded on to the plates 0.5cm from the base line and developed in a tank containing chloroform 20ml: methanol 10ml: propan-2-ol 5ml: acetone (redistilled) 1ml. The major bands were then carefully scraped off and eluted into solvents, the ratio of solvents being the same as the running solvent. HPLC grade solvents were used for all parts of the procedure. The three compounds were analysed using a range of bioassays and identified by means of spectroscopic techniques

2.5. *G.aparine* - Silica column

Silica gel 60 was used in a column (size 50 x 2cm) to fractionate the crude chloroform extract of *G.aparine*, which proved to be most active in the screening assay, inhibition of chemically induced erythema (TPA) on the mouse ear.

1.7g of the chloroform extract was dissolved into 1ml or near as possible of chloroform and loaded onto the column. As with previous work all column chromatography work was carried out in a fume cupboard. The extract was pipetted on to the column and adsorbed carefully into the top layer of the silica gel. Acid washed sand was then placed on top (2cm thickness) followed by cotton wool to prevent any disturbances of the column. Elution of the column began with 100% chloroform with a gradual increase in polarity by adding ethyl acetate in the following percentages 2, 5, 10, 50, 80 and 100% ethyl acetate and continuing with 10, 20, 50, 80 and finally a 100% methanol wash. The quantity of solvent used for each gradient step varied according to elution of compounds as illustrated using analytical T.L.C (silica). Fifty 100ml fractions were collected and grouped into eleven based on their similarities from analytical T.L.C. The fractions were tested separately for inhibition of chemically induced ear erythema; two fractions were identified as containing potentially active compounds.

2.5.1. *G.aparine* - Sorbisil Column

A column 50x1cm was prepared using sorbisil (silica gel C60, 40-60 microns) mixed with 100% chloroform. Aluminium foil was used to cover the column to prevent possible degradation of the compounds by light. 0.17g of fraction four from the silica column of *G.aparine* was dissolved into chloroform and loaded onto the column. Acid washed sand and cotton wool was then placed on top of the column. The column was eluted with 90% chloroform and 10% ethyl acetate (1000ml) which removed the majority of chlorophyll. The percentage of ethyl acetate in the eluting solvent was then increased from 10, 15, 20 and finally to 50%. The fractions were again grouped together according to their similarities based on the analytical T.L.C., six fractions were obtained and again tested using the screening assay technique.

2.5.2. *G.aparine* - LH20 Column

A column 40 x 1.5cm was packed using LH20 sephadex gel in a solvent of 30% chloroform and 70% methanol (HPLC grade). Again aluminium foil was used to cover the column to protect the compounds from the light. Fraction four (0.023g) obtained from the sorbisil column was dissolved into a mixture of chloroform and methanol (30%:70% respectively) and pipetted on to the top of the column. A small piece of cotton wool was then placed on top to prevent any disturbance to the column. Sand was not used as with a thorough washing the column could be used again. A non-gradient solvent system of 30% chloroform and 70% methanol was used, 100 ml fractions were collected until the point at which the compounds were eluted. Eleven 5ml fractions were collected and grouped into six fractions according to the analytical T.L.C. The six fractions were tested again using the screening assay.

2.5.3. *G.aparine* - Preparative T.L.C.

The active compound isolated from the LH20 column was purified using preparative T.L.C., using silica plates 0.2cm in thickness. Initially the solvent system acetone 1: hexane 1 was used to purify the compound from the remaining chlorophyll. Further purification was required using the solvent system ethyl acetate 70%: hexane 30%, to

isolate three compounds. The three compounds were analysed using the various bioassays described below and identified by means of spectroscopic techniques.

2.5.4. *G.aparine* - Analytical H.P.L.C.

From the proton NMR data it was established that compound two from the chloroform extract of *G.aparine* was a mixture of two compounds. This was undetectable using mass spectrometry and UV analysis alone. Using the method of Khachik *et al.*, (1995) separation of the two compounds using HPLC was attempted.

Analytical HPLC separation was carried out on a stainless steel C_{18} reversed-phase column, 25cm in length, 0.5cm internal diameter and packed with 3-10 micron particles, using a C_{18} waters pre-column. The samples were injected manually into a 20 µl loop using a 50 µl micro-syringe.

The solvents used were HPLC grade. Each one was degassed using a sonicator for 30 minutes prior to use. Analytical separation of the compounds was attempted by employing a combination of isocratic and gradient chromatography. An isocratic mixture of acetonitrile (85%), methanol (10%) and hexane-dichloromethane (1:1)(5%) (containing 0.1% diisopropylethylamine) at time 0 was followed by a linear gradient beginning at time 10 minutes completing at time 40 minutes. The final ratio of mixture was acetonitrile 45%: methanol 10%, hexane-dichloromethane (1:1) 45%. The flow rate was 0.7ml/minute and chromatograms were monitored at wavelengths of 250, 300, 350, 400, 450 and 500nm using the photodiode array detector to determine separation of the two compounds and the presence of impurities. Elutes were collected manually. Data were processed using PDA software version 6.22 and chromatograms printed using a Waters 5200 printer.

2.6. Spectroscopic Techniques

UV spectroscopy, mass spectrometry and nuclear magnetic resonance were used to identify the isolated compounds and confirm their molecular structures.

2.6.1. UV Spectroscopy

All spectra were obtained from an SP8-100 ultraviolet spectrophotometer (PUE UWICAM) using standard quartz cells. The solvent used was HPLC grade methanol for all compounds. For flavonoid compounds the addition of one drop (1mM solution) of either NaOH, $AlCl_3$, $AlCl_3$ + HCl, NaOAc or NaOAc/ H_3BO_3 was used to identify shifts in the UV spectrum characteristic to the compounds. The addition of 1mM solution of HCl to compound 315A was used to confirm as to whether it was an epoxide by the presence of 13-40nm shift increase.

2.6.2. Mass Spectrometry (MS)

Fast atom bombardment (FAB) was used to establish the MS and accurate mass of the compounds within this study, the MH+ (molecular ion) being the most abundant ion. The MS of the compounds was measured on a VG Analytical Ltd. ZAB IF spectrometer at room temperature; this procedure was carried out by Mr M.Cocksedge, at the London School of Pharmacy.

2.6.3. Nuclear Magnetic Resonance (NMR)

All spectra were run on a 400MHz Bruker AM 400 spectrometer. The NMR experiments were carried out by Mrs Jane Hawkes, Chemistry Department, Kings College, University of London. The pure compounds were dissolved in deuterated chloroform ($CDCl_3$). One drop of tetramethylsilane (TMS, Sigma) was added to the compound solution to act as an internal standard. The chemical shifts in the proton (^1H) NMR were quoted in parts per million and coupling constants (J) in Hertz (Hz). The following symbols were used to identify the protons: (s) represents a singlet, (d) represents a doublet, (dd) represents a double doublet, (t) represents a triplet, (q) represents a quartet (b) represents a broad singlet and (m) represents a multiplet.

Strongly coupled protons connectivities were obtained from correlated spectroscopy (COSY). Non-bonding proton-proton interactions were obtained from the nuclear overhauser and exchange spectroscopy (NOESY).

A carbon nuclear magnetic resonance (^{13}C-NMR) spectrum was attained by preparing the pure compounds as described in ^{1}H-NMR using TMS as an internal standard. The chemical shifts were quoted in parts per million. Distortionless enhancement polarisation transfer techniques (DEPT) was used to distinguish between signals for quaternary carbons and all others

2.7. Biological Assays

A selection of biological assays was used in this study to establish the potential anti-inflammatory activity of the compounds isolated. The in-vivo assays include inhibition of chemically induced erythema on the mouse ear (used as the bioassay guided technique) and inhibition of physically induced erythema on the dorsal skins of rats (used only with isolated compounds). In-vitro assays include inhibition of blood platelet aggregation and inhibition of normal and TPA induced proliferation of Swiss 3T3 mouse fibroblast cells.

2.8. Inhibition of Chemically Induced Erythema on the Mouse Ear

Inhibition of chemically induced erythema on the mouse ear by 12-O-tetradecanoyl phorbol-13-acetate (TPA) was initially used as a screening test for biological guided fractionation, as inflammation is an important part of the disease psoriasis. Chemically induced erythema of the mouse ear is an easy and effective test as mouse ears are translucent making the erythema response quite visible.

Materials

The TPA was obtained from Sigma Chemicals (USA) and dissolved into HPLC grade acetone (BDH). Micro-pipettes were obtained from Drummonds (UK) and the mice from Charles-River (UK).

2.8.1. Animals

Four or six CD1 female mice weighing 20g were housed together on wood shavings as bedding. Standard pelleted food and water were available ad libitum, the temperature was 22-23°C and the room was lit from 0700 hours to 1900 hours.

2.8.2. Assay Protocol for Inhibition of Chemically Induced Erythema

Assay One

Assay one was used for the bioassay guided fractionation of the compounds. In the initial screening of the crude extracts two concentrations were used only (10 and 100μg/ear). Five micro litre aliquots of the test solution were applied by means of a micropipette (Drummonds) to the outer surface of the left mouse ear. Twenty minutes later 0.1μg/ear TPA (0.16μM) was applied to the same ear in a single 5μl aliquot. The ears were examined at two, three, four, six , seven and twenty-hours after the application of TPA. The maximum inflammatory response to TPA occurs at six hours after application. One group of mice were used as a negative control group receiving 0.1μg/ear of TPA only.

Anti-inflammatory activity was based on the redness of ears, although quantification maybe argued as being subjective, erythema can be assessed on an all or none basis. The absence of erythema may be described as a positive anti-inflammatory response, whereas a negative response would be recorded if the vasodilatation of major blood vessels were apparent or redness of the ear between blood vessels. Further studies on the pure isolated compounds included a dose response study, testing the compounds at 0.1, 0.5, 1, 5, 10, 20, 50 and 100μg/ear. The following symbols were used to describe the anti-inflammatory response;

+ erythema absent in 100% of mice, positive anti-inflammatory response

- erythema present in less than 50% of mice

- - erythema present in 50% or more of the mice

- - - erythema present in 100% of the mice, negative anti-inflammatory response

Assay Two

A variation to assay one was used whereby application of TPA was applied twenty minutes prior to the application of the isolated active compound. The ears were again examined at two, three, four, six , seven and twenty-hours after the application of TPA. One group of mice was used as a negative control group receiving 0.1μg/ear of TPA. Dose response studies were established for all pure compounds, testing at the same concentrations used in assay one.

Mice were reused a total of three times; after the third experiment mice were disposed off using the Home-Office schedule 1 procedure.

2.9. Blood Platelets

Materials

Tri-sodium citrate, citric acid, glucose and sodium hydroxide pellets used in the buffer solution were all supplied by BDH (UK) and the pH meter was supplied by Mettler Delta. Butterfly needles (type 21) were supplied by 3S Health Care and the sterile disposable syringes by BDH, both the bench centrifuge and the biofuge (Micro Centaur) were supplied by MSE (UK). Presept tablets were supplied by Johnson and Johnson medical Care (UK). Acetylsalicylic acid, colchicine, ADP, TPA and a 25% solution of glutaraldehyde solution were all supplied by Sigma. Philips XL20 scanning electron microscope was used to take photographs of platelets.

2.9.1. Assay Protocol For Inhibition Of Blood Platelet Aggregation

Citrate Buffer

A trisodium citrate buffer was used as an anticoagulant, acting by lowering the Ca^{2+} levels preventing thrombin release. The trisodium citrate buffer contains citric acid 72 mM (303mg), trisodium citrate 90 mM (529mg) and glucose 100 mM (360mg). All were dissolved into 20ml of de-ionised water. The pH was adjusted to 6.5 by adding sodium hydroxide pellets or solution. A fresh buffer solution was prepared on each occasion.

Blood Collection

Male human blood was taken by forearm venipuncture from healthy volunteers who had denied medication for the previous 14 days. Two 60ml plastic sterile syringes containing 5ml of citrate buffer solution were filled with 45ml of blood, the concentration of the buffer being 10% of the total volume. The total volume of blood and buffer was therefore 100ml.

Preparation of Platelet-Rich-Plasma and Platelet-Poor-Plasma

The blood was then transferred to two Falcon tubes which were gently inverted to mix the buffer and blood. The citrated blood was centrifuged at 1,000 rpm for twenty minutes in a bench centrifuge to separate the blood in to an upper and lower layer. The upper layer (supernatant) was referred to as platelet-rich-plasma (PRP) a translucent straw-coloured serum, the lower level containing the erythrocytes was discarded. 1ml of the PRP was placed into an Eppendorf tube and spun for a further 10 minutes at 11,000rpm using a biofuge to give platelet-poor-plasma (PPP). The PRP and PPP were then left for 30 minutes to settle.

Platelet Aggregation

A Payton dual channel aggregometer was used to measure the inhibition of platelet aggregation. The temperature was adjusted to 37°C; the module range to 4 and the number of revolutions per minute set at 800. The PPP was used to adjust the machine for 100% light transmission and PRP for 0% light transmission.

500µl of PRP was placed in a cuvette with a metal stirrer and placed into one of the channels for two minutes to allow the PRP to warm up to 37°C. A negative control to inhibition of platelet aggregation was established by adding 2.5µl of distilled acetone and 2.5µl quantity of either TPA (0.25µg, 0.405µM) or ADP (16µg, 37.44µM). Only 1% of the total volume (500µl) could be added. A greater volume of acetone was found to give inaccurate results. A positive control response was established by adding an already known inhibitor of platelet aggregation, acetyl salicylic acid and colchicine to both TPA and ADP induced aggregation, and establishing a dose response curve.

After two minutes of the test compounds (2.5µl) being added to the PRP, TPA was added to channel one and ADP to channel two. Inhibition of platelet aggregation was tested at two minutes for TPA and one minute for ADP. Aggregation was however monitored for three minutes. The crude extracts were all tested at 10 and 100µg. A dose response study was established for each active crude extract testing positive for inhibition against chemically induced erythema. Concentrations used in the dose response study for the active extracts and the pure compounds were 0.1, 0.5, 1, 5, 10, 20, 50, 100 and 200µg. Each dose was repeated three times per platelet sample and platelets were kept no longer than three hours. All contaminated equipment and waste materials were disposed of by autoclaving. Some equipment was sterilised using a solution of Presept (Johnson & Johnson Medical, UK).

Platelet aggregation was measured as the percentage change in light transmission as recorded. Inhibition of aggregation was measured as the loss of reduction in height of the curve as produced for a 100% platelet aggregation induced either by TPA or ADP. The standard deviation was calculated for each set of results to give an indication of the extent of variation in each group as a whole. Assuming the values were normally distributed a one sided paired students t-test was used to test the null hypothesis H_o, the alternative hypothesis being H_1 :

H_o:$\mu_d \geq$ O. The population mean of the differences is zero or more ,i.e. the compounds have no effect on platelet aggregation or increase the percentage of aggregation.
H_1:$\mu_d <$ O. The population mean of the differences is less than zero, i.e. the compounds decrease the percentage of aggregation.

A paired T-test was used to accept or reject the null hypothesis as the two sets of data were not independent from each other. If the t-value was tabulated below 0.01 (p-value) then there was good evidence at the 1% level to suggest that the compounds decrease the percentage aggregation as induced by TPA or ADP. The levels of significance were recorded at the 1% and 5% level.

A Philips xL20 scanning electron microscope was used to take photographs of blood platelets in the non-aggregated and aggregated state (x 5000 magnification). A pellet of platelets was obtained from 1ml of PRP by centrifuging for 10 minutes at 11,000rpm using a biofuge. The platelets were then stored in a 2% solution of glutaraldehyde using the citrate buffer solution. Scanning electron microscope photographs were taken by Mr D.McCarthy, London School of Pharmacy, University of London.

2.10. Swiss 3T3 Mouse Fibroblast Cell Culture

The aim of the study was to establish the ability of purified compounds, derived from the bioactivity guided fractionation, to inhibit the natural proliferation of 3T3 Swiss fibroblasts or when induced by TPA.

Materials

The cell line was donated by Professor Parker of ICRF. The Dulbecco's Modified Eagle Medium (DMEM) supplemented with sodium pyruvate and D-glucose, heat inactivated foetal bovine serum (FBS) and kanamycin were all supplied by Life Technologies (UK). Tissue culture flasks were supplied by Greiner, the CO_2 220 incubator and laminar flow cabinet by Flow Laboratories. Trypsin-EDTA solution and Typan-Blue solution was supplied by Sigma. The inverted microscope (TMS) was supplied by Nikon (UK).

2.10.1. Preparation of Culture Medium

The cell line was grown in Dulbecco's Modified Eagle Medium supplemented with sodium pyruvate and D-Glucose, with the addition of 10% FBS and the anti-fungal agent kanamycin (0.05μg/500ml media). The media was kept at 4°C and discarded after one month if not used. The FBS and kanamycin were stored at -20°C.

2.10.2. Initiation and Maintenance of Cultures

Stock cultures were maintained in 250ml flasks at 37°C in a humidified atmosphere of 10% CO_2, 90% air . The cell cultures were maintained in a sub-confluent state by passing every four days (density, 0.5-5 x 10^5 cells/ml). After twelve passages the cells were replaced by a new batch (passage 2-4) to eliminate any increase in spontaneous differentiation. All work was carried out in aseptic conditions within a laminar flow cabinet. Waste cultures and contaminated equipment were disposed of by decontamination with hypochlorite solution.

2.10.3. Trypan Blue Exclusion Test

The viability of the cell cultures were assessed using the Trypan Blue stain test. This test is based on the principle that live cells do not take up certain dyes, whereas dead cells do.

Cells were removed from the standard tissue culture flask using a 1% solution of Trypsin-EDTA solution and resuspended in 10ml of media (previously warmed in the incubator to 37°C) and pipetted off into a Falcon tube. The cells were then spun for five minutes at 2,000 rpm using a bench centrifuge , the existing media removed and the cells again re-suspended in the 10% FBS media and the cells dispersed by vigorous pipetting. 100µl of this suspension was taken and added to an equal volume of 0.4% Trypan-Blue and allowed to stand for 15 minutes. A small amount of Tryphan-Blue cell solution was transferred to both chambers of an hemocytometer by carefully touching the edge of the coverslip. Using an inverted microscope, starting in chamber one, the centre 1mm square and the four corner squares were counted for viable and non-viable (stained) cells. If 10% or more of the cells appeared clustered the procedure was repeated after vigorous pipetting of the solution. The average cells per ml, total number of cells and the % cell viability was counted using the equations below. The % cell viability was always maintained above 95%.

Cells per ml = the average count per square x dilution factor x 10^4 (count 10 square)

Total Cells = cells per ml x the original volume of fluid from which cell samples were removed

%Cell Viability = total viable cell (unstained) ÷ by total cells (stained and unstained) x 100%

2.10.4. Preparation of Cells For Assay

Sterile 6 x 24 well (1ml) plates were used for the proliferation assay. Cells were removed from the standard tissue culture flask using a 1% solution of EDTA and resuspended in 10ml of media and pipetted of into a Falcon tube. The cells were then spun for five minutes at about 2,000 rpm, the existing media removed and the cells again resuspended in 10ml of 10% FBS medium and dispersed by vigorous pipetting. The 10ml of suspension was then added to 135ml of 10% FBS medium in a sterile glass jar and mixed. 1ml of the media cell suspension (1 x 10^4cells/ml)was then placed into each well and the plates incubated at 37°C at 10% CO_2 for three days, until the cells were confluent.

2.10.5. [^{3}H]-Thymidine Incorporation

Materials

[^{3}H]- thymidine and dimethyl sulphoxide (DMSO) were supplied by Sigma (U.K.). The phosphate buffered saline (PBS), trichloroacetic acid (TCA) and sodium hydroxide were supplied by BDH and the sodium dodecyl sulphate by ICN. The scintillation vials were supplied by National Diagnostics, the scintillation fluid (Ecolite) by ICN and a Beckman Scintillation Counter (LS6000TA) was used .

Protocol

Cells were dosed with the test compounds two days prior to the addition of [^{3}H]-thymidine. 72µl was added to 144ml of 1% FBS media to dose the corresponding six plates, therefore 0.5µl of [^{3}H]-thymidine was added per well. 100µl of the media was taken to measure the radioactive dose (37Bq=1mCi) and added to scintillation vials containing 4ml of scintillation fluid. The radioactive media was then transferred to 144 sterile specimen tubes and dosed with the test compounds before adding to the cells. This took place in a laminar flow cabinet, although the air flow was switched off to prevent personal contamination. After the cells were dosed they were returned to the incubator and pulsed with the radiolabelled thymidine (0.5µCi/ml) for four hours. After pulsing the cells were washed twice with 0.5ml (per well) ice cold 10% PBS, and incubated for 30 minutes using 1ml (per well) of ice cold 5% TCA at room temperature. The TCA was then removed and another 1ml of 5% TCA added for five minutes. After the TCA was removed for the second time 0.3M NaOH and 0.1% sodium dodecyl sulphate was added to each well for 30 minutes (1ml/well). The contents of each well were added to scintillation vials containing 4ml of scintillation fluid rotamixed, rested for thirty minutes and each sample counted for four minutes using a scintillation counter. Three different assays were carried out using this protocol for inclusion of [^{3}H]-thymidine.

2.10.6. Inhibition of Cell Proliferation Assays

Cytotoxicity Testing of Crude Extracts and Pure Compounds

After 72 hours of incubation and the cells were confluent a concentration gradient of 0.01, 0.1, 1.0, 10 and 20 µg of the test compounds or extracts were added to the cells, in 2.5µl quantities plus 2.5µl DMSO. The 2.5µl DMSO was added so a comparison could be made in the results with further dose response studies whereby 2.5µl of TPA was used to induce cell proliferation. The 24 well plates were divided into six rows of four, therefore the first row of wells were left untreated as the control. In the majority of samples (all pure compounds) tested the vehicle solution was sterile DMSO, however in some cases sterile water (water extract) and re-distilled acetone (hexane extract) had to be used. The cells were incubated as before for a further 48

hours at which point the assay protocol for [^{3}H]-thymidine incorporation assay was carried out. The radioactive media was dosed with exactly the same doses as at the start of the experiment.

Dose Response Study of TPA Induced Proliferation

A dose response study was carried out on the Swiss 3T3 fibroblast cells to establish the concentration of TPA required to ensure that the proliferation of cells would be induced in all experiments. The cells were dosed three days after seeding when the cells were confluent with a 5µl quantity of TPA (dissolved in DMSO). The concentrations tested were 0.05, 0.1, 0.3, 0.5 and 0.7 µm as established from previous research reports from other people. After 48 hours of incubation with TPA the [^{3}H]-thymidine incorporation assay was carried out, the radioactive media being dosed with the appropriate concentrations of TPA.

Inhibition of TPA Induced Proliferation

After 72 hours of incubation and when the cells were confluent, a dose response study was established testing the inhibition of TPA induced proliferation on Swiss 3T3 mouse fibroblasts. A known quantity of the compound (0.01, 0.1. 1, 10, 20µg) was added in 2.5µl to the media with a 2.5µl dose of TPA (0.5µm), therefore a total of only 5µl was added to the media. The objective of this experiment was to establish if the compounds could successively inhibit the TPA induced proliferation of cells especially at doses whereby they were not cytotoxic to normal cell growth. After 48 hours of incubation the cells were tested using the [^{3}H]-thymidine incorporation assay to establish the percentage of cell growth as compared to the control.

Inhibition of TPA Induced Proliferation, After Prior Exposure to Test Compounds For 24 Hours

After 72 hours of incubation, the cells were dosed with a known quantity (0.01, 0.1. 1, 10, 20µg) of the test compound in 2.5µl solution, plus 2.5µl of DMSO. After a further 24 hours of incubation the media was removed and replaced with fresh media dosed with the corresponding test compound plus a 2.5µl aliquots of TPA(0.5µm).

The objective of this experiment was to observe whether prior incubation with the test compound had any effect on the TPA induced proliferation of the cells. After a further 48 hours the cells were assayed for the percentage increase or decrease in cell growth as compared to the control using the [^{3}H]-thymidine incorporation assay.

In each of the three assays the dosage of compounds was tested in quadruplicate and the experiments repeated three times. The standard error of mean was calculated for each dose tested. Assuming the values were normally distributed a two-sample students t-test (two-sided) was used to test the null hypothesis H_o, the alternative hypothesis being H_1 :

H_o:μ_d = O. The population mean of the differences is zero ,i.e. the compounds had no effect on the tissue culture.
H_1:$\mu_d \neq$ O. The population mean of the differences is more or less than zero, i.e. the compounds either decrease or increase the proliferation of cells.

A two-sample T-test was used as the sets of data were independent from each other. Using the t-test the null hypothesis could either be rejected or accepted. The levels of significance were recorded at the 1% and 5% level.

2.11. Physical Erythema

Ultraviolet ray B induced dermatitis in the rat was chosen as a model for human psoriasis.

Materials

Male Wistar rats were supplied by the School of Pharmacy. Hypnorm was supplied by Janssen (UK). The transilluminator (TM20E) and UVX radiometer were supplied UV Products Limited. A Lertz Base Sledge Microtone (1400 model) was used to take specimens. The 1% eosin and haemotoxylin solutions were supplied by Sigma. Nikon Microplat FXA Microscope (plan apo objective at x4 and x10) was used to take photographs.

2.11.1. Preparation of Animals

Male Wistar rats were used in this assay as previous research conducted by Nakaguma *et al.* (1995) concluded that this strain of rat responded more quickly to UVB light than other strains tested. Five male rats, each weighing 180g, were used per test group and housed together using wood shavings as bedding; standard pelleted food and water were available *ad libitum* and the room temperature kept at 22-23°C. Twenty four hours prior to the experiment animals were anaesthetised using 0.2ml Hypnorm and the dorsal area of the body clipped and shaved carefully using a sharp razor blade, washed and then dried.

2.11.2. Assay Procedure For Inhibition Of Physically Induced Erythema

A transilluminator lamp (302nm) was used to provide the source of UVB light (5x20 watt bulbs), the protective covering was removed from the lamp so a greater intensity of light could be achieved by exposing only the bulbs. The transilluminator was then rotated and placed onto a specially made stand, so that the light source was directed downwards.

Rats were anaesthetised with 0.2ml Hypnorm and placed onto a box and then covered by a piece of black polythene with two rectangles ($2x1cm^2$) cut out so as to expose two areas of the dorsal skin without harming the rest of the animal. Once the lamp had stabilised, this was established using a UVX radiometer, the rat was placed beneath the lamp for a time period of 390 seconds. For personal safety, the surround of the transilluminator was also covered by black polythene to prevent exposure of myself and colleagues to the UVB light. After 390 seconds the rat was removed from the UV light source and the areas of exposure marked using a permanent marker pen.

A pilot study was initially done to establish the intensity of light required to induce erythema and proliferation of the cells. The intensity of light was altered by changing the distance of the animals from the light source. The intensities of light were as follows $1.74J/cm^{-2}$, $1.90J/cm^{-2}$, $2.10J/cm^{-2}$, $2.40J/cm^{-2}$ and $2.66J/cm^{-2}$. On the fifth

day histological post mortem sections were taken and stored in a 10% solution of formaldehyde. All sections were processed by Dr Alex Brown at the department of Morbid Anatomy and Histopathology at the Royal London Hospital. Each specimen was stained using 1% solution of haematoxylin and eosin. Photographs of the skin sections were take by Mr.D.McCarthy using a Nikon Microplat FXA microscope at x4 and x10 magnification.

From the pilot study it was established that the UV exposure required to achieve the degree of proliferation of cell layers and thickening of the epidermal layer was $2.4J/cm^{-2}$. When testing the compounds exactly the same procedure was used except immediately after UVB exposure 100μg of the test compound in a suitable vehicle solution (HPLC acetone) was applied to an area of UV exposed skin and a control area. The vehicle solution was applied to the control UVB exposed area. The application of the test compounds was repeated daily for five days, when histologically post mortem sections were taken.

There were four skin samples taken from each animal, each one receiving different treatments. The skin samples were as follows: a control sample receiving no treatment, a skin sample with UVB exposure only, a skin sample exposed to UVB with application of the test compound afterwards and a skin sample with application of the test compound only. Observations of the skin morphology during the five days was recorded, for example the presence of scaling or erythema of the skin. From each skin sample two sections were mounted per slide. Skin samples were taken from the central point of any treatment. The two skin sections taken from each sample were placed onto a slide and examined under a microscope at x40 magnification. In each section taken from the animals the numbersof cells in the epidermal layer were counted at random in ten different areas and the epidermal layer thickness measured. Five animals were used per group.

The mean and standard deviation was calculated for the four different skin samples from each animal. The standard error of mean was calculated for each group of five animals. The difference was calculated for treated skin samples as compared to the control skin samples with no treatment at all. The data were then analysed using one

sided paired t-test using Microsoft EXCEL. The experimental design was such that a paired t-test could be carried out on the data so as to eliminate the natural variation in the number of cells in the epidermal layer and the thickness of the epidermal layer, that would normally occur in different rats. The following hypotheses were used:

H_o:$\mu_d \leq$ O. The population mean of the differences is zero or less, i.e. the skin treatment had no effect on the number of cells in the epidermis or the number of cells was less than in the control

H_1:$\mu_d \geq$ O. The population mean of the differences is more than zero, i.e. the skin treatments increased the number of cells in the epidermis

The UVB exposed skin with the test compounds was then compared to the control skin with UVB exposure only, to determine if the test compounds had inhibited UVB induced erythema. The hypothesis tested were:

H_o:$\mu_d \geq$ O. The population mean of the differences is zero or more, i.e. the application of the test compound to UVB irradiated skin had no effect on the number of cells in the epidermis or the number of cells increased

H_1:$\mu_d \leq$ O. The population mean of the differences is less than zero, i.e. the application of test compounds to UVB irradiated skin decreased the number of cells in the epidermis as compared to UVB irradiated skin only

Parallel hypotheses could be made for the thickness of the epidermal layer. The level of significance in both cases was determined at the 1 and 5% level.

CHAPTER THREE

RESULTS

3.1. Screening of sequential extracts obtained from *G.glabra*, *G.aparine*, *T.pratense* and *B.perennis*

The initial screening programme evaluated the species *Glycyrrhiza glabra* and *Trifolium pratense* both from the Leguminosae family, *Galium aparine* from the Rubiaceae and *Bellis perennis* from the Compositae in three short term *in vivo* and *in vitro* biological assays. The experiments included inhibition of chemically induced erythema on the mouse ear, which was subsequently used as the technique for bioassay guided fractionation, inhibition of chemically induced blood platelet aggregation and cytotoxicity testing using Swiss 3T3 mouse fibroblast cells. The objective of the study was to substantiate past reports that these plants possess anti-psoriatic activities. Those which do express such an activity were based on the fact that psoriasis exhibits inflammatory and proliferative symptoms which the tests have incorporated.

The four plants were sequentially extracted using five solvents that increased in polarity. The total yields obtained from the 100g of plant material extracted were as follows: *G.glabra* 22.85g, *G.aparine* 20.84g, *T.pratense* 27.48g and *B.perennis* 24.03g.

3.1.1. Inhibition of chemically induced erythema using crude plant extracts

Inhibition of erythema on the mouse ear is a very simple procedure and the translucent ears of the mice make the reddening of the ear quite visible. The concentration of TPA used to induce the erythema was established using results of past research (Williamson & Evans, 1981: Evans & Schmidt, 1979) and testing either side of the optimum dose producing a 100% response. This ensured that in all test animals, in the absence of inhibitors, an erythema response would be achieved

each time. The procedure used for initial screening of the extracts and used for biological fractionation was described as assay one, whereby TPA was applied after the test extracts.

Although some animals showed a negative response, the degree of erythema could be affected by the compounds tested. For example some animals responded to the TPA by vasodilatation of major blood vessels only and the compounds were therefore recorded as slightly inhibiting erythema. However other animals responded by the vasodilatation of major blood vessels with a severe redness of the ear. This was recorded as a negative response and as pronounced erythema. The anti-inflammatory activity of the test compounds was recorded on an all or none basis. If there was any sign of erythema no matter how minor it was recorded as negative response of inhibition. The inhibitory response was therefore recorded as a percentage of the number of mice exhibiting anti-inflammatory activity.

Table 3.1.1. lists the anti-inflammatory effects of the crude extracts from the four plants chosen at six hours after the application of TPA, the time at which TPA produces its maximum effect.

From the five extracts obtained from *G.glabra* the ethyl acetate extract expressed the most significant anti-inflammatory activity followed by the chloroform extract. At 100μg/ear both extracts completely inhibited erythema at six hours after the application of TPA and a positive inhibitory response was still present at 24 hours. A dose response study was done to establish the IC50 (dose required to produce a 50% inhibitory response to the TPA). At 20μg/ear the ethyl acetate extract inhibited the erythema completely at six hours and at twenty four hours. At 15μg/ear one mouse showed an erythema response and at 5μg/ear three mice out of the four had an inflammatory response. The IC50 of the ethyl acetate extract was established between 5 and 10μg/ear.

Table 3.1.1. **Anti-inflammatory Screening of the Crude Plant Extracts Against Chemically (TPA) Induced Erythema on the Mouse Ear**

Plant Extracts	*Glycyrrhiza glabra*		*Galium aparine*		*Trifolium pratense*		*Bellis perennis*	
	Concentration µg							
	100	10	100	10	100	10	100	10
Hexane	-	- -	- - -	- -	-	- - -	-	- - -
Chloroform	+	- -	+	+	-	- - -	- - -	- - -
Ethyl Acetate	+	-	+	- - -	- - -	- - -	- -	- - -
Ethanol	+	- -	- - -	- - -	- - -	- - -	- - -	- - -
Water	- - -	- - -	- - -	- - -	+	-	+	-

Number of mice per group was four

\+ No sign of irritancy, a positive anti-inflammatory response in 100% of mice

\- Inhibitory activity in 50% or more of mice

\- - Inhibitory activity in less than 50% of the mice

\- - - Inhibitory activity not recorded in any of the mice

Observations recorded at six hours, length of time taken for TPA to induce a maximum erythema response

The chloroform extract of *G.aparine* has a strong anti-inflammatory activity at both 100 and 10µg/ear at six hours. However the following day at 24 hours inhibition was only noticeable with those dosed at 100µg/ear. Some anti-inflammatory activity was also exhibited by the ethyl acetate extract at 100µg/ear. A dose response study was established using the chloroform extract and an IC50 estimated between 5 and 10µg/ear.

T.pratense exhibited some anti-inflammatory activity with both the chloroform and water extract, but in either case inhibitory activity was not expressed at 24hours. At 10µg/ear the chloroform extract did not exhibit anti-inflammatory activity and the mice tested with a 10µg/ear dose of water extract a slight erythema was observed at six hours. None of the crude extracts from *B.perennis* showed a significant inhibitory response to the TPA induced erythema. The water extract at 100µg/ear inhibited

erythema up until six hours only. At 10μg/ear a slight erythema was observed. The ethanol extract at 100μg/ear also exhibited some anti-inflammatory activity. No further fractionationsof the *T.pratense* and *B.perennis* extracts were undertaken on the basis of these results.

3.1.2. Inhibition of blood platelet aggregation using crude plant extracts

The inhibition of blood platelet aggregation was measured using a Payton dual channel aggregometer. This allowed measurements of ADP and TPA induced aggregation to be carried out simultaneously. The induction of platelet aggregation by TPA and ADP increases the amount of light transmission through the platelet rich plasma. The inhibition of platelet aggregation was monitored using a chart recorder and calculated as the decreased height of the curve as compared to the control producing a 100% platelet aggregation, induced either by TPA or ADP. The inhibition in aggregation was therefore monitored by the reduction of light transmission as compared to the control.

Each extract obtained from the four plant species was tested at 10 and 100μg. Table 3.1.2. lists the percentage inhibition of TPA and ADP induced platelet aggregation and the standard deviations. *G.glabra* presented the least inhibitory response to aggregation, although inhibition to TPA and ADP induced aggregation differed considerably. The hexane, chloroform and ethyl acetate extracts all expressed similar results to TPA induced aggregation, the average percentage of inhibition being 21% when tested at 100μg. ADP induced aggregation was strongly inhibited by the chloroform extract at 100μg. A 43% inhibition was recorded. The hexane and ethyl acetate extracts exhibited a 36 and 30% inhibition respectively at this dosage.

G.aparine also expressed strong anti-platelet aggregation activity within both the hexane and chloroform extracts. The hexane extract inhibited TPA induced aggregation by 31 and 55% at 10 and 100μg correspondingly, ADP aggregation was also similarly inhibited. The chloroform extract also inhibited the ADP and TPA

Table 3.1.2. Inhibition of TPA and ADP Induced Blood Platelet Aggregation by the Crude Plant Extracts

Species	Extract	Concentration µg	% Inhibition of TPA Platelet Aggregation	SD	%Inhibition of ADP Platelet Aggregation	SD
Glycyrrhiza glabra	Hexane	100	22	0.12	36	0.10
		10	5	0.42	14	0.21
	Chloroform	100	22	0.12	43	0.23
		10	12	0.12	15	0.17
	Ethyl Acetate	100	20	0.26	30	0.36
		10	14	0.00	22	0.12
	Ethanol	100	6.8	0.26	11	0.12
		10	-1.5	0.06	11	0.00
	Water	100	7.6	0.15	11	0.10
		10	-3	0.25	-5	0.10
Galium aparine	Hexane	100	55	0.06	47	0.32
		10	31	0.10	40	0.12
	Chloroform	100	42	0.44	49	0.44
		10	12	0.15	23	0.15
	Ethyl Acetate	100	26	0.10	23	0.17
		10	15	0.12	7	0.06
	Ethanol	100	9	0.06	19	0.06
		10	9	0.10	13	0.06
	Water	100	14	0.21	13	0.06
		10	10	0.06	12	0.25
Trifolium pratense	Hexane	100	51	0.15	56	0.15
		10	40	0.21	37	0.38
	Chloroform	100	52	0.10	66	0.12
		10	35	0.06	44	0.29
	Ethyl Acetate	100	36	0.06	50	0.06
		10	31	0.10	32	0.40
	Ethanol	100	25	0.06	24	0.38
		10	13	0.06	25	0.26
	Water	100	8	0.23	0.6	0.06
		10	3	0.25	-8.8	0.10
Bellis perennis	Hexane	100	45	0.10	40	0.15
		10	29	0.23	24	0.30
	Chloroform	100	30	0.20	33	0.15
		10	21	0.17	18	0.21
	Ethyl Acetate	100	23	0.06	24	0.06
		10	16	0.06	21	0.06
	Ethanol	100	9	0.06	19	0.62
		10	7	0.15	12	0.32
	Water	100	-7	0.10	7	0.06
		10	-9	0.12	9	0.20

Results are the mean of a triplicate experiment
Inhibition of platelet aggregation measured as the loss of reduction in height of the curve as produced for a 100% aggregation induced either by TPA or ADP

induced platelet aggregations at both 100 and 10μg. The ethyl acetate, ethanol and water extracts also inhibited platelet aggregation.

T.pratense exhibited the highest percentage of anti-platelet aggregation activity, the hexane extract inhibited TPA induced aggregation by 40 and 51% at 10 and 100μg respectively. The chloroform extract inhibited ADP induced aggregation by 44 and 66%. Significant inhibition was expressed by both the hexane and chloroform extracts in ADP and TPA induced aggregation.

B.perennis expressed a high percentage of inhibition by the hexane extract (100μg) against both TPA and ADP induced aggregation (45 and 40% respectively). The percentage of inhibition reduced as the polarity of the extracts increased. The water extract exhibited no inhibition against ADP induced aggregation and the percentage of TPA induced aggregation was enhanced by 7% at 100μg.

3.1.3. Cytotoxicity testing of the crude plant extracts

[^{3}H]-thymidine incorporation assay was employed as a sensitive and reliable test to measure the proliferation of Swiss 3T3 mouse fibroblasts in the presence of the crude plant extracts to establish their cytotoxic activity .

Each result is the mean of three experiments conducted in quadruplicates. Table 3.1.3. lists the percentage increase or decrease in cell proliferation as compared to the control. Media only (without cells) dosed with [^{3}H]-thymidine was used as an additional internal control. The inhibitory response of the test compounds was calculated as the percentage decrease as compared to the control samples. A control group was used on each micro-titre plate.

G.glabra exhibited cytotoxic activity for the hexane, chloroform, ethyl acetate and ethanol extracts. All cells apart from those dosed with the water extract ceased proliferating and became detached from the base of the micro-titre plates when dosed with 20μg. At 1μg the ethanol extract promoted growth by 39%, whilst the

Species	Extract	Concentrations µg/ml				
		20	10	1	0.1	0.01
Glycyrrhiza glabra	Hexane	-98	-90	-44	29	2
	Chloroform	-95	-98	-53	-10	-2
	Ethyl Acetate	-98	-97	-70	-8	-9
	Ethanol	-83	-77	39	31	45
	Water	-44	0	-20	-19	0
Galium aparine	Hexane	52	-7	7	-1	-1
	Chloroform	-85	-62	-16	-13	0
	Ethyl Acetate	-30	-16	-13	0	42
	Ethanol	-56	-65	-52	-11	-22
	Water	-35	11	15	10	11
Bellis perennis	Hexane	26	22	34	63	60
	Chloroform	-77	-32	4	61	73
	Ethyl Acetate	-81	-88	62	23	5
	Ethanol	-90	-91	-13	-19	-7
	Water	-65	-32	-28	-16	-18
Trifolium pratense	Hexane	-4	-14	10	19	18
	Chloroform	-99	-98	-18	-9	-15
	Ethyl Acetate	-94	-78	-67	-25	-26
	Ethanol	-77	-76	37	23	-25
	Water	-43	19	19	97	98

Data represents the mean of three experiments conducted in quadruplicates
Micro-titre plates were incubated with test compounds for 48 hours and the percentage increase or decrease of cells (-) measured using the [^{3}H]-thymidine incorporation assay

Table 3.1.3. Inhibition of Swiss 3T3 Fibroblast Cell Proliferation by Crude Plant Extracts

hexane, chloroform and ethyl acetate extracts inhibited growth by 44, 53 and 70% respectively. At a 0.1μg dose the percentage cytotoxicity decreased for all extracts of *G.glabra,* except for the ethanol extract which enhanced the cell proliferation by 31% and 45% at 0.01μg.

The hexane extract of *G.aparine* enhanced proliferation by 52% at 20μg, which decreased at lower concentrations. The chloroform extract showed a strong cytotoxic effect. At 20μg an 85% inhibitory response to cell growth was recorded. A dose dependent relationship was evident and at 0.01μg normal proliferation of the cells was apparent. Both the ethyl acetate and ethanol extracts were cytotoxic at 20μg. The ethanol extract remained cytotoxic at 0.01μg and a 22% inhibitory response was recorded. The water extract exhibited a slight inhibition to cell proliferation at 20μg (35%) and at lower doses the proliferation of cells increased.

T.pratense exhibited the strongest cytotoxicity activity of the four plant species tested. The chloroform, ethyl acetate, ethanol and water extracts of *T.pratense* at 20μg inhibited cell proliferation by 99, 94, 77 and 43% respectively. The hexane extract only produced a 4% inhibition. All extracts inhibited the proliferation of cells at 20μg and at 0.01μg the chloroform, ethyl acetate and ethanol extracts remained toxic at 15, 26 and 25% respectively. However the water extract promoted the proliferation of cells by 19% at 1μg. At 0.1μg and less the water extract promoted cell proliferation by 97%.

At 10μg the ethyl acetate and ethanol extracts of *B.perennis* was extremely cytotoxic (88 and 91% respectively). The water and chloroform extracts were similarly cytotoxic at 20μg. At 0.01μg both the hexane and chloroform extracts induced the proliferation of cells by 60 and 73% , whilst the ethanol and water extracts inhibited the proliferation by 7 and 18%.

3.2. Bioassay guided fractionation of isoliquiritigenin from *G.glabra*

Following the initial fractionation of *G.glabra* using solvent extraction and identification of the ethyl acetate extract as possessing anti-inflammatory activity, column chromatography using silica gel and a gradient solvent system of chloroform and methanol was used to further fractionate the crude ethyl acetate extract (Figure 3.2.). From this column a total of one hundred and fifty fractions 10ml each were collected to form twelve fractions according to their similarities as displayed using analytical T.L.C. The twelve fractions were then screened for anti-inflammatory activity as before using inhibition of chemically induced erythema as the bioassay technique (Table, 3.2.).

Table 3.2. Anti-inflammatory screening of fractions obtained from column chromatography of the ethyl acetate extract of *G.glabra*

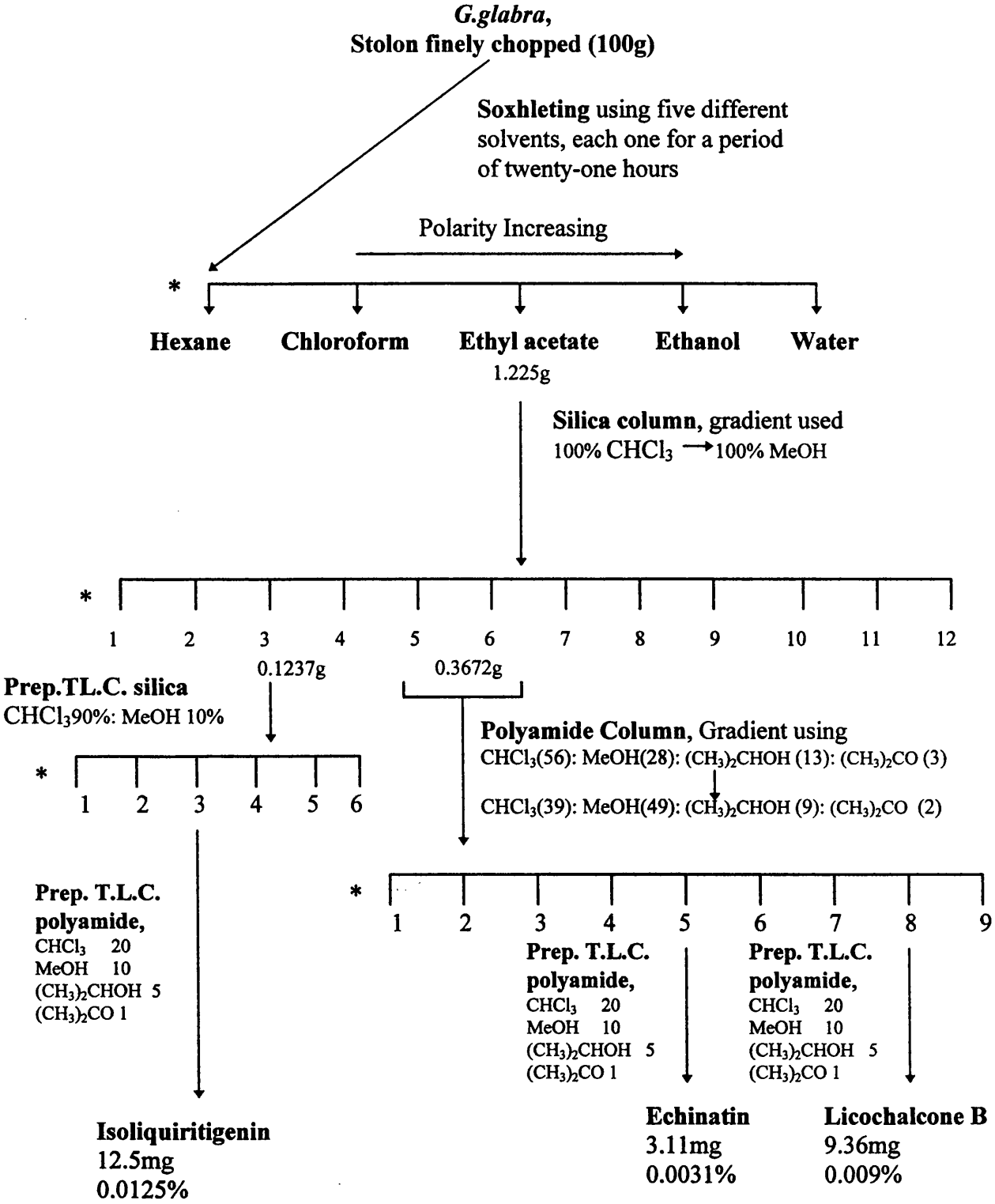
Concentration	Fractions - Erythema Response											
	1	2	3	4	5	6	7	8	9	10	11	12
10μg	---	---	---	--	+	+	---	-	-	---	--	---
20μg	--	--	+	--	+	+	-	-	-	--	--	--

Number of mice per group was four
+ No sign of irritancy, a positive anti-inflammatory response in 100% of mice
- Inhibitory activity in 50% or more of mice
- - Inhibitory activity in less than 50% of the mice
- - - Inhibitory activity not recorded in any of the mice
Observations recorded at six hours the length of time taken for TPA to induce a maximum erythema response

By using the bioassay guided techniques it was established that at least three fractions exhibited strong anti-inflammatory activity: fraction three, five and six. Fractions five and six were subsequently grouped together because of similarities expressed on the silica T.L.C. plate.

Fraction three was further separated using preparative silica T.L.C. The solvent system 90% chloroform and 10% methanol was used to develop the T.L.C. plate (Figure 3.2.). Six bands were scraped from the plate and the compounds eluted using the same solvent system. The six fractions were then tested for anti-

Figure 3.2. **Bioassay Guided Fractionation and Isolation of Active Anti-psoriatic Compounds From *G. glabra***



* = Fractions tested using the bioassay guided technique, inhibition of chemically (TPA)induced erythema.

inflammatory activity using the bioassay technique. Fraction three from the preparatory T.L.C. was identified as the fraction responsible for the anti-inflammatory activity.

Fraction three was identified as being slightly impure as established from the T.L.C. Using polyamide plates and the solvent system chloroform 20: methanol 10: propan-2-ol and acetone 1 it was apparent that one major compound was present. In daylight the compound appeared as a bright yellow spot and under UV (365nm) as a brown absorbing spot. The analysis of the compounds on the polyamide plates using spray reagents was not possible. Minor compounds present as revealed using ultraviolet light (365nm) were blue (R_f 50), yellow (R_f 33) green (R_f 20) and green (R_f 13). Purification of the compound was achieved using the same solvent system as used for analytical TLC (chloroform 20: methanol 10: propan-2-ol: acetone 1). The compound was subsequently identified as isoliquiritigenin.

3.2.1. Identification of isoliquiritigenin

Isoliquiritigenin, (2', 4, 4'-trihydroxychalcone)

Yellow oil, R_f 54 (chloroform 5: acetone 8: toluene7)

UV λ_{max}^{MeOH} nm(logε) 262 (0.183), 360 (0.419); $_{max}^{MeOH + MeONa}$ nm(logε): 253 (0.132), 280sh, 319sh, 349sh, 425 (0.539); $_{max}^{MeOH + AlCl_3}$ nm(logε): 258sh, 321, 382, 424(0.449); $_{max}^{MeOH + AlCl_3/HCl}$ nm(logε): 319sh, 376sh, 421(0.442); $_{max}^{MeOH + NaOAc}$ nm(logε): 281 sh, 340, 350sh, 393; $_{max}^{MeOH + NaOAc}$ nm(logε): 286, 353sh, 380, 443, 476sh.

^{1}HNMR (400MHz, Acetone):δ 6.37 (1H,d,J=2.4,H-3'), 6.47 (1H, dd, J=8.8, 2.4Hz, H-5'), 6.93 (2H, d, J=8.6Hz, H-3 and 5), 7.74(2H, d, J=8.6Hz, H-2 and 6), 7.76(1H,d,J=15.2Hz, H-α), 7.84(1H, d, J=15.3Hz, H-β), 8.12 (1H, d, J=8.9Hz, H-6').

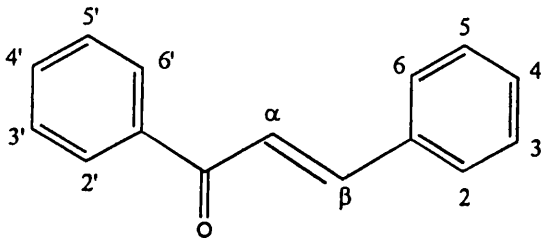
^{13}CNMR (400MHz, Acetone):δ 104.18(d, C3'), 109.1(d, C-5'), 114.9(s, C1'), 117.2(2C, d, C-3 and C-5), 118.7(d, C-α), 127.9(s, C-1), 132.2 (2C, d, C-2 and C-6), 133.7 (d, C-6'), 145.5(d, C-β), 161.4 (s, C-4), 165.9 (s, C-2'), 168.0(s, C-4'), 193.2(s, C=O).

Isoliquiritigenin (2', 4, 4'-trihydroxychalcone) is a typical chalcone. Chalcones are yellow phenolic pigments, C6-C3-C6, which lack a central heterocyclic ring. Isoliquiritigenin displays a bright yellow spot in daylight and gave an intense brown colour in UV (365nm) when using silica T.L.C. plates (R_f 54, chloroform 5, acetone 8, toluene 7). On spraying with sulphuric acid the intensity of the spot increased in daylight and under UV light (365nm) became a dull yellow colour. On spraying with KOH isoliquiritigenin remained bright yellow in daylight and under UV (365) was an orange brown colour.

The identification of isoliquiritigenin was achieved by spectroscopic techniques including mass spectrometry, one and two dimensional NMR and UV analysis. The use of fast atom bombardment mass spectrometry (FABMS) technique with 3-nitrobenzyl alcohol (MNOBA) and sodium (Na^+) as a matrix gave an $[M]^+$ ion peak at *m/z* 257.06 (100%). This corresponded to the molecular formula $C_{15}H_{12}O_4 + M^+$. A corresponding ion was found at 279 corresponding to $C_{15}H_{23}O_4 + Na + M^+$(9%). Other ions afforded were 239 (8%,) corresponding to the loss of a hydroxyl group, 150 (7%), 137 (88%) 120 (14%) and 108 (8%).

The proton nuclear magnetic resonance (^{1}HNMR) of isoliquiritigenin was matched to that of published literature (Aida, 1990). The two rings within the chalcone molecules are identified as the A ring, primed numbers, and the B ring, unprimed numbers, as illustrated below (Figure 3.2.1.).

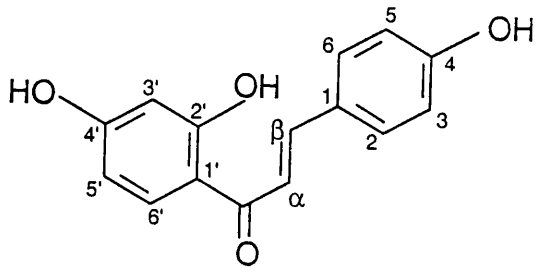
Figure 3.2.1. Molecular Structure Of Chalcone



The ^{1}HNMR spectrum (acetone) of isoliquiritigenin revealed the presence of seven aromatic protons of which four appeared as A_2B_2 signals (δ 7.74 (d, J=8.6, 2H), 6.93

(d, J=8.6, 2H)). The proton at position 5' of isoliquiritigenin (Figure 3.2.1.1.) was identified on the ^{1}HNMR as a double-doublet coupling to the proton 6' as an A-B system (ortho coupling) and long range coupling to the proton at 3' (meta coupling) (δ 6.37 (dd, J= 2.4 and 8.8Hz, 1H)) (Table 3.2.1., Figures 3.2.1.1 and 3.2.2.2.). No singlets were identified. The shift at δ8.12 was assigned to the aromatic proton at 6', the signal corresponds to a doublet coupling with the proton at position 5'. The pair of doublet signals at δ 7.76 (J=15.2Hz) and 7.84 (J=15.2Hz) were assigned to the Hα and Hβ of the trans-unsaturated bond.

Figure 3.2.1.1. Molecular Structure Of Isoliquiritigenin



The ^{13}C-NMR was measured using the technique of distortionless enhancement through polarisation transfer (DEPT) to identify CH_3, CH_2, CH and quaternary carbons. The spectrum of isoliquiritigenin exhibits a total of 13 signals (Table 3.2.1.1, Figure 3.2.1.3.), two of which are high intensity and are assigned to C-2, 6 (δ 132.20) and C-3,5 (δ117.20). The ^{13}C-NMR exhibited only a single shift at δ193.26 corresponding to C=O. The three carbon atoms with hydroxyl groups C4, 2' and 4' were grouped together in the spectrum at δ161.1, 165.97 and 168.01 respectively, and were identified as quaternary carbons from the DEPT spectrum (Figure 3.2.1.3.), as were C1' and 1. As the ^{13}C-NMR data and proton-NMR data matched that of published literature there was no need for further NMR experiments.

The major absorbing band (I) of isoliquiritigenin in methanol, as measured using a spectrophotometer, was at 366nm. As established from published literature the major band (I) of chalcones occurs in the range of 340nm-390nm. A minor peak within the 220-270nm region was referred to as band II. Isoliquiritigenin exhibits shoulder peaks at 258 and 300nm. On the addition of sodium methoxide (NaOMe) a colour

change occurred from a light translucent yellow to a very dark translucent yellow, a characteristic of these compounds. A bathochromic shift of band I occurred from 367nm to 430nm (60nm shift), due to the presence of the free 4-hydroxyl group. Band I in the UV spectrum of isoliquiritigenin underwent a large bathochromic shift in the presence of $AlCl_3$/HCl. This was due to the hydroxyl group present at C2'. Band I appears at 421nm a shift of 54nm from 367nm.

Table 3.2.1. ^{1}H-NMR Spectral Data of Isoliquiritigenin

Acetone, 400MHZ, TMS = 0.0000ppm, Figure 3.2.1.2.

Proton	δ	Aida *et al.*, (1990) (Me_2CO-d_6)
α	7.76	7.76
β	7.84	7.85
2	7.74	7.75
3	6.93	6.47
5	6.93	6.47
6	7.74	7.75
3'	6.37	6.38
5'	6.47	6.47
6'	8.12	8.12

Table 3.2.1.1. ^{13}C-NMR (DEPT) Spectral Data for Isoliquiritigenin

Acetone, 400MHZ, TMS = 0.00ppm, Figure 3.2.1.3.

Carbon	δ	Markham & Ternai (1976)($CDCl_3$)	Aida *et al.*, (1990) (Me_2CO-d_6)
C=0	193.26	191.4	192.9
β	145.59	143.8	145.1
α	118.71	117.8	118.4
1	127.98	125.8	127.6

Continued:

Table 3.2.1.1. ^{13}C-NMR (DEPT) Spectral Data for Isoliquiritigenin

Acetone, 400MHZ, TMS = 0.00ppm, Figure 3.2.1.3.

Carbon	δ	Markham & Ternai (1976)(DMSO-d_6)	Aida *et al.*, (1990) Me_2CO-d_6
2	132.21	130.6	131.7
3	117.20	115.8	116.8
4	161.41	159.9	161.0
5	117.20	115.8	116.8
6	132.21	130.6	131.7
1'	114.93	113.2	114.6
2'	165.97	164.6	165.6
3'	104.18	102.6	103.8
4'	168.01	165.4	167.6
5'	109.11	107.9	108.7
6'	133.71	132.3	133.3

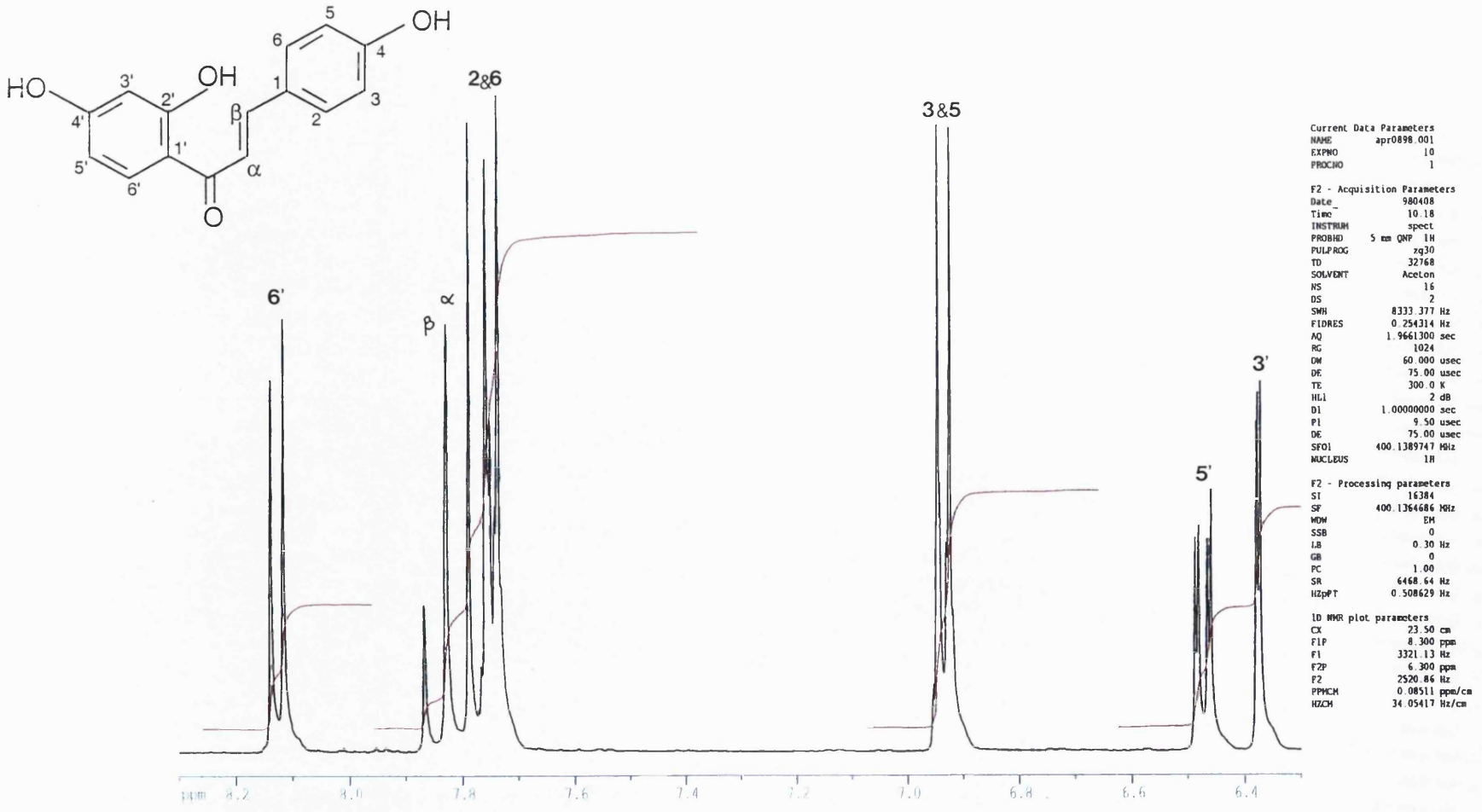


Figure 3.2.1.2. **^{1}H-NMR Spectrum (400MHz, Acetone) of Isoliquiritigenin, 2', 4, 4'-trihydroxychalcone**

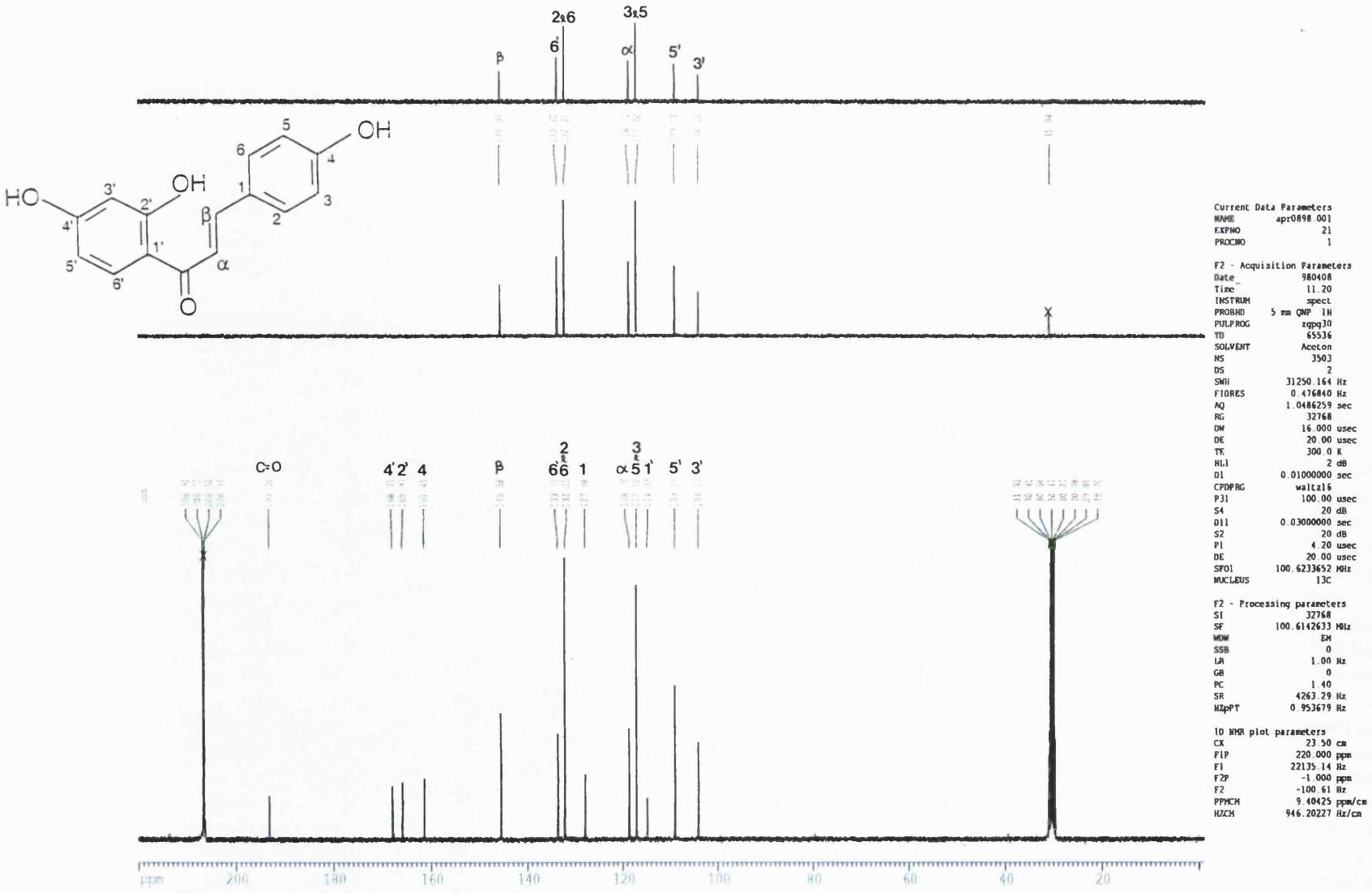


Figure 3.2.1.3. ^{13}C-NMR Spectrum (400MHz, Acetone) of Isoliquiritigenin, 2', 4, 4'-trihydroxychalcone

3.2.2 Bioassay guided fractionation of licochalcone B and echinatin from *G.glabra*

Fractions 5 and 6 from the silica column were fractionated further on the basis of previous bioassay results which established their anti-inflammatory activity (Table, 3.2.) . Using a range of chromatographic techniques a polyamide column was established as the best method to further separate the compounds. Using a solvent system chloroform 56: methanol 28: propan-2-ol 13 and acetone 3 and gradually increasing the polarity to chloroform 39: methanol 49: isopropan-2-ol and acetone 2, a total of nineteen fractions 5ml each were collected and grouped together according to T.L.C. analysis. Silica T.L.C. plates were now effectively used (solvent, chloroform 90: propan-2-ol 10) to analyse the different fractions and sprayed with sulphuric acid to identify the compounds. Each fraction was tested separately at 20μg using the bioassay technique. Three active fractions were identified (Table 3.2.2.).

Table 3.2.2. Anti-inflammatory screening of fractions obtained from the polyamide column (fractions 5 and 6 from the ethyl acetate extract) of *G.glabra*

Concentration	Fractions - Erythema Response								
	1	2	3	4	5	6	7	8	9
20μg	- -	- -	- -	-	+	-	+	+	- -

Number of mice per group was four
+ No sign of irritancy, a positive anti-inflammatory response in 100% of mice
- Inhibitory activity in 50% or more of mice
- - Inhibitory activity in less than 50% of the mice
- - - Inhibitory activity not recorded in any of the mice
Observations were recorded at six hours, the length of time taken for TPA to induce maximum erythema response

Fractions 7 and 8 were grouped together as both contained the same major compound. In daylight this appeared as a brown spot and in UV (365nm) yellow. Upon spraying with 60% sulphuric acid the spot turned orange in daylight and in UV (365nm) became dull brown (R_f 47, chloroform 5: acetone 8: toluene 7). Fraction 5 also consisted of one major compound. In daylight the spot appeared yellow and in

UV (365nm) green/blue and upon spraying with sulphuric acid turned to orange/pink in daylight and bright yellow under UV (R_f 47, chloroform 5: acetone 8: toluene 7). Purification of both compounds was achieved using exactly the same methods, preparative polyamide plates with the solvent system chloroform 5: methanol 20: isopropan-2-ol: acetone 3. The two compounds were identified as retrochalcones, licochalcone B and echinatin, by means of spectroscopic techniques as described below.

3.2.3. Identification of licochalcone B

Licochalcone B (3, 4, 4'-Trihydroxy-2-methoxychalcone)

Yellow needles, R_f 36 (chloroform 5: acetone 8: toluene7)

UV λ_{max}^{MeOH} nm(logε) 262 (3.71), 366 (4.27); $_{max}^{MeOH + MeONa}$ nm(logε): 254 (0.238), 385 (0.290); $_{max}^{MeOH + AlCl_3}$ nm(logε): 276 (0.292), 379 (0.271); $_{max}^{MeOH + AlCl_3/HCl}$ (logε): 300(0.241),359 (0.33);

^{1}HNMR (400MHz, Me_2CO-d_6):δ 3.86(3H, s, OMe), 6.70 (1H,d,J=8.5,H-5), 6.96 (2H, d, J=8.6 Hz, H-3' and 5'), 7.32 (1H, d, J=8.5Hz, H-6), 7.71(1H,d,J=15.6Hz, H-α), 7.97 (1H,d,J=15.6Hz, H-β), 8.05 (2H, d, J=8.7Hz, H-2'and 6')

^{13}CNMR (400MHz, Me_2CO-d_6):δ 61.60 (CH_3), 112.53 (C-5), 116.10(C-3'and 5'), 119.80(C-6), 120.51 (C-α), 121.30 (C-1), 131.55(C-1'), 131.63(C-2'and 6'), 139.15(C-3), 139.18(C-β), 149.21(C-2), 149.85(C-4),162.45 (C-4'), 188.18(C=O)

The use of FABMS technique with MNOBA + Na^+ as a matrix showed a prominent ion at 286.08 with an abundance of 52% ($C_{16}H_{14}O_5$). Other abundant ions were found at 309 abundance of 22% corresponding to $C_{16}H_{14}O_5$ + Na + M^+, also at 255 (38%) corresponding to $C_{16}H_{14}O_5$-OMe and at 121 (100%).

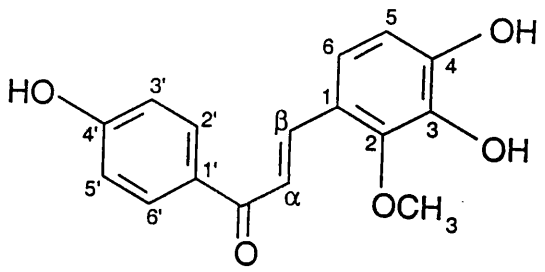
In daylight licochalcone B was identified as a brown spot using silica T.L.C., in UV (365nm) licochalcone appeared yellow and in UV 254nm as a dark absorbing spot. On spraying with H_2SO_4 licochalcone B turned brown orange in daylight and in UV (365nm) a dull brown colour. A dark absorbing spot remains in UV (254nm). On

spraying with KOH there was no colour change in daylight and in UV (365nm) the colour became a dull brown.

The ^{1}H-NMR of licochalcone B was matched to data already published in the literature (Kajiyama *et al.*, 1992). The NMR spectrum (acetone) of licochalcone B revealed the protons, of which four appeared as A_2B_2 signals (δ 6.96 (d, J=8.6Hz, 2H), 8.05 (d, J=8.7Hz, 2H)) and two as AB signals (δ 6.70 (d, J=8.5Hz), 7.32 (d, J=8.5Hz)) (Table 3.2.3., Figures 3.2.3. and 3.2.3.1.) A pair of doublet signals at δ 7.71 (J=15.6Hz) and 7.97 (J=15.6Hz) were assigned to Hα and Hβ. The methyl group at position 2 was assigned to the shift at δ3.86 (s, 3H) and its position confirmed using the experimental technique of two dimensional nuclear overhauser effect spectroscopy (NOESY) (Table 3.2.3.1., Figure, 3.2.3.2.).

The ^{13}C-NMR DEPT spectrum of licochalcone B exhibits a total of 14 signals (Table 3.2.3.2., Figure 3.2.3.3.), two of which are high intensity and are assigned to C-2', 6'(δ131.63) and C-3', 5' (δ116.10). The ^{13}C-NMR spectrum exhibited only one single shift at δ61.6006 corresponding to the CH_3 group. The shift low field at δ188.1864 was assigned to the C=O group. The two signals at δ139.18 and δ120.51 were assigned to C-β and α respectively. The three carbons with hydroxyl groups show signal shifts at δ162.45, δ149.85 and δ116.10 and are assigned to C-4', 4 and 3 respectively; they are all quaternary carbons. Remaining quaternary carbons at δ149.21, δ131.55, δ121.3 are assigned to C-2, 1' and 1 respectively.

Figure 3.2.3. Molecular Structure Of Licochalcone B



The two dimensional spectra NOESY was used to confirm the positioning of protons in the ^{1}H-NMR proton spectrum. Although the data had been matched to previous data, the NOESY spectrum does illustrate the positioning of the methyl group by indicating those protons close in space. The methyl protons are coupled through space to the C-β, whilst the protons connected to C-6 are coupled to C-α and less prominently to the C-β.

The major absorbing band (band I) of licochalcone B (MeOH) was measured at 366 nm (0.3310) and band II at 262 (0.371). On the addition of sodium methoxide (NaOMe) the solution turned yellow. A shift in band I from 366 to 400nm was recorded (34nm); this technique confirmed the presence of a free 4-hydroxyl group. The presence of the B-ring ortho-dihydroxyl groups at positions C-3 and C-4 were identified by a 41nm bathochromic shift of band I when in methanol with the addition of $AlCl_3$, relative to the position of band 1 in methanol with $AlCl_3$/HCl.

Table 3.2.3. ^{1}H-NMR Spectral data of Licochalcone B

Acetone, 400MHZ, TMS = 0.0000ppm, Figure 3.2.3.1.

Proton	δ	Kajiyama *et al.* (1992) (CD_3OD)
OMe	3.86	3.85
α	7.71	7.64
β	7.97	7.98
5	6.70	6.65
6	7.32	7.23
2'	8.05	7.99
3'	6.96	6.89
5'	6.96	6.89
6'	8.05	7.99

Table 3.2.3.1. NOESY ^{1}H-^{1}H NMR Spectral data of Licochalcone B

Acetone, 400MHZ, TMS = 0.0000ppm, Figure 3.2.3.2.

^{1}H-^{1}H connections	δ Connections
α - 2' & 6'	7.71 - 8.05
α - 6	7.71 - 7.32
β - α	7.97 - 7.71
β - 6	7.97 - 7.32
β - 2'	7.97 - 8.05
5 - 6	6.70 - 7.32
6 - α	7.32 - 7.71
6 - β	7.32 - 7.97
6 - 5	7.32 - 6.70
2' & 6' - α	8.05 - 7.71
2' & 6' - 3' & 5'	8.05 - 6.96
3' & 5' - 2' & 6'	6.96 - 8.05

Table 3.2.3.2. ^{13}C-NMR (DEPT) Spectral Data for Licochalcone B

Acetone, 400MHZ, TMS = 0.0000ppm, Figure 3.2.3.3.

Carbon	δ	Kajiyama *et al.* (1992) (MeOH-d_4)
C=O	188.18	191.4
α	120.51	120.6
β	139.18	141.1
1	121.30	121.5
2	149.21	150.0
3	139.15	139.7
4	149.85	150.8
5	112.53	112.7
6	119.80	120.3
1'	131.55	131.3
2'	131.63	132.2

Continued:

Table 3.2.3.2. ^{13}C-NMR (DEPT) Spectral Data for Licochalcone B

Acetone, 400MHZ, TMS = 0.00ppm, Figure 3.2.3.3.

Carbon	δ	Kajiyama *et al.* (1992) (MeOH-d_4)
3'	116.10	116.4
4'	162.45	163.7
5'	116.10	116.4
6'	131.63	132.2
CH_3	61.60	61.8

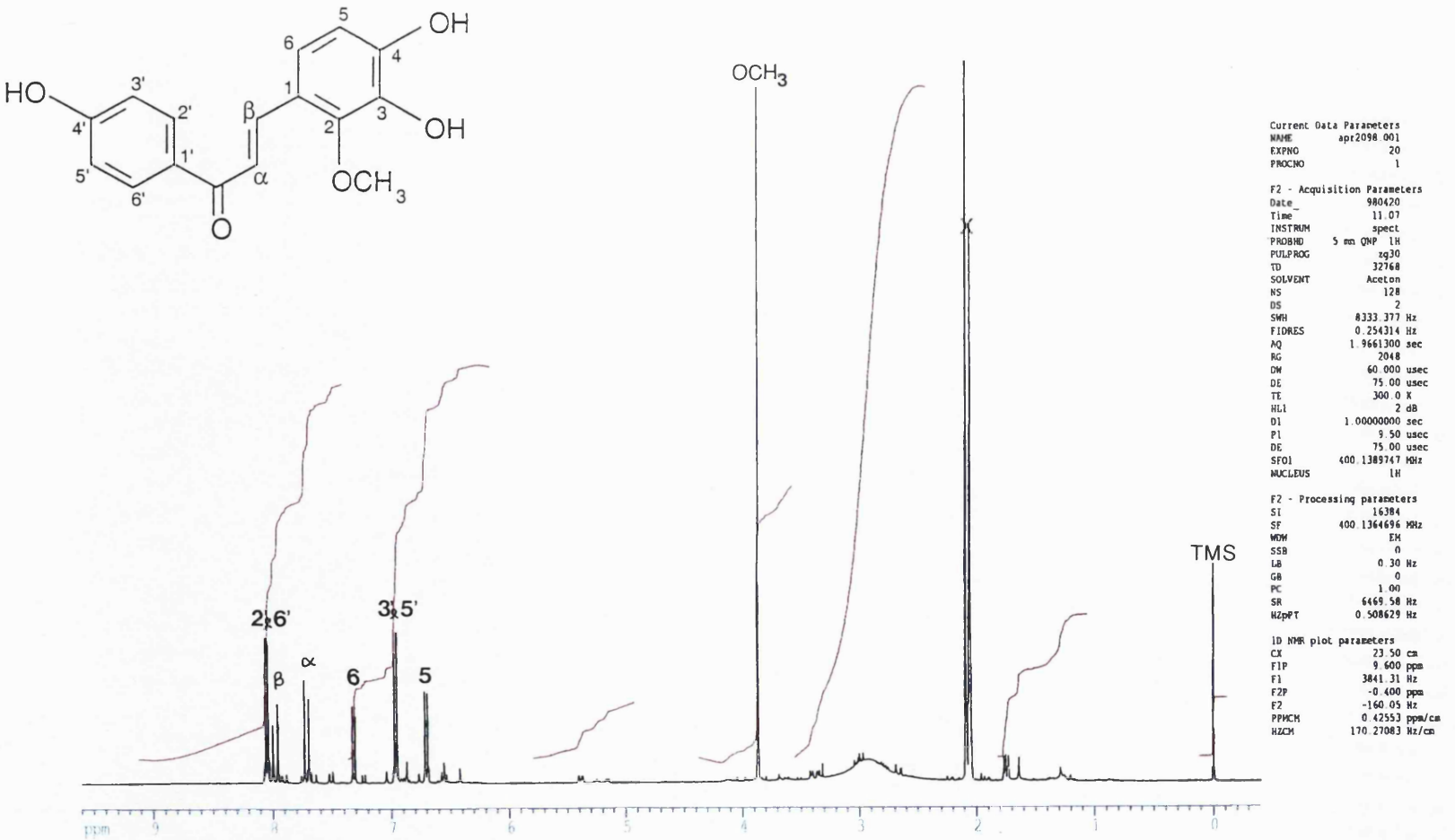
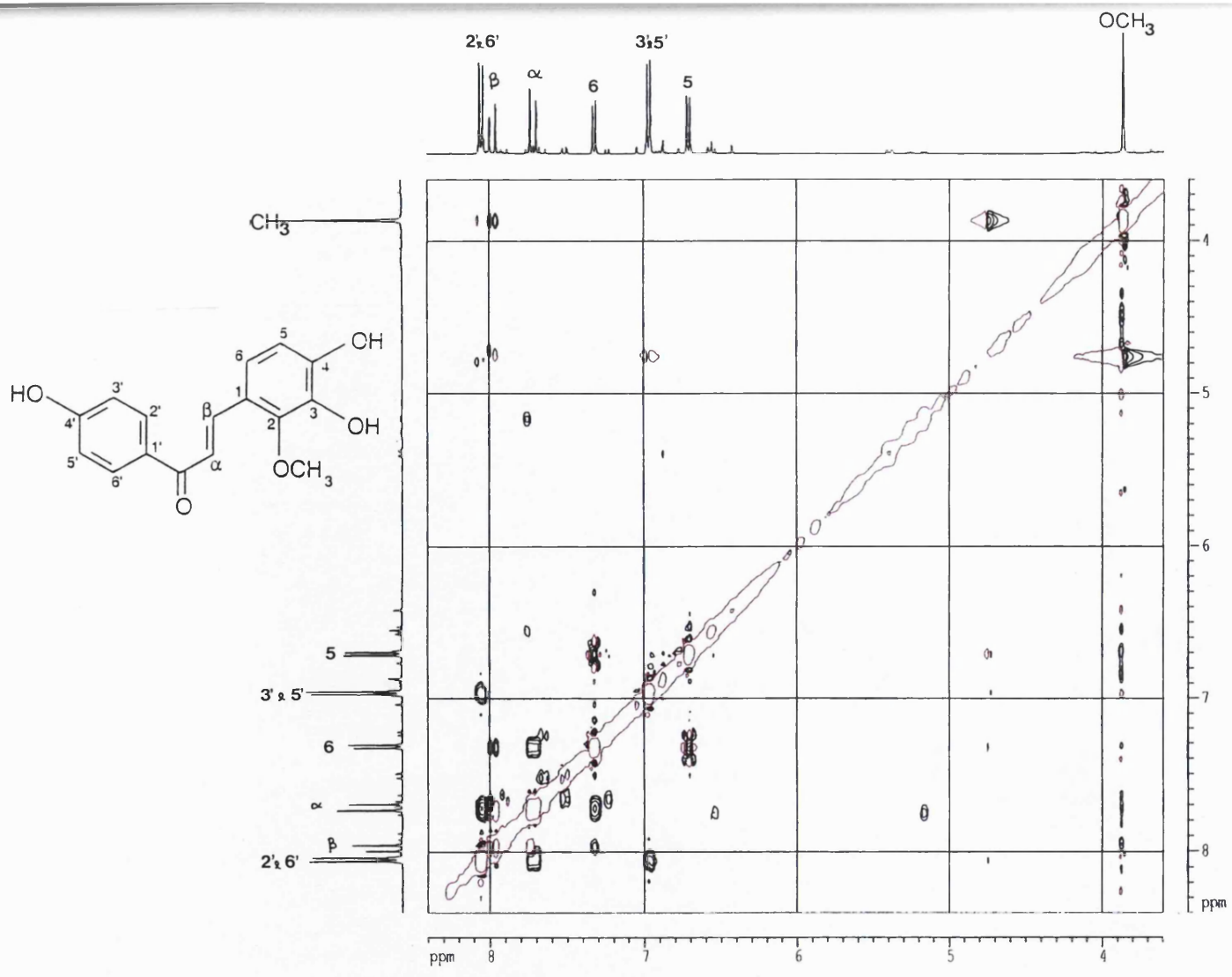


Figure 3.2.3.1. **^{1}H-NMR Spectrum (400MHz, Acetone) of Licochalcone B, 3, 4, 4'-trihydroxy-2-methoxychalcone**



GG2 NOESY

Current Data Parameters

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EXPNO	14
PROCNO	1

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PROBHD	5 mm QNP 1H
PULPROG	noesytp
TD	1024
SOLVENT	Aceton
NS	64
DS	4
SWH	3968.254 Hz
FIDRES	3.875248 Hz
AQ	0.1290740 sec
RG	4096
DW	126.000 usec
DE	175.71 usec
TE	300.0 K
HL1	2 dB
D1	0.96313542 sec
P1	9.50 usec
D0	0.00000300 sec
D8	1.00000000 sec
DE	175.71 usec
SFO1	400.1383684 MHz
NUCLEUS	1H
INO	0.00012600 sec

F1 - Acquisition parameters

NDO	2
TD	256
SFO1	400.1384 MHz
FIDRES	15.500992 Hz
SW	9.917 ppm

F2 - Processing parameters

SI	512
SF	400.1364696 MHz
WDW	QSINE
SSB	2
LB	0.00 Hz
GB	0
PC	1.00
SR	6469.58 Hz
HzPPT	7.750496 Hz

F1 - Processing parameters

SI	512
MC2	TPPI
SF	400.1364696 MHz
WDW	QSINE
SSB	2
LB	0.00 Hz
GB	0

2D NMR plot parameters

CX2	15.00 cm
CX1	15.00 cm
F2PLO	8.400 ppm
F2LO	3361.15 Hz
F2PHI	3.600 ppm
F2HI	1440.49 Hz
F1PLO	8.400 ppm
F1LO	3361.15 Hz
F1PHI	3.600 ppm
F1HI	1440.49 Hz
F2PPMCM	0.32000 ppm/cm
F2HZCM	128.04367 Hz/cm
F1PPMCM	0.32000 ppm/cm
F1HZCM	128.04367 Hz/cm

Figure 3.2.3.2. **NOESY ^{1}H-^{1}H-NMR Spectrum (400MHz, Acetone) of Licochalcone B, 3, 4, 4'-trihydroxy-2-methoxychalcone**

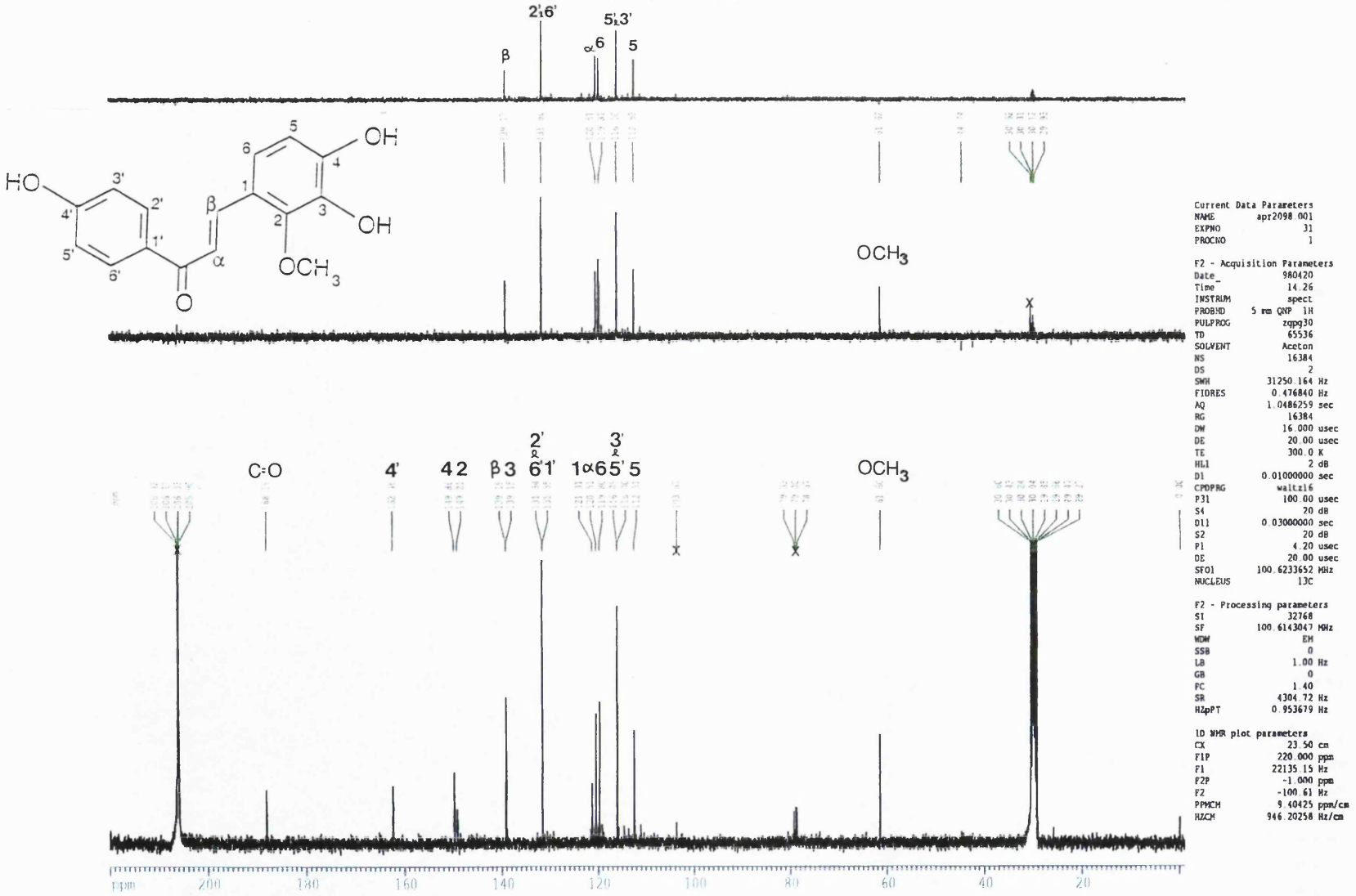


Figure 3.2.3.3. ^{13}C-NMR Spectrum (400MHz, Acetone) of Licochalcone B, 3, 4, 4'-trihydroxy-2-methoxychalcone

3.2.4. Identification of echinatin

Echinatin (4, 4'-Dihydroxy-2-methoxychalcone)

Yellow needles, R_f 47 (chloroform 5: acetone 8: toluene7)

UV λ_{max}^{MeOH} nm(logε): 230 (0.213), 312 (0.228), 369 (0.322);

$_{max}^{MeOH + MeONa}$ nm (logε): 252 (0.213), 264 (0.219), 434 (0.413)

^{1}HNMR (400MHz, Me_2CO-d_6):δ 3.91(3H, s, OMe), 6.51 (1H,dd,J=2.2 and 8.8, H-5), 6.55 (1H, d, J=2.2Hz, H-3), 6.96 (2H, d, J=9.1 Hz, H-3' and 5'), 7.32 (1H, d, J=8.5Hz, H-6), 7.68(1H,d,J=15.8Hz, H-α), 7.69 (1H,d,J=7.84Hz, H-6), 8.02 (2H, d, J=9.1Hz, 2'and 6'), 8.47(1H, d,J=15.7Hz, H-β).

^{13}CNMR (400MHz, Me_2CO-d_6):δ 56.00 (CH_3), 99.9(C-3), 109.2 (C-5), 116.30(C-3'and 5'), 116.70(C-1), 119.40 (C-α), 131.40 (C-1'), 131.60 (C-6), 132.10(C-2' and 6'), 141.30(C-β), 162.10(C-2), 163.00(C-4), 163.40(C-4'), 191.70(C=O).

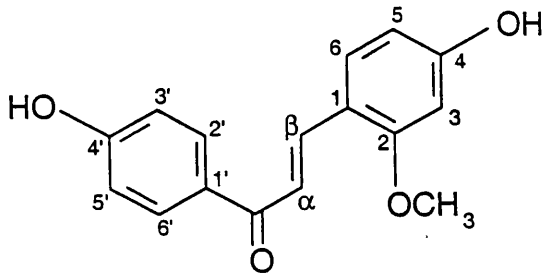
Using FABMS technique with MNOBA + Na as a matrix the ion 271 was afforded, this corresponds to $C_{16}H_{14}O_4$ + M^+. The abundance was 100%. Other ions found were at 293 (26%) corresponding to [Na^+], 255 (31%)[M-CH_3] and 239 (34%)[M- O CH_3].

Echinatin was visible on T.L.C.(silica) in daylight as a yellow spot. Under UV (365nm) the spot appeared as a green/blue fluorescence and in UV (254nm) as a dark absorbing spot. On spraying with H_2SO_4 echinatin turned orange/pink in daylight, UV(365nm) bright yellow and remained as a dark absorbing spot under UV(254nm). On spraying with KOH a bright yellow colour remained in daylight and under UV(365nm) turned a yellow/green colour.

The ^{1}H-NMR data obtained for echinatin was matched to data already in the published literature (Kajiyama *et al.*, 1992) (Table 3.2.4., Figures 3.2.4. and 3.2.4.1.). The structure of echinatin is very similar to licochalcone B and hence the ^{1}H-NMR spectrum was also similar in parts. A pair of doublet signals at δ7.68 and δ8.47 (J=15Hz) were ascribed to the trans protons on the double bond (CO-CH=CH-phenyl). There are seven aromatic protons of which signals at δ6.55, 6.51 and 7.69

are assigned to respective protons at 3, 5 and 6 on the B-ring. The signal at δ6.51, assigned to proton 5, is a double doublet an indication of ortho-coupling to proton 6 and meta-coupling to proton 3. Both protons 6 and 3 are doublets. Four of the aromatic protons appear as A_2B_2 signals (δ8.02(d, J=9.1), 6.95 (d, J=9.1)). The signals at δ6.51 and δ8.02 support the presence of the 4'-hydroxyl group in the A ring. A single shift at δ3.91 integrating for three protons, is assigned to the methyl group. As in licochalcone B the methyl group is situated upfield.

Figure 3.2.4. Molecular Structure Of Echinatin



Although the data were confirmed with published data of echinatin a two dimensional spectra COESY was carried out to illustrate the geometry of the molecule (Figure 3.2.4.2). A COESY spectrum determines the coupling across bonds of the different protons, for example cross peaks at δ6.95 and δ8.02 confirmed the coupling between 3' & 5' and 2'& 6'. Other cross peaks arose between the protons at α and β, and between the protons at 5 and 6. No cross peaks were assigned to the protons of the methyl group.

The UV spectrum for echinatin when measured in methanol was 369nm (0.3229), 312nm (0.228) and 236nm (0.239), the major absorbing band I being 369nm. With the addition of sodium methoxide the solution went yellow and band I shifted to 434nm (0.413) a 65nm increase indication of a free-4 hydroxyl group. The other peaks shifted to 360, 264 and 252nm. With the addition of $AlCl_3$ and $AlCl_3$/HCl there was no change in the spectrum, an indication of the absence of phenolic hydroxyls near to the carbonyl group.

Table 3.2.4. ^{1}H-NMR Spectral data of Echinatin

Acetone, 400MHZ, TMS = 0.0000ppm, Figure 3.2.4.1.

Proton	δ	Kajiyama et al., (1992) (Me_2CO-d_6)
OMe	3.91	3.91
α	7.68	7.70
β	8.47	8.06
3	6.55	6.56
5	6.51	6.52
6	7.69	7.71
2'	8.02	8.04
3'	6.95	6.96
5'	6.95	6.96
6'	8.02	8.04

Table 3.2.4.1. COESY ^{1}H-^{1}H NMR (DEPT) Spectral Data for Echinatin

Acetone, 400MHZ, TMS = 0.0000ppm, Figure 3.2.4.2.

^{1}H-^{1}H Connection	δ Connections
α - β	7.68 - 8.47
β - α	8.47 - 7.68
5 - 6	6.51 - 7.69
6 - 5	7.69 - 6.51
2'&6'- 3'&5'	8.02 - 6.95
3'&5'- 2'&6'	6.95 - 8.02

Table 3.2.4.2. ^{13}C-NMR (DEPT) Spectral Data for Echinatin, taken from Kajiyama et al., (1992)

MeOH-d_4,100MHZ, TMS = 0.0000ppm,

Carbon	δ
C=O	191.70
α	119.40
β	141.30
1	116.70
2	162.10
3	99.90
4	163.00
5	109.20
6	131.60
1'	131.40
2'	132.10
3'	116.30
4'	163.40
5'	116.30
6'	132.10
OMe	56.00

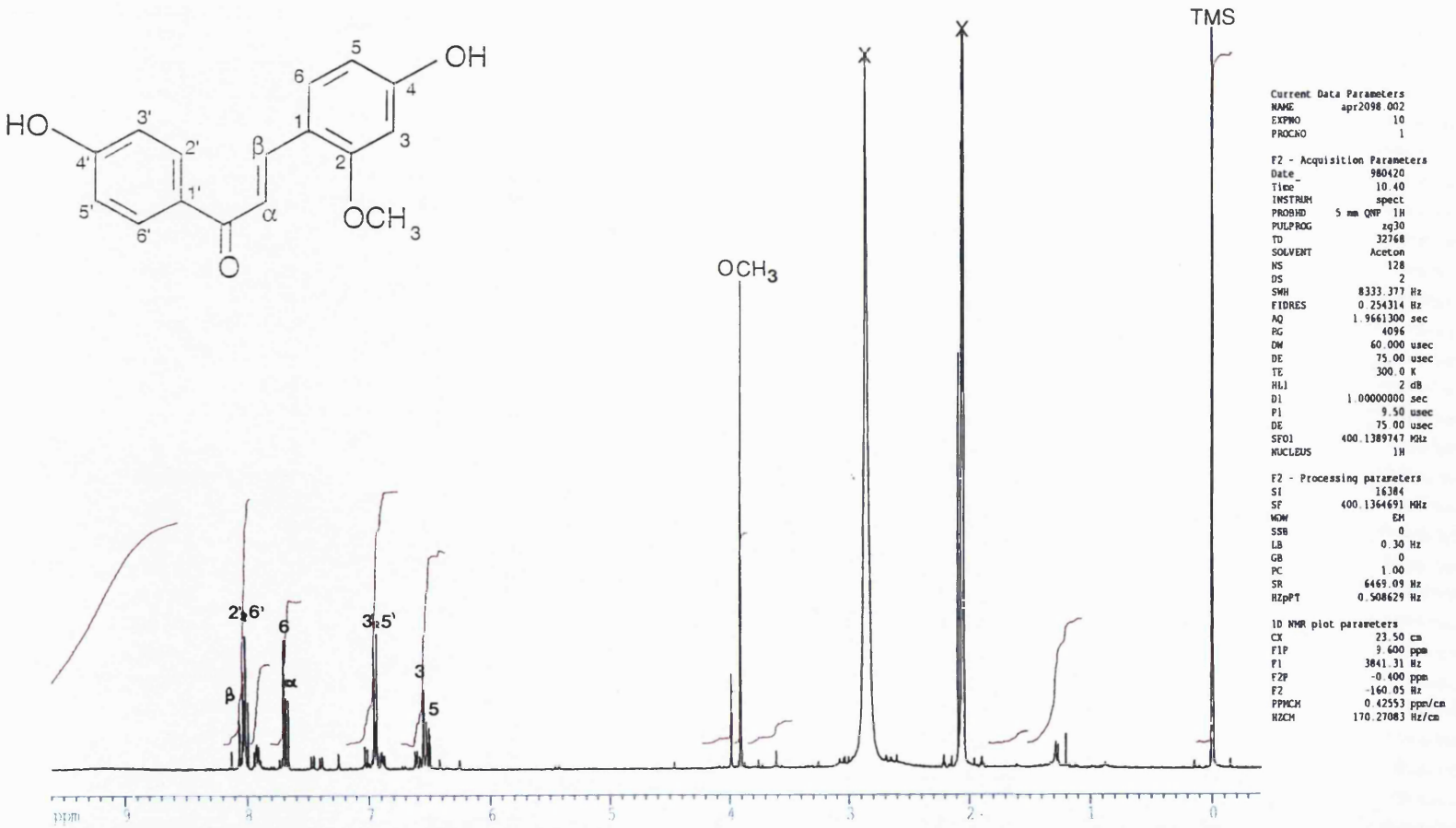
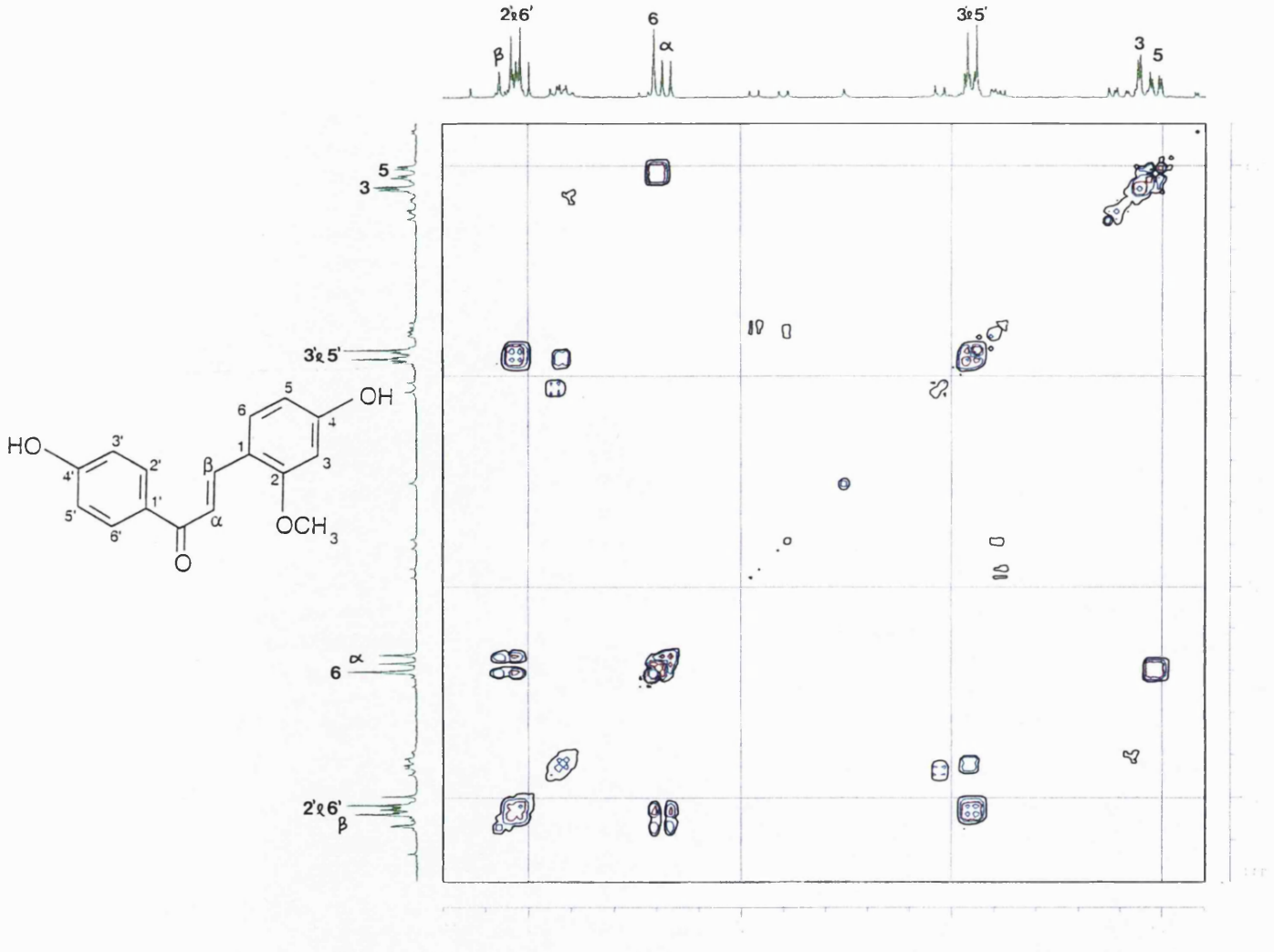


Figure 3.2.4.1. **^{1}H-NMR Spectrum (400MHz, Acetone) of Echinatin, 4 , 4'-dihydroxy-2-methoxychalcone**



G19 COSY45

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DE	172.86 usec
TE	300.0 K
HL1	2 dB
D1	0.97132617 sec
P1	9.50 usec
D0	0.00000300 sec
DS	172.86 usec
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F1 - Acquisition parameters

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SW	9.256 ppm

F2 - Processing parameters

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WDW	SINE
SSB	2
LB	0.00 Hz
GB	0
PC	1.40
SR	4469.09 Hz
HzPPT	3.614898 Hz

F1 - Processing parameters

SI	1024
MC2	QF
SF	400.1364691 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

2D NMR plot parameters

CX2	15.00 cm
CX1	15.00 cm
F2PPM	8.200 ppm
F2LO	3281.12 Hz
F2PHI	6.100 ppm
F2HI	2560.87 Hz
F1PPM	8.200 ppm
F1LO	3281.12 Hz
F1PHI	6.100 ppm
F1HI	2560.87 Hz
F2PPMCM	0.12000 ppm/cm
F2HZCM	48.01638 Hz/cm
F1PPMCM	0.12000 ppm/cm
F1HZCM	48.01638 Hz/cm

Figure 3.2.4.2. COESY ^{1}H-^{1}H-NMR Spectrum (400MHz, Acetone) of Echinatin, 4 , 4'-dihydroxy-2-methoxychalcone

3.2.5. Inhibition of chemically induced erythema on the mouse ear by isolated *G.glabra* compounds

Inhibition of chemically induced erythema on the mouse ear was done using two different assay techniques. In assay 1 the chemical irritant TPA was applied 20 minutes after the application of the compounds and in assay 2 application of the TPA took place 20 minutes before the application of test compounds. Apart from the application time of TPA the assay techniques were exactly the same. In each test group six mice were used. The inhibitory response to erythema was categorised as being slight or absent. A slight inhibition to erythema was shown by the vasodilatation of veins in the ear only, whilst an absence of inhibition was observed as vasodilatation of veins with a reddening of the ear as well. In either case the compound had failed to inhibit the erythema and the result was recorded as negative. The inhibition to erythema was therefore described as: + no signs of erythema in all mice tested (positive anti-inflammatory response), - inhibition present in 50% or more mice, - - inhibition present in less than 50% of the mice and - - - no inhibition of erythema in 100% of mice (negative anti-inflammatory response). The IC50 was estimated between the dose where 50% or more exhibited inhibition to erythema and 50% or less exhibited inhibition to erythema.

Isoliquiritigenin exhibited an IC50 response, at six hours, between 20 and 50 μg/ear when application of TPA was applied 20 minutes after the compound or before the compound. However the time taken for the onset of erythema was reduced in animals where application of TPA was before the compound (Tables 3.2.5. and 3.2.5.1.). For example isoliquiritigenin, at 20 μg/ear, (observations recorded at 4 hours) exhibited a positive inhibitory response when applied before TPA, but when TPA was applied prior to isoliquiritigenin one mouse out of the group of six exhibited an erythema response.

Licochalcone B exhibited a strong anti-inflammatory response to the TPA when applied 20 minutes before TPA. The IC50 was estimated between 5 and 10μg/ear. Total inhibition of erythema was observed at 20μg/ear or more. However when TPA was applied before licochalcone B the IC50 increased to 50-100μg/ear. Although

Dose µg/ear	Isoliquiritigenin			Licochalcone B			Echinatin			β-Glycyrrhetinic Acid (Sigma) - Control			Indomethacin (Sigma) - Control			Dithranol (Sigma) - Control		
	3hr	4hr	6hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr
100	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-
50	+	+	-	+	+	+	+	+	+	-	- -	-	+	+	+	+	-	-
20	+	+	- -	+	+	+	+	+	-	+	+	- -	+	+	+	+	-	- -
10	+	-	- -	+	-	-	+	+	-	- -	-	- -	+	-	+	+	+	-
5	-	-	- -	+	-	- -	+	+	-	- -	- - -	- - -	+	- -	- -	-	- -	- -
1	-	- -	- - -	-	-	- -	-	-	- - -	+	- -	- - -	+	+	-	+	-	- -
0.5	-	- -	- - -	+	+	- - -	+	-	- -	- -	- -	- - -	+	- -	- - -	- -	- -	- - -
0.1	-	- -	- - -	- -	-	- - -	-	- -	- -	- -	- -	- - -	+	- -	- - -	- -	- -	- - -

Number of mice per group was six

\+ Inhibition of erythema present in all mice

\- Inhibition of erythema in +50% of the mice

\- - Inhibition of erythema present in less than 50% of mice

\- - - No inhibition of erythema

Observations recorded at 3, 4 and 6 hours

Table 3.2.5. **Inhibition of Chemically Induced Erythema on the Mouse (CD1) Ear By *G.glabra* Compounds Applied 20 Minutes Prior to TPA**

Dose µg/ear	Isoliquiritigenin			Licochalcone B			Echinatin			β-Glycyrrhetinic Acid (Sigma) - Control			Indomethacin (Sigma) - Control			Dithranol (Sigma) - Control		
	3hr	4hr	6hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr
100	+	+	+	+	+	+	+	+	+	+	-	--	+	+	+	-	+	---
50	+	-	-	+	+	---	+	+	+	--	--	---	+	+	+	+	+	--
20	+	-	--	+	+	---	+	+	-	-	-	--	+	+	+	+	--	---
10	-	-	--	+	-	--	+	-	--	-	--	--	+	-	+	--	--	-
5	-	--	--	+	-	--	+	-	--	--	--	---	+	--	--	-	--	---
1	-	--	---	+	--	--	-	-	---	-	--	---	+	+	-	--	---	--
0.5	-	--	---	+	--	---	-	---	---	--	---	---	+	--	---	-	--	---
0.1	-	--	---	-	--	---	-	---	---	--	---	---	+	--	---	--	---	---

Number of mice per group was six

\+ Inhibition of erythema present in all mice

\- Inhibition of erythema in +50% of the mice

-- Inhibition of erythema present in less than 50% of mice

--- No inhibition of erythema

Observations recorded at 3, 4 and 6 hours

Table 3.2.5.1. **Inhibition of Chemically Induced Erythema on the Mouse (CD1) Ear by *G.glabra* Compounds Applied 20 Minutes After TPA**

when dosed with both 20 and 50μg/ear at 4 hours there was total inhibition to erythema, but at 6 hours there was a complete absence of inhibitory activity.

Echinatin exhibited strong anti-inflammatory activity. When observed at 6 hours the IC50 required to inhibit erythema was between 1 and 5 μg/ear with application of TPA 20 minutes after echinatin. Erythema was completely inhibited up until six hours when mice were dosed with 5μg/ear or more. The application of TPA before echinatin increased the dose required for an IC50 to 10 and 20μg/ear. Inhibition was observed as being totally absent in animals treated with a 0.1, 0.5 and 1μg/ear dose at four and six hours. Total inhibition to erythema was only recorded at three hours for all doses above 1μg/ear. However at six hours a total inhibitory response was recorded in all animals treated with 50μg/ear and more.

β-glycyrrhetinic acid exhibited anti-inflammatory activity when applied before the TPA, the IC50 being between 20 and 50μg/ear at six hours. However at doses below 10μg/ear erythema was present in all animals at six hours after application and at four hours erythema was present in 50% or more of the animals. The application of TPA before β-glycyrrhetinic acid increases the percentage of animals showing an erythema response and thus a failure to inhibit inflammation. The IC50 was estimated above 100μg/ear. In all animals dosed with 100μg/ear or less erythema was present in at least 50% or more of the animals at six hours and was present in all animals dosed with 5μg/ear and less. At three hours a considerable number of the mice were already showing an erythema response. Those animals treated with 5μg/ear or less, 50% or more of the animals exhibited an erythema response.

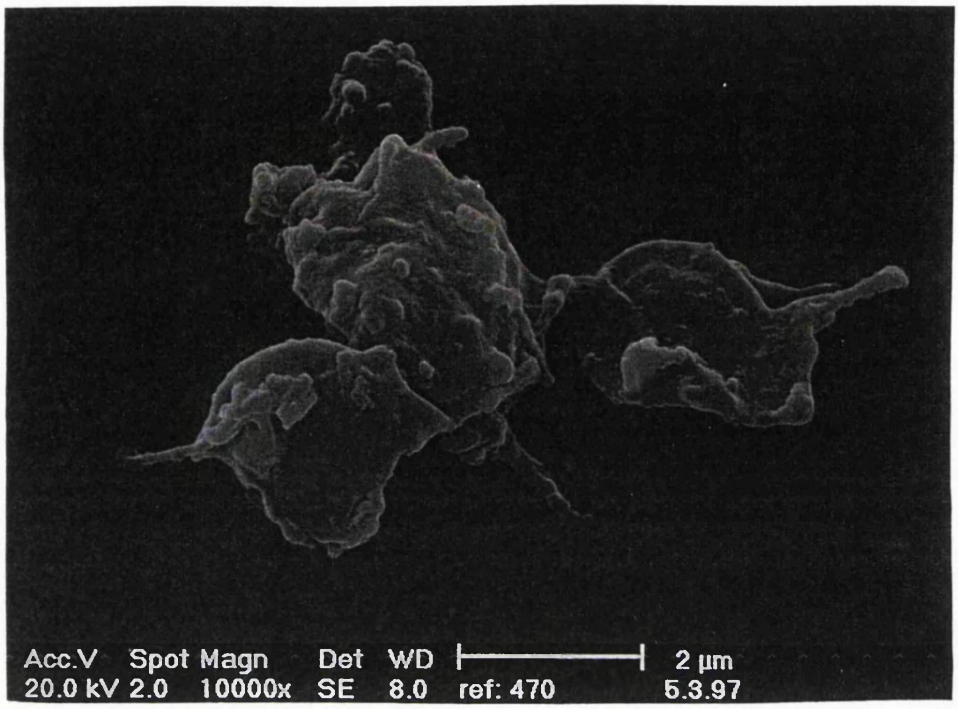
Indomethacin was used as a positive control anti-inflammatory agent. Strong anti-inflammatory activity was exhibited at six hours in those animals dosed with 10μg/ear or above. Whether TPA was applied either before or after indomethacin it had no effect. No erythema activity was detected in any mice at three hours after the application of TPA. Inflammatory activity was observed in all mice dosed at 0.1 and 0.5μg/ear at six hours after application. At four hours 50% or more of the mice exhibited an erythema response, when treated with 0.1 and 0.5μg/ear.

Dithranol is used as an anti-psoriatic drug often in combination with UV-B radiation or coal tar. Dithranol when applied before TPA exhibited a mild anti-inflammatory activity (IC50 20 and 50μg/ear). In mice treated with a dose of 50 and 100μg/ear one mouse out of the six exhibited erythema in the form of vasodilatation of the veins and reddening of the ears. Other mice were recorded as having a mild erythema with only vasodilatation of the veins. Total inhibition to erythema at three hours was only recorded in animals treated with 10μg/ear or more. The application of TPA before the dithranol increased the percentage of mice showing an erythema response considerably. At six hours mice treated with 100μg/ear or less exhibited an erythema response. Total inhibition to the erythema was only observed in animals treated with 100 and 50μg/ear at three and four hours after application.

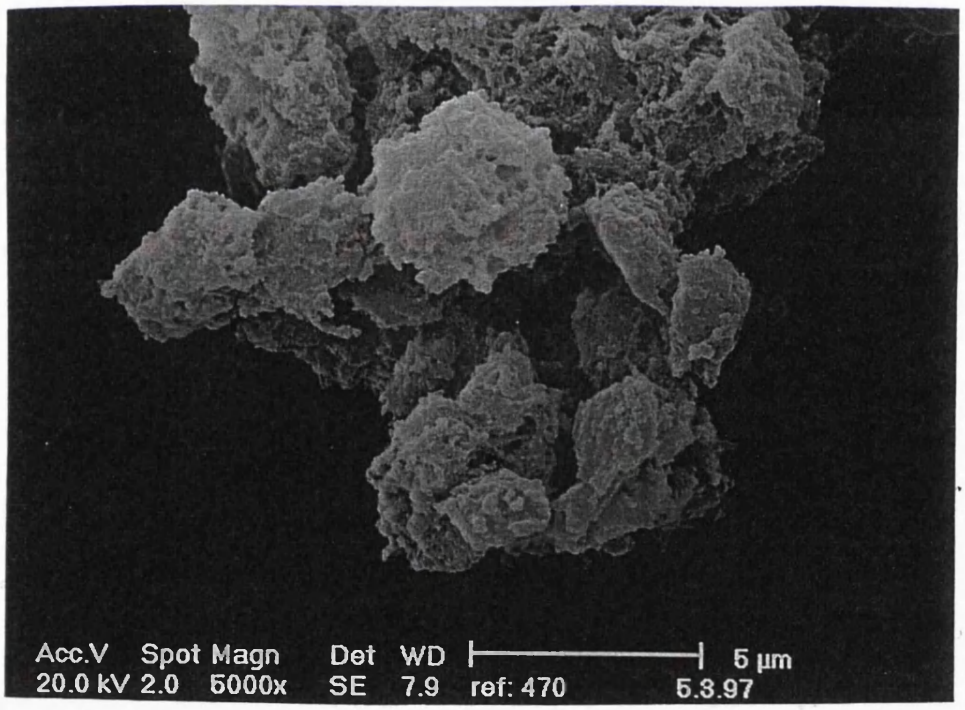
3.2.6. Inhibition of TPA and ADP induced blood platelet aggregation by isolated *G.glabra* compounds

Figure 3.2.6. shows blood platelets in a non-aggregated and aggregated state as seen using a scanning electron microscope. Inhibition of blood platelet aggregation was very much dependent on the chemical responsible for the induction of the aggregatory response (Tables 3.2.6. & 3.2.6.1., Figures 3.2.6.1. & 3.2.6.2.), some compounds were able to inhibit one type of the aggregation and not the other. β-Glycyrrhetinic acid, colchicine and aspirin were used for control experiments as they have been documented in the literature as exhibiting anti-platelet aggregation activity. In some cases the aggregatory response was actually enhanced. This occurred in samples where the concentration of the test compound was relatively low.

Isoliquiritigenin exhibited inhibition to both ADP and TPA induced aggregation. At 200μg/500μlPRP inhibition against ADP induced aggregation was 18%. This was recorded as being highly significant, the level of significance was tabulated below 1% as calculated using a one sided paired T-test (Table 3.2.6., Figure 3.2.6.1.). TPA induced aggregation when dosed at this concentration was inhibited by 8.2%, significant at the 5% level (Table 3.2.6.1., Figure 3.2.6.2.). Isoliquiritigenin exhibits



a) Normal Blood Platelets (x10000)



b) Aggregated Blood Platelets (x5000)

Figure 3.2.6. Photographs of Human Blood Platelets
Scanning Electron Microscope (Philips xL20)

Dose µg/500µlPRP	Isoliquiritigenin		Licochalcone B		Echinatin		β-Glycyrrhetinic Acid		Colchicine		Aspirin Acetylsalicylic Acid	
	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.
200	18.0**	0.06	22.0**	0.00			24.3**	0.06	8.2*	0.20	26.6**	0.00
100	8.2*	0.06	14.0**	0.12	1.7	0.06	22.6**	0.06	4.3*	0.12	19.3**	0.25
50	10.4*	0.00	15.6**	0.00	0.9	0.20	19.9**	0.00	3.5	0.26	18.1**	0.06
20	-8.2	0.17	10.4**	0.12	-1.7	0.10	17.8*	0.17	8.2*	0.36	16.3**	0.00
10	-0.7	0.00	2.2	0.10	-6.1	0.06	18.5*	0.00	3.5	0.10	20.0**	0.10
5	0.0	0.10	0.7	0.06	-2.6	0.12	16.0*	0.00	5.9*	0.10	15.0**	0.26
1	-8.2	0.15	0.0	0.17	-3.5	0.21	15.1*	0.15	5.9*	0.00	12.1**	0.10
0.5	-15.6	0.00	0.3	0.15	-6.1	0.12	0.0	0.00	2.7	0.23	13.5**	0.15
0.1	-14.8	0.06	0.1	0.10	-2.6	0.12	1.8	0.06	0.4	0.06	17.5**	0.06

Number of measurements per group was three
One-sided Paired T-test
* 5% level of significance
** 1% level of significance

Table 3.2.6. **Inhibition of ADP Induced Platelet Aggregation By *G.glabra* Compounds**

Dose µg/500µlPRP	Isoliquiritigenin		Licochalcone B		Echinatin		β-Glycyrrhetinic Acid		Colchicine		Aspirin Acetylsalicylic Acid	
	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.
200	8.2*	0.12	29.0**	0.00			16.5*	0.06	37.7**	0.06	-0.1	0.00
100	6.6*	3.80	17.0**	0.12	32.2**	0.06	11.5*	0.10	24.6*	0.12	0.8	0.00
50	5.8	0.06	12.0**	1.10	26.4**	0.15	13.1*	0.06	13.0	0.17	-0.1	0.60
20	-2.5	0.12	1.9	0.06	0.0	0.26	13.1*	0.15	-4.3	0.10	-0.1	0.00
10	-3.6	0.12	3.8	0.20	1.1	0.23	8.1*	0.06	-2.9	0.15	-2.0	0.00
5	0.0	0.10	2.8	0.06	1.1	0.12	7.6	0.06	-5.8	0.06	-2.0	0.12
1	-5.8	0.10	2.8	0.12	-1.1	0.15	7.1	0.12	-10.1	0.06	-3.0	0.00
0.5	-14.8	0.10	1.4	0.21	0.0	0.10	6.9	0.00	-5.8	0.06	-2.5	0.06
0.1	-19.7	0.06	-4.3	0.10	-1.1	0.21	6.9	0.10	-13.0	0.26	-1.1	0.10

Number of measurements per group was three
One-sided Paired T-test
* 5% level of significance
** 1% level of significance

Table 3.2.6.1. **Inhibition of TPA Induced Blood Platelet Aggregation By *G.glabra* Compounds**

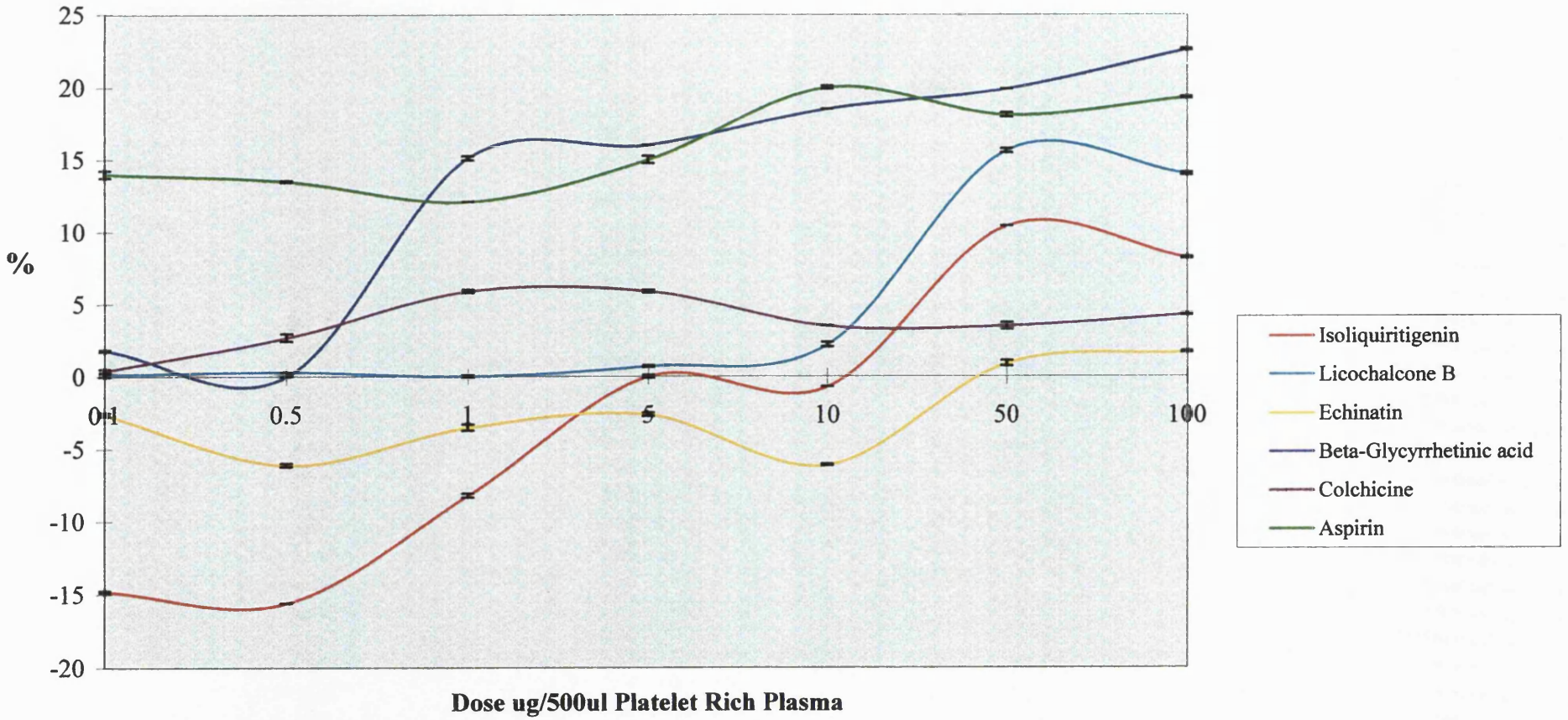


Figure 3.2.6.1. **Inhibition of ADP Induced Blood Platelet Aggregation By *G.glabra* Compounds**

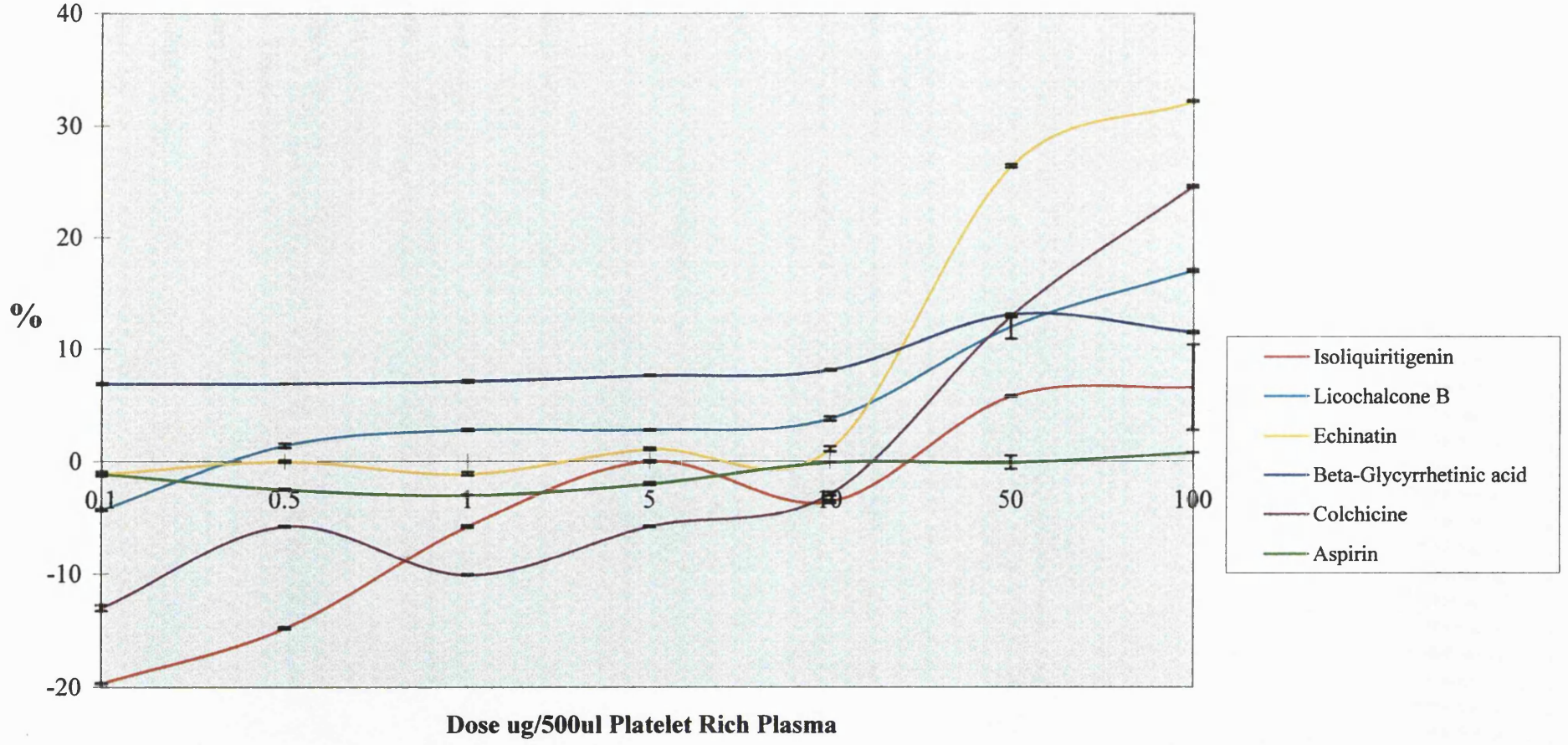


Figure 3.2.6.2. **Inhibition of TPA Induced Blood Platelet Aggregation By *G.glabra* Compounds**

an inhibitory response to both forms of aggregation at concentrations above 20μg/500μlPRP; below this concentrations the percentage inhibition for ADP and TPA was measured as -8.2 and -2.5% respectively. At 0.1μg/500μlPRP the level of inhibition was reduced to -14.8 and -19.7% for ADP and TPA respectively.

Licochalcone B inhibits platelet aggregation to both TPA and ADP induced significantly at doses of more than 20μg/500μlPRP. At 200μg/500μlPRP Licochalcone B inhibited TPA induced aggregation by 29% which was calculated as being highly significant (P<1%); inhibition against ADP induced aggregation was also recorded as being highly significant, 22%. At concentrations below 10μg/500μlPRP inhibition to both types of chemically induced aggregation was reduced to levels between 0 and 5%.

Echinatin exhibits no inhibition to ADP induced aggregation; at concentrations of 50μg/500μlPRP and above, inhibition to aggregation was only recorded between 0.9 and 1.7% , at lower concentrations the aggregation was actually enhanced from -1 to 6.1%. However at 50 and 100μg/500μlPRP TPA induced aggregation was inhibited significantly by 26% (P<1%) and 32% (P<1%) respectively; at lower concentrations the inhibition to platelet aggregation ceases. The TPA induced aggregation was not enhanced at low doses of echinatin unlike other compounds.

β-Glycyrrhetinic acid is a well documented anti-inflammatory compound and the ability to inhibit both types of chemically induced aggregation was recorded. ADP induced aggregation was inhibited significantly at doses 1μg/500μlPRP and above (Figure 3.2.6.1.), comparable to that of aspirin. At 200μg/500μlPRP ADP induced platelet aggregation was inhibited by 24.3% and at 10μg/500μlPRP the percentage of inhibition slightly reduced to 18.5%. Both were recorded at the 1% significance level. There was no inhibitory response to ADP aggregation by β-glycyrrhetinic acid at doses below 0.5μg/500μlPRP. TPA induced aggregation was inhibited in a dose dependent manner by β-glycyrrhetinic acid. At 200μg/500μlPRP aggregation was inhibited by 16.5%. At 50μg/500μlPRP this was reduced slightly to 13.1%. A dosage between 10 and 0.1μg/500μlPRP the inhibition of aggregation does not

decrease dramatically and the percentage of inhibition remains between 6.9 and 8.1%.

Colchicine is a well documented platelet aggregation inhibitor especially against phorbol ester induced aggregation. The inhibitory response against ADP induced aggregation was insignificant at 200µg/500µlPRP measuring as 8.2%. At 100µg/500µlPRP the percentage of inhibition was halved to 4.3% (Figure 3.2.6.1.). The percentage of inhibition remained between 3-6% at doses of 0.5-100µg/500µlPRP. Inhibitory activity against TPA induced aggregation by colchicine was more active than ADP induced aggregation. At 200µg/500µlPRP platelet aggregation was inhibited by 37% (P<1%). At 100µg/500µlPRP inhibitory activity was reduced to 24.6% and at 50µg/500µlPRP to 13%. At 10µg/500µlPRP there was no inhibitory activity expressed, but TPA induced aggregation was enhanced. For example at 0.1µg/500µlPRP the percentage aggregation was recorded as -13%.

Acetylsalicylic acid is also a well documented inhibitor to platelet aggregation, although not against TPA induced aggregation. ADP induced aggregation was significantly inhibited even at the very low doses tested. At 200µg/500µlPRP the percentage inhibition recorded was 26.6% (P<1%). The percentage of inhibition was reduced to 16.3% at 20µg/500µlPRP and at 0.1µg/500µlPRP was recorded at 14%. These inhibitory responses were recorded at the 1% level of significance.

3.2.7. Inhibition of Swiss 3T3 fibroblast cell proliferation using *G.glabra* compounds

A dose response study of the crude ethyl acetate extract, the three compounds isolated from *G.glabra* and β-glycyrrhetinic acid (used as positive inhibitory control) at concentrations of 0.01, 0.1, 1, 10 and 20µg was established for their cytotoxicity activity on Swiss 3T3 mouse fibroblast cells. Each result was calculated as the mean of three experiments each one conducted in quadruplicate. The inhibition of cell

proliferation was calculated as the percentage decrease in proliferation as compared to the control measured using the [^{3}H]-thymidine incorporation protocol.

The crude ethyl acetate extract and isolated compounds from *G.glabra* exhibited extreme cytotoxicity at 20μg whereby all cells became detached from the microtitre plate with correspondingly no [^{3}H]-thymidine incorporation (Figure 3.2.7., Table 3.2.7.). The IC50 was estimated as the dose required to inhibit 50% of the normal growth.

Dose μg/1ml	Isoliquiritigenin (1μg=3.90μM)		Licochalcone B (1μg=3.49μM)		Echinatin (1μg=3.70μM)		β-Glycyrrhetinic Acid (1μg= μM)		Ethyl acetate *G.glabra*	
	%	S.E.M	%	S.E.M	%	S.E.M	%	S.E.M	%	S.E.M
0.01	10	5	16*	2	3	4	28**	13	24	15
0.1	18*	13	13	1	28*	6	52**	7	13	14
1	31**	8	41**	7	82**	2	23**	6	70**	9
10	44**	12	55**	4	94**	0	98**	0	97**	1
20	99**	0	86**	2	99**	0	99**	0.6	98**	1

* 5% level of significance
** 1% level of significance
S.E.M. Standard error of mean

TABLE. 3.2.7. Inhibition of Swiss 3T3 fibroblast cell proliferation by *G.glabra* compounds

The crude ethyl acetate extract of *G.glabra* inhibited the proliferative activity of the cells. At doses of 10 and 20μg the cell proliferation was completely inhibited. At a dose of 1μg the inhibitory activity remained at the 1% significance level giving a 70% inhibition. A dose of 0.01μg produced no inhibitory activity and a normal proliferation of cells was achieved.

Isoliquiritigenin exhibits strong inhibitory activity to cell proliferation. At 20μg only a 1% of cell growth was recorded. However at a 10μg dose the percentage of growth

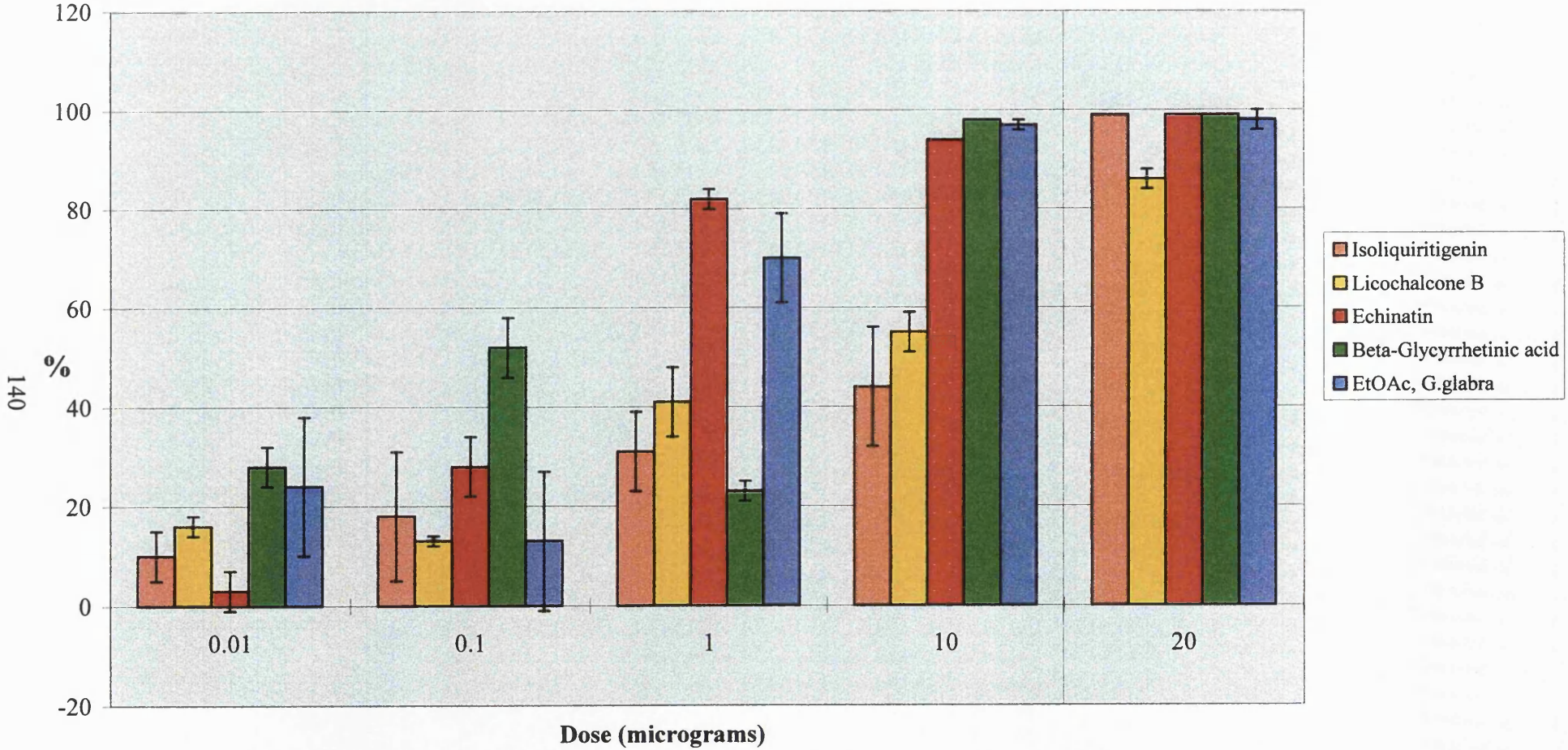


Figure 3.2.7. **Inhibition of Swiss 3T3 Fibroblast Proliferation By *G.glabra* Compounds**

was calculated at 56%, the inhibitory activity being 44%, although this was still at he 1% level of significance from the control. The IC50 for isoliquiritigenin was estimated between 10 and 20μg. At 0.01 and 0.1μg the inhibition to cell proliferation was reduced to 10 and 18% respectively.

Licochalcone B exhibited strong cytotoxicity activity inhibiting cell proliferation by 55% and 86% at 10 and 20μg respectively. At 1μg the percentage of inhibition remained high at 41% (P<1%). The IC50 value was estimated between 1 and 10μg. Licochalcone B still exerts inhibitory response to normal cell proliferation by 13 and 16 % at 0.1 and 0.01μg dose.

Echinatin exhibited cytotoxic activity at 1, 10 and 20 μg the percentage inhibition was 82, 94 and 99% respectively (P<1%). The inhibition to cell proliferation decreased to 28% at 0.1μg, the IC50 was estimated between 0.1 and 1μg (0.370-3.70μM). At a dose of 0.01μg only a 3% inhibitory response to TPA induced proliferation was recorded.

β-glycyrrhetinic acid also exerted strong anti-proliferative activity against the normal proliferation of Swiss 3T3 fibroblast cells. Total inhibition to proliferation of the cells was recorded at 10 and 20μg. The inhibitory response decreased to 23% at 1μg. The IC50 was estimated between 1 and 10μg. However at 0.1μg the inhibitory response to proliferation increased to 52%(P<1%), at 0.01μg the response decreased to 28%.

3.2.7.1. Inhibition of TPA induced Swiss 3T3 fibroblast cell proliferation when *G.glabra* compounds and TPA added at the same time point

This assay was used to test the inhibitory activity of the compounds isolated from *G.glabra* and β-glycyrrhetinic acid against TPA induced cell proliferation when added at the same time point.

The molar concentration of TPA required was established using a dose response study of the induction of cell proliferation by TPA. A range of concentrations were tested between 0.001 and 1μM. The required concentration of TPA should induce proliferation of the cells to a percentage whereby any further increase in the dose would have no effect on the cell proliferation and the percentage increase of cells was constant from one assay to the next. At 0.001μM there was no induction of cell proliferation, and at 0.01μM there was a 557% increase in cell proliferation. At 0.5μM the percentage increase was recorded as 1114%, at 0.7μM it was 1097% and at 1μM 1387%. 0.5μM was chosen as the appropriate concentration.

Isoliquiritigenin inhibited the TPA induced proliferation of cells at 20μg at the 1% level. The percentage inhibition at both 10 and 20μg was recorded as 28%. At 1 and 0.1μg a 4% inhibition was calculated. At 0.01μg the inhibition was recorded as 6% (Table 3.2.7.1., Figure 3.2.7.1.).

Licochalcone B exhibited strong anti-proliferative activity against TPA induced cell growth. At 20μg the percentage inhibition was recorded as 70% (P<1%). At a 10μg dose the inhibition reduced to 32% (P<1%). At doses of 0.1 and 1μg the inhibitory activity remained at 15%. However when dosed with 0.01μg there was no inhibition to growth but an enhancement of cell proliferation as compared to the control, the increase in cell proliferation was recorded as 26%(P<1%).

The inhibitory response to TPA induced proliferation by echinatin was similar to that expressed in normal cell growth. At 10 and 20μg the percentage of cell proliferation was recorded as 1% as compared to cell growth induced by TPA only (P<1%). The cells had died and become detached from the microtitre wells (Figure 3.2.7.1., Table 3.2.7.1.). At 1μg the percentage inhibition to proliferation decreased to 42%, remaining significant at the1% level. At 0.1μg the inhibition decreased to 15% and at 0.01μg fibroblast proliferation was enhanced by 5% .

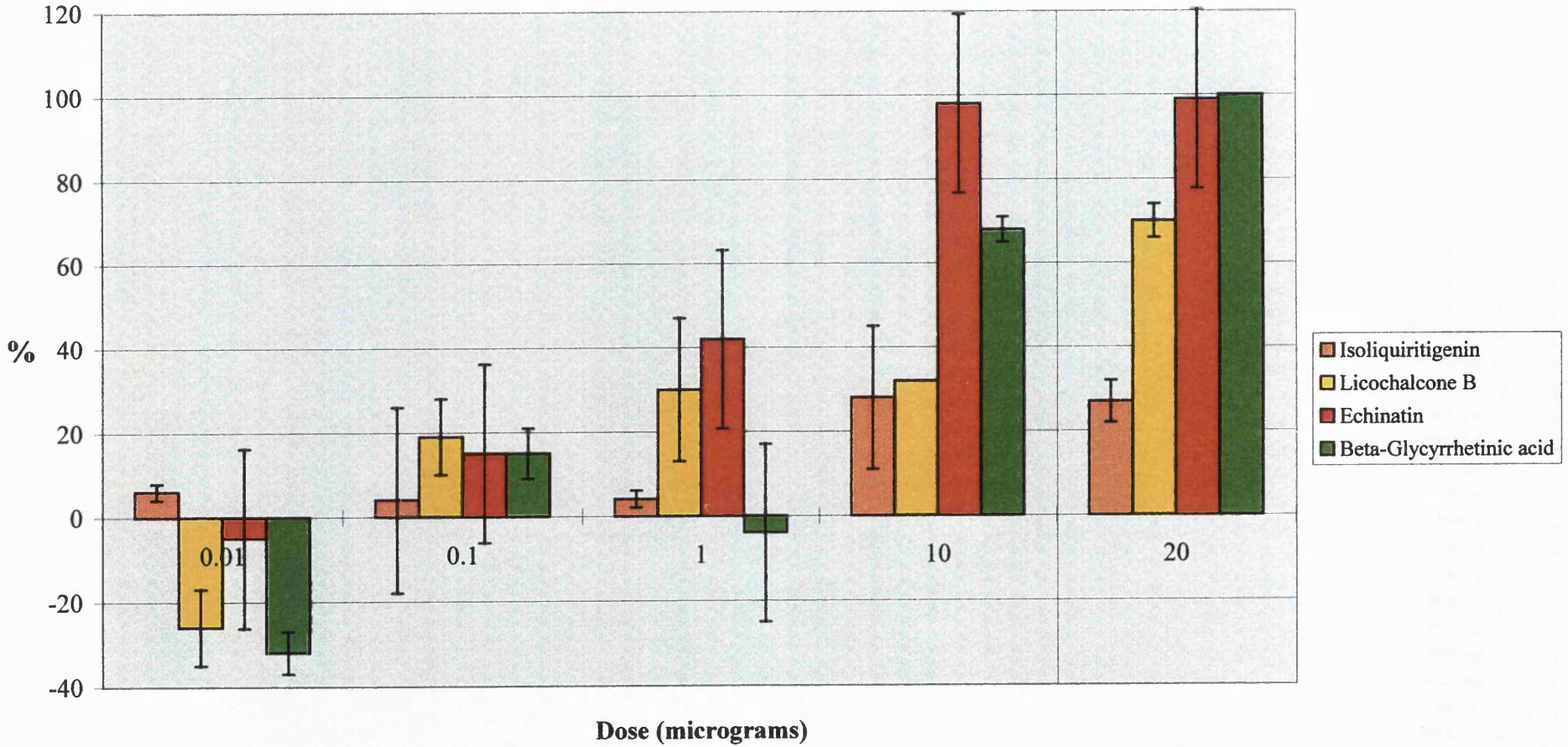


Figure 3.2.7.1. **Inhibition of TPA Induced Swiss 3T3 Fibroblast Cell Proliferation When TPA and *G.glabra* Compounds Added at the Same Time Point**

Dose µg/1ml	Isoliquiritigenin		Licochalcone B		Echinatin		β-Glycyrrhetinic Acid	
	%	S.E.M.	%	S.E.M.	%	S.E.M.	%	S.E.M.
0.01	6	2	-26**	9	-5	13	-32*	5
0.1	4	22	19*	9	15	11	15*	6
1	4	2	30**	17	42**	2	-4	21
10	28*	17	32**	0	99**	1	68**	3
20	28**	5	70**	4	99**	0	100**	0

* 5% level of significance
** 1% level of significance
S.E.M. Standard error of mean

TABLE. 3.2.7.1. Inhibition of TPA induced cell proliferation when *G.glabra* compounds and TPA are added at the same time point

β-glycyrrhetinic acid was able to inhibit the TPA induced proliferation significantly at 10 and 20µg/1ml, the percentages being 68 and 100% respectively (P<1%)(Table 3.2.7.1., Figure 3.2.7.1.). At 1µg the cell proliferation was enhanced by 4%, but then at 0.1µg inhibition to cell proliferation was recorded at 15%. An enhancement of cell proliferation (32%) was again recorded at 0.01µg.

3.2.7.2. Inhibition of TPA induced Swiss 3T3 fibroblast cell proliferation when *G.glabra* compounds added 24 hours prior to the TPA

This assay was used to test the inhibitory activity of the chalcones, isolated from *G.glabra* and β-glycyrrhetinic acid against TPA induced cell proliferation when added to the media 24hours prior to the addition of TPA (figure 3.2.7.2. & table 3.2.7.2.)

Isoliquiritigenin when dosed at concentrations of 1, 10 and 20µg/1ml inhibited cell proliferation by 49, 45 and 60% respectively (P<1%). At 0.1µg the percentage inhibition was 29% (P<1%) and at 0.01µg 31% (P<1%). A 1% level of significance was tabulated for all concentrations tested.

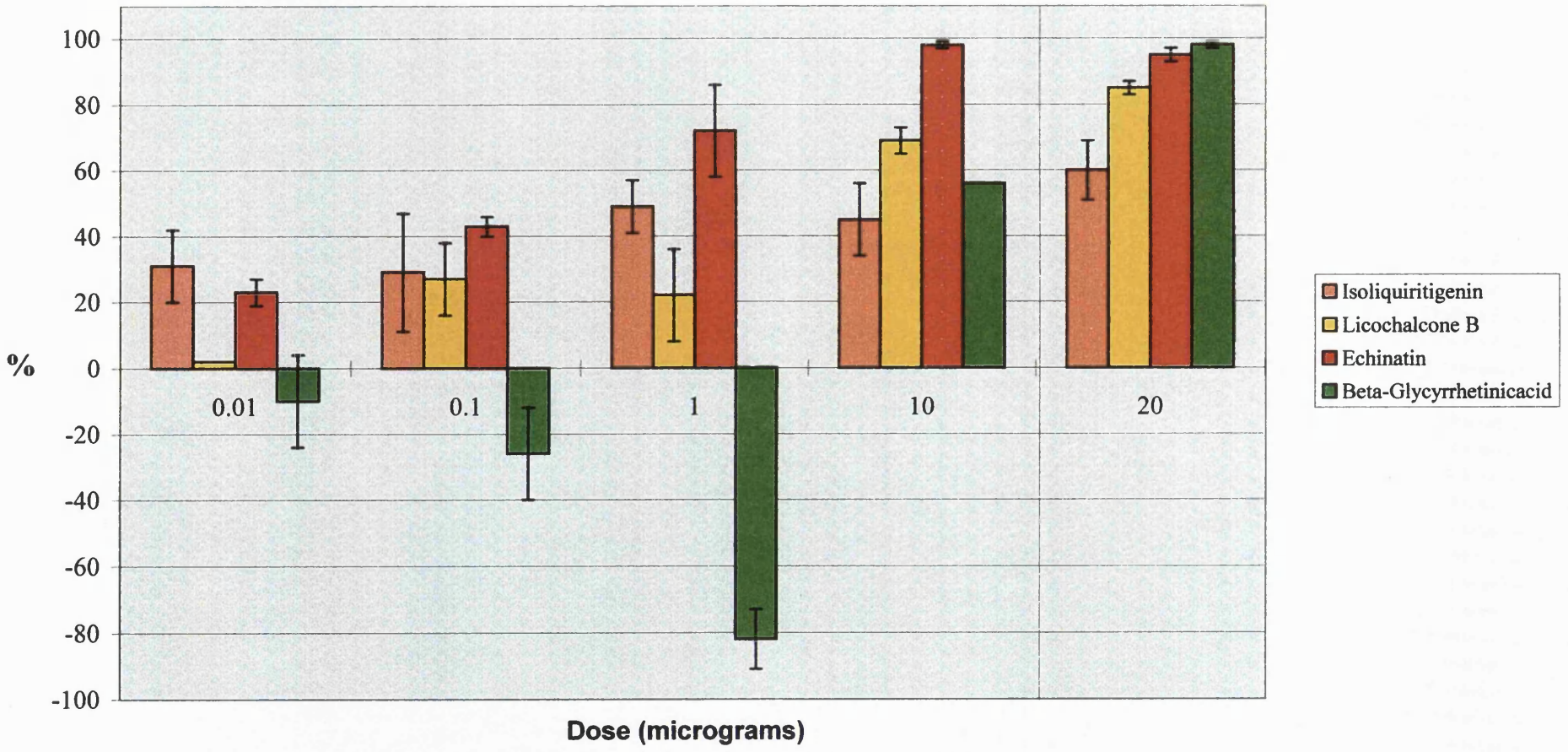


Figure 3.2.7.2. **Inhibition of TPA Induced Swiss 3T3 Fibroblast Cell Proliferation When *G.glabra* Compounds Added 24 Hours Prior to TPA**

Dose µg/1ml	Isoliquiritigenin		Licochalcone B		Echinatin		β-Glycyrrhetinic Acid	
	%	S.E.M.	%	S.E.M.	%	S.E.M.	%	S.E.M.
0.01	31**	11	2	0	23**	4	-10*	14
0.1	29**	18	27*	11	43**	3	-26*	14
1	49**	8	22*	14	72**	14	-82**	6
10	45**	11	69**	4	98**	1	56**	0
20	60**	9	85**	2	98**	2	98**	1

* 5% level of significance
** 1% level of significance
S.E.M. Standard error of mean

TABLE. 3.2.7.2. Inhibition of TPA induced Swiss 3T3 fibroblast cell proliferation when *G.glabra* compounds are added 24 hours prior to TPA

The inhibition to TPA induced cell proliferation by licochalcone B at 10 and 20µg was recorded as 69 and 85% respectively (P<1%). At 1µg the percentage inhibition reduced to 22% (P<5%) but slightly increases to 27% at 0.1µg. The lowest concentration, 0.01µg, had no effect on cell proliferation.

Echinatin was as in the previous two assays the most active in inhibiting the proliferation of the cells. At 10 and 20µg the percentage inhibition was 98 and 98% respectively (P<1%). At 1µg the percentage inhibition decreased to 72%. At 0.1µg and 0.01µg the percentage inhibition was 43% and 23% respectively.

The response of β-glycyrrhetinic acid TPA proliferation when added 24 hours prior to the TPA was unexpected. At 20µg the cells all died and became detached from the micro-titre plate. At 10µg the inhibitory response was 56%. However at 1µg there was no inhibition but a considerable increase in the proliferation as compared to the control, 82% (P<1%). At 0.1 and 0.01µg there was also an increase in the proliferation of cells as compared to the control; the percentage increases were recorded as 26 and 10% respectively.

3.2.8. Inhibition of UVB physically induced erythema using *G.glabra* compounds

The isolated compounds isoliquiritigenin and licochalcone B from *G.glabra*, β-glycyrrhetinic acid and the crude ethyl acetate extract from *G.glabra* were examined for their anti-inflammatory activity against physically induced erythema, a response similar to that of psoriasis. UVB (302nm) induced radiation was used to induce the inflammation. Echinatin was not tested due to insufficient quantities of material obtained to conduct a full experiment.

The anti-inflammatory response of the test compounds was established by measuring the epidermal layer thickness and counting the number of cells in the epidermal layer. Under normal conditions in rat skin there is usually one layer of cells within the epidermis excluding the basal layer.

In each assay five animals were used per test group. The individual measurements of epidermal thickening and the number of cells for each test area was subsequently calculated as the percentage of the control from each individual rat (table 3.2.8.). This was done to ensure a uniformity in results. The experimental design was such that the data were paired, which allowed natural variation in the number of epidermal cells and the epidermal thickness from different rats to be eliminated.

The crude ethyl acetate fraction of *G.glabra* did inhibit UVB induced inflammation. The thickness of the epidermal layer increased by 71% when exposed to UVB radiation (9.78μm). This was calculated as being significant at the 1% level, using a one sided students T-test. With application of the crude extract this measurement decreased to 29% (7.38 μm), a 59% decrease in epidermal thickening as compared to the UVB control (Figure 3.2.8.2.). The number of epidermal cells also increased similarly with UVB radiation; an increase of 89% ($P<1\%$) was recorded. This decreased to 21.62% ($P<5\%$) with the application of the crude extract, a difference of 75% ($P<1\%$) (Figure 3.2.8.1.& 3.2.8.2.). Application of the crude extract without

	Control		U.V.		U.V. + Compound		Compound Only	
	Thickness (μm)	Number Cells	Thickness (μm)	Number Cells	Thickness (μm)	Number Cells	Thickness (μm)	Number Cells
***G.glabra* -EtOAc Fraction**								
Average	5.70	1.48	9.78	2.80	7.38	1.80	6.46	1.62
S.E.M.	0.46	0.25	1.83	0.75	1.03	0.36	0.81	0.23
%Difference from control			71 **	89 **	29 **	21 *	13 *	9
% Inhibition from UVB					59 *	75 **		
***G.glabra* -Isoliquiritigenin**								
Average	6.60	1.60	10.60	2.86	7.76	1.84	6.86	1.58
S.E.M.	0.56	0.23	2.11	0.32	0.51	0.51	0.36	0.20
%Difference from control			60 **	78 **	17 *	15	3.9	-1
% Inhibition from UVB					71 *	81 **		
***G.glabra* - Licochalcone B**								
Average	6.44	1.46	15.36	3.92	8.80	1.94	6.04	1.48
S.E.M.	0.94	0.21	1.23	0.53	3.55	0.79	1.22	0.23
%Difference from control			138 **	168 **	36	32	-6	1
% Inhibition from UVB					73 **	80 **		
β- Glycyrrhetinic Acid								
Average	6.40	1.35	12.30	3.27	9.87	2.45	7.27	1.70
S.E.M.	1.59	0.21	1.90	0.59	2.00	0.34	1.55	0.29
%Difference from control			92 **	142 **	54 *	81 **	13	25
% Inhibition from UVB					41 **	42 **		

One sided Paired T-test

*** = 5% significance from control/UVB, ** = 1% significance from control/UVB**

Table 3.2.8. Inhibition of Physically Induced Erythema By *G.glabra* Compounds

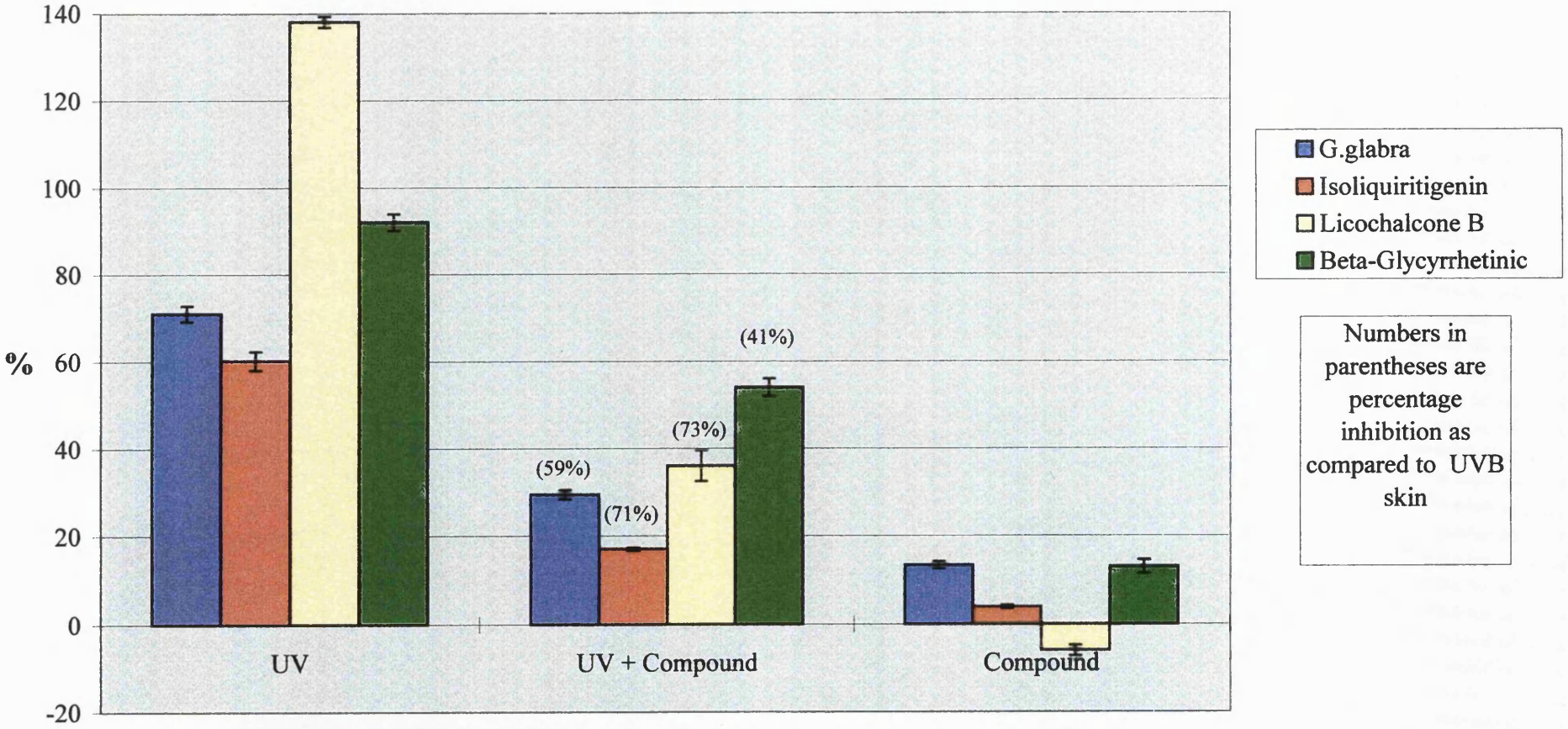


Figure 3.2.8. **Effects of *G.glabra* Compounds on UVB Induced Wistar Rat Epidermal Skin Thickening**

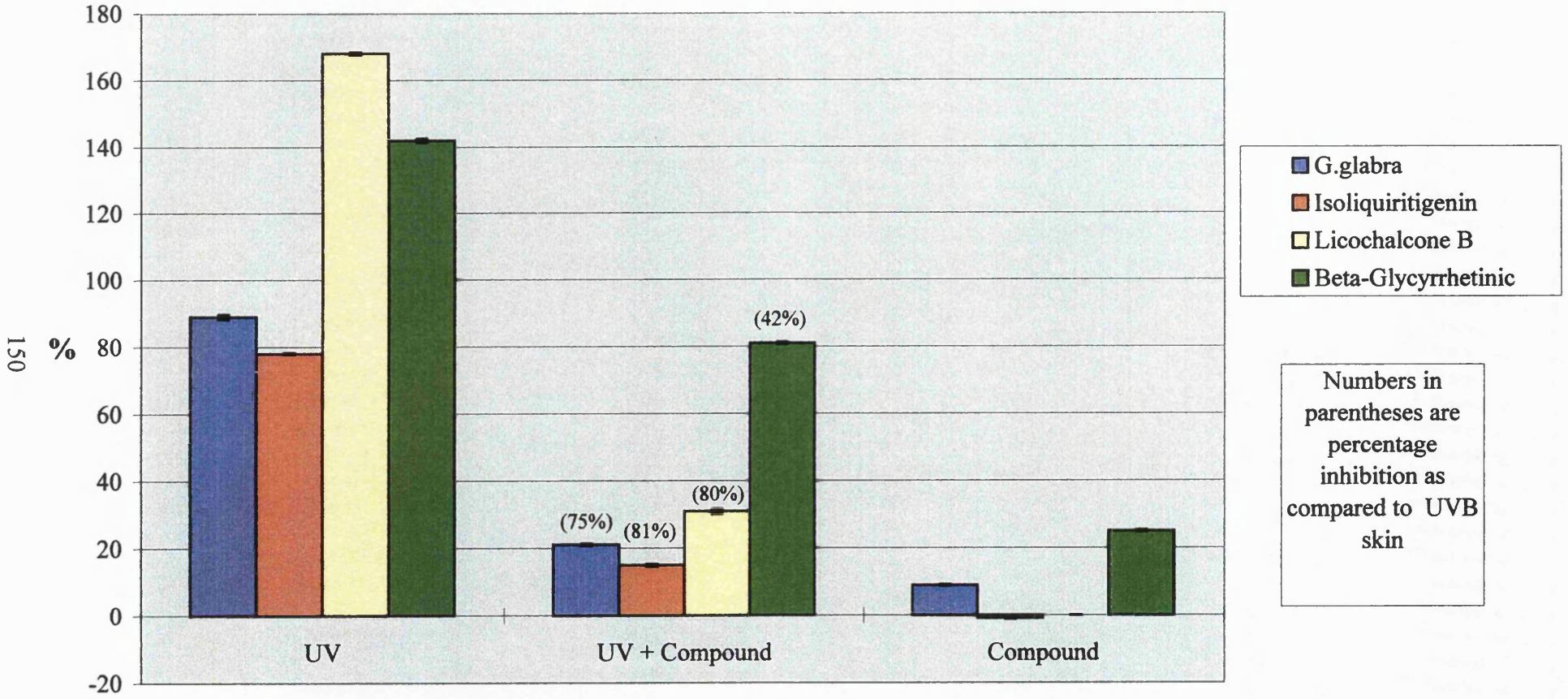


Figure 3.2.8.1. **Effects of *G.glabra* Compounds on UVB Induced Wistar Rat Epidermal Skin Proliferation**

UVB exposure increased the thickness and number of cells within the epidermis but neither was significant from the control.

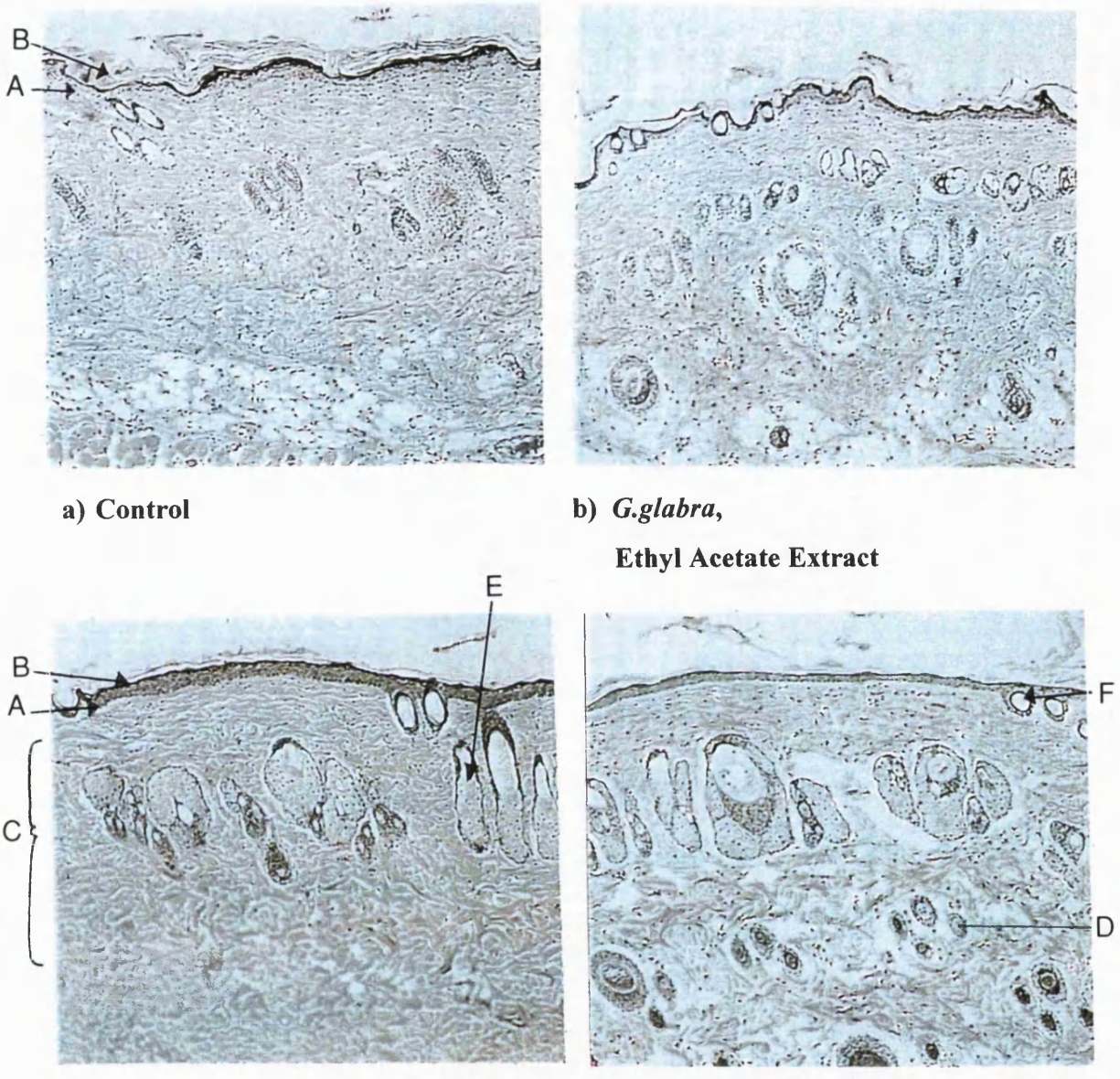
Isoliquiritigenin also exhibited anti-inflammatory activity to UVB induced dermatitis. The percentage increase in epidermal thickening due to UVB exposure as compared to the control was 60% (P<1%). With the application of isoliquiritigenin to UVB exposed skin the percentage increase was 17% (P<5%), a 71% decrease from the UVB control section (P<1%) (Figure 3.2.8.2.). The number of epidermal cells similarly increased with UVB radiation from 1.6 to 2.86, a 78% increase (P<1%). However with application of isoliquiritigenin this decreased by 81% to a 15% (1.84 cells) increase as compared to non-irradiated skin (Figure 3.2.8.1 & 3.2.8.3.). Isoliquiritigenin had no effect on the number of epidermal cells and the epidermal thickness when applied to the unexposed skin.

Licochalcone B exhibited strong anti-inflammatory activity against UVB induced inflammation. In skin exposed to UVB only the epidermal thickening increased to 15.36um, 138% more than the control (P<1%) With the application of licochalcone B the epidermal thickening caused by UVB was reduced to 8.80um, still 36% more than the control but 73% (P<1%) less than the skin exposed to UVB only. The number of epidermal cells increased and decreased parallel to the number of epidermal cells counted The average number of cells in the normal skin was measured as 1.46, in skin exposed to UVB light the mean number of epidermal cells increased to 3.92 a 168% (P<1%) increase (Figure 3.2.8.1. & 3.2.8.4.). With the application of licochalcone B to UVB exposed skin the number of epidermal cells increased to 1.94, 32% more than the control. However the number of epidermal cells was calculated as 80% less than the UVB exposed skin only (P<1%). The application of licochalcone B to normal skin did not have any significant increases or decreases to the thickness or the number of cells in the epidermis.

β-glycyrrhetinic acid inhibited UVB induced inflammation but not to the same levels as the previous compounds reported. The epidermal thickening for UVB exposed skin was measured as 12.30μm, a 92% (P<1%) increase as compared to the control 6.40μm (Figure 3.2.8.). With the application of β-glycyrrhetinic acid the thickness

was measured as 9.87μm, a reduction of 41%. The average number of epidermal cells counted in the epidermal layer for the untreated skin was 1.35 and the UVB exposed skin 3.27, a 142% increase. However when UVB exposed skin was treated with β-glycyrrhetinic acid the number of cells counted decreased to 2.45, an 81% increase as compared to the control (P<1%), but a 42 % reduction as compared to UVB exposed skin only (Figure 3.2.8.1& 3.2.8.5). With application of β-glycyrrhetinic acid only to unexposed skin there was a slight increase in the number of cell layers counted as with the epidermal thickness, they were both found to be significant from the control at the 5% level.

Histologically, those areas of rat skin exposed to UVB light appeared quite different to unexposed skin (figures 3.2.8.2, 3.2.8.3., 3.2.8.4. and 3.2.8.5.) and shared many expected similarities with human psoriatic skin. The light microscope pictures were taken at a magnification which showed the increased epidermal proliferation of the cells as well as other histological changes associated with UVB induced erythema Figure 3.2.8.2.c clearly shows a thickened epidermis as compared to non-irradiated skin (figure 3.2.8.2.a) and irradiated skin with *G.glabra* extract applied (figure 3.2.8.2.d). Although the *G.glabra* inhibited the proliferation of epidermal cells other histological changes associated with UVB appeared unaffected. The blood vessels in the upper dermis became dilated and tortuous; usually lymphocytes and macrophages can be found in the surrounding area (figure 3.2.8.2.c). Also noticeable are the development of microabscesses in the stratum corneum, following infiltration of PMNs. These can be seen on all UVB skin treated or untreated with the test compounds (figures 3.2.8.2.c&d, 3.2.8.3.c&d, 3.2.8.4.c&d and 3.2.8.5.c&d). Clear areas are associated with an accumulation of water. In some experiments it can be observed that application of the test compound as compared to the control actually enhanced cell proliferation, for example figure 3.2.8.5.b.



a) Control

b) *G.glabra*, Ethyl Acetate Extract

c) UVB

d) UVB & *G.glabra* Ethyl Acetate Extract

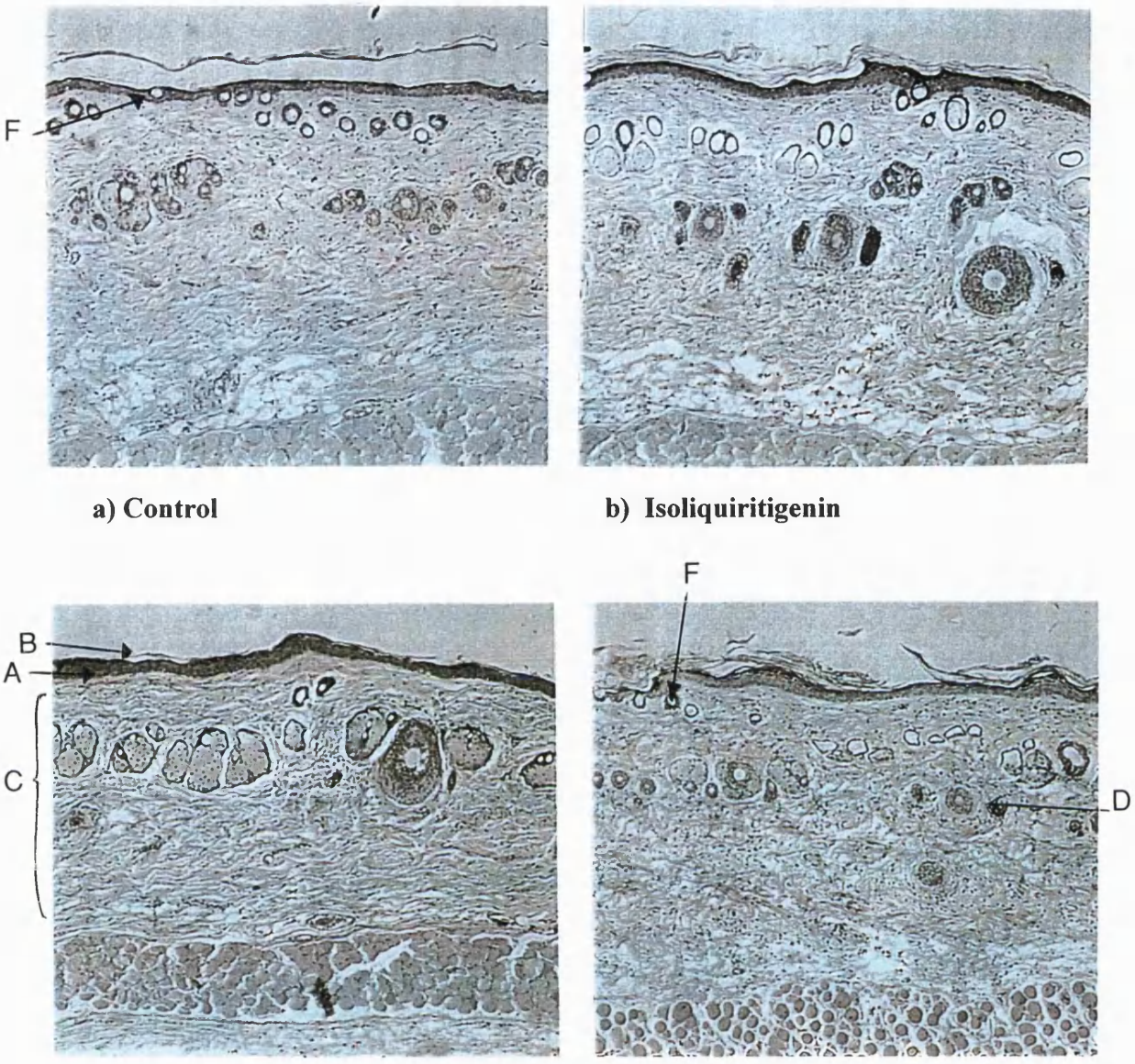
Stain: Haematoxylin and Eosin

Magnification x4

Key

A=Epidermis
B=Stratum Corneum
C=Dermis
D=Tortuous Capillaries
E=Hair Follicles
F= Microabscess

Figure 3.2.8.2. Histological Appearance of Male Wistar Rat Skin Five Days After Treatment With UVB Irradiation and *G.glabra*, Ethyl Acetate Extract



a) Control **b) Isoliquiritigenin**

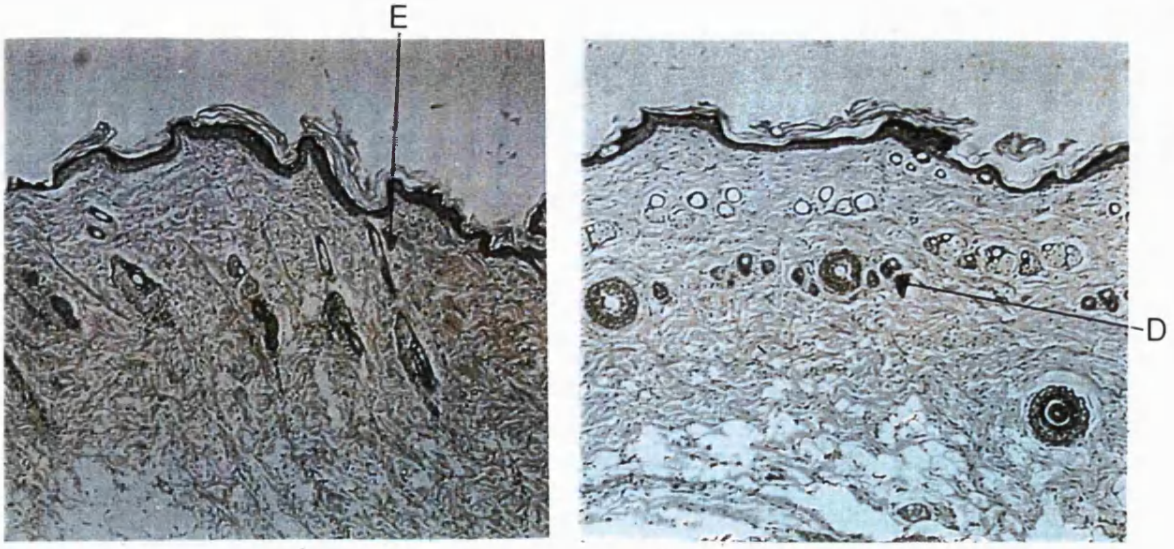
c) UVB **d) UVB & Isoliquiritigenin**

Stain: Haematoxylin and Eosin Magnification x4

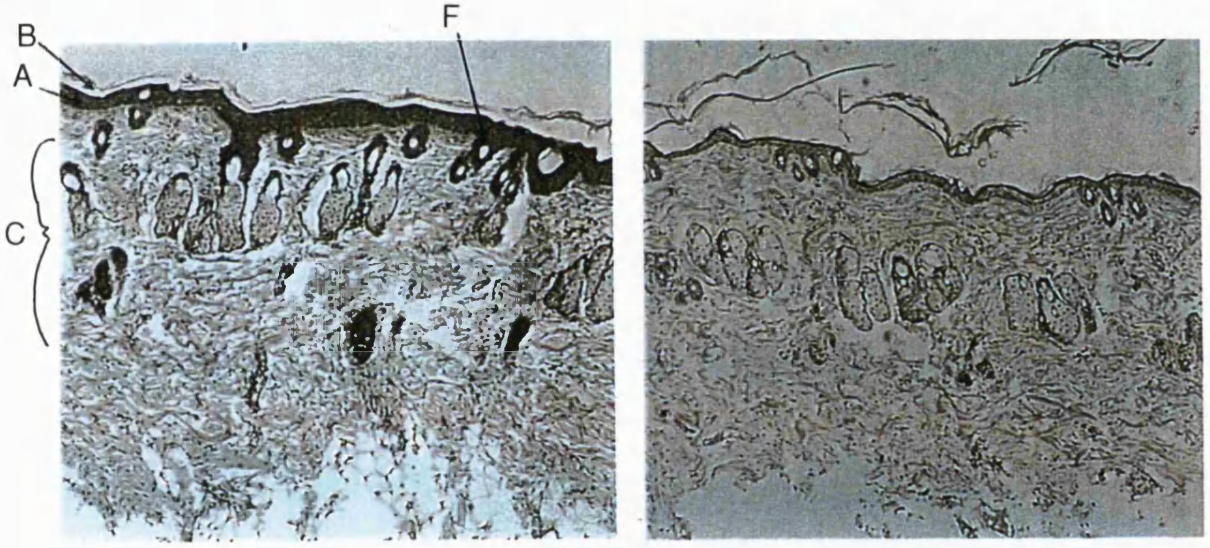
Key

A=Epidermis
B=Stratum Corneum
C=Dermis
D=Tortuous Capillaries
E=Hair Follicles
F= Microabscess

Figure 3.2.8.3. Histological Appearance of Male Wistar Rat Skin Five Days After Treatment With UVB Irradiation and Isoliquiritigenin



a) Control **b) Licochalcone B**



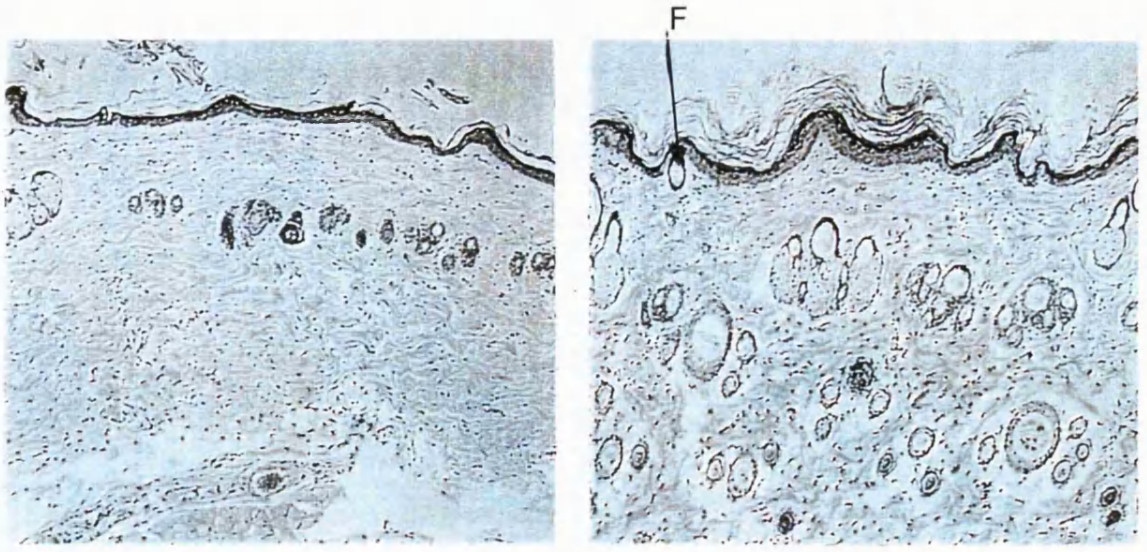
c) UVB **d) UVB & Licochalcone B**

Stain: Haematoxylin and Eosin Magnification x4

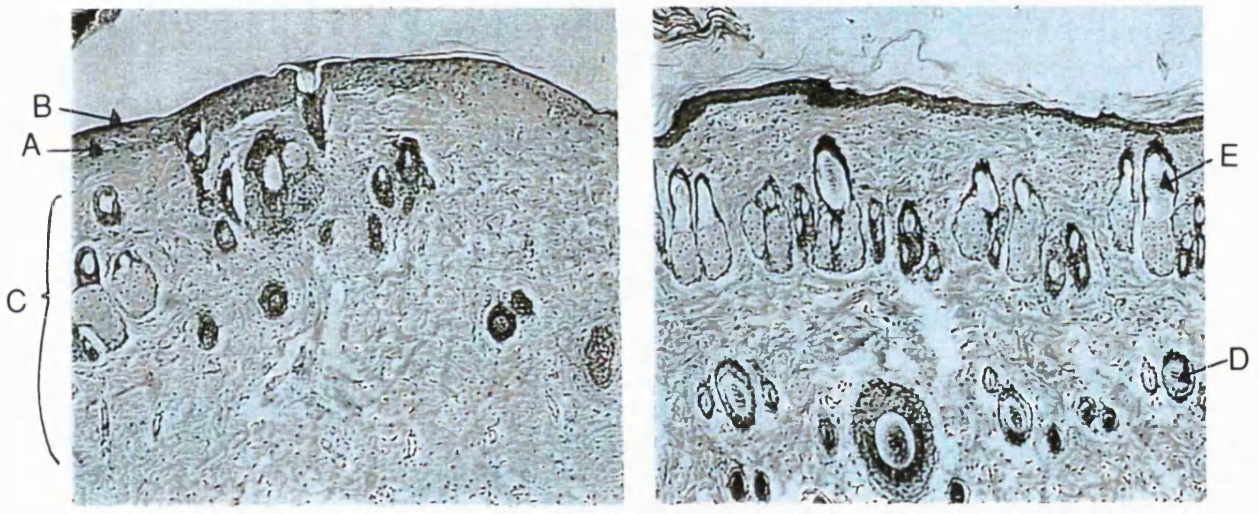
Key

A=Epidermis
B=Stratum Corneum
C=Dermis
D=Tortuous Capillaries
E=Hair Follicles
F=Microabscess

Figure 3.2.8.4. Histological Appearance of Male Wistar Rat Skin Five Days After Treatment With UVB Irradiation and Licochalcone B



a) Control **b) β-Glycyrrhetinic Acid**



c) UVB **d) UVB & β-Glycyrrhetinic Acid**

Stain: Haematoxylin and Eosin Magnification x4

Key

A=Epidermis
B=Stratum Corneum
C=Dermis
D=Tortuous Capillaries
E=Hair Follicles
F=Microabscess

Figure 3.2.8.5. Histological Appearance of Male Wistar Rat Skin Five Days After Treatment With UVB Irradiation and β-Glycyrrhetinic Acid

3.3. Bioassay guided fractionation of lutein, anhydrolutein I, 2',3'-anhydrolutein II and 3-hydroxy-3'-methoxy-β-ε-carotene

The initial screening assay of the five extracts from *G.aparine*, using inhibition of chemical (TPA) erythema on the mouse ear identified the chloroform extract as exhibiting significant anti-inflammatory activity (IC50 5-10μg)(Table 3.3.). Using a silica column (70-230 microns, 50 x 20cm) 1.7g of the chloroform extract was subsequently fractionated. A gradient solvent system was used increasing in polarity from 100% chloroform through to 100% ethyl acetate and finally 100% methanol. Fifty-four fractions were collected in total, each one measuring 10ml. Using analytical T.L.C. the fractions were combined to form only eleven according to the colour and RF values of the compounds. The eleven fractions were screened for anti-inflammatory activity (Table 3.3.) and fractions four and seven identified as possessing anti-inflammatory activity (Figure 3.3.).

Table. 3.3. Bioassay screening of fractions from silica column of the chloroform extract of *G.aparine*

Dose	Fractions										
μg	1	2	3	4	5	6	7	8	9	10	11
20	- - -	-	-	+	- - -	- - -	+	-	- -	- - -	- - -

Number of mice per group was four

\+ No sign of irritancy, a positive anti-inflammatory response in 100% of mice

\- Inhibitory activity in 50% or more of mice

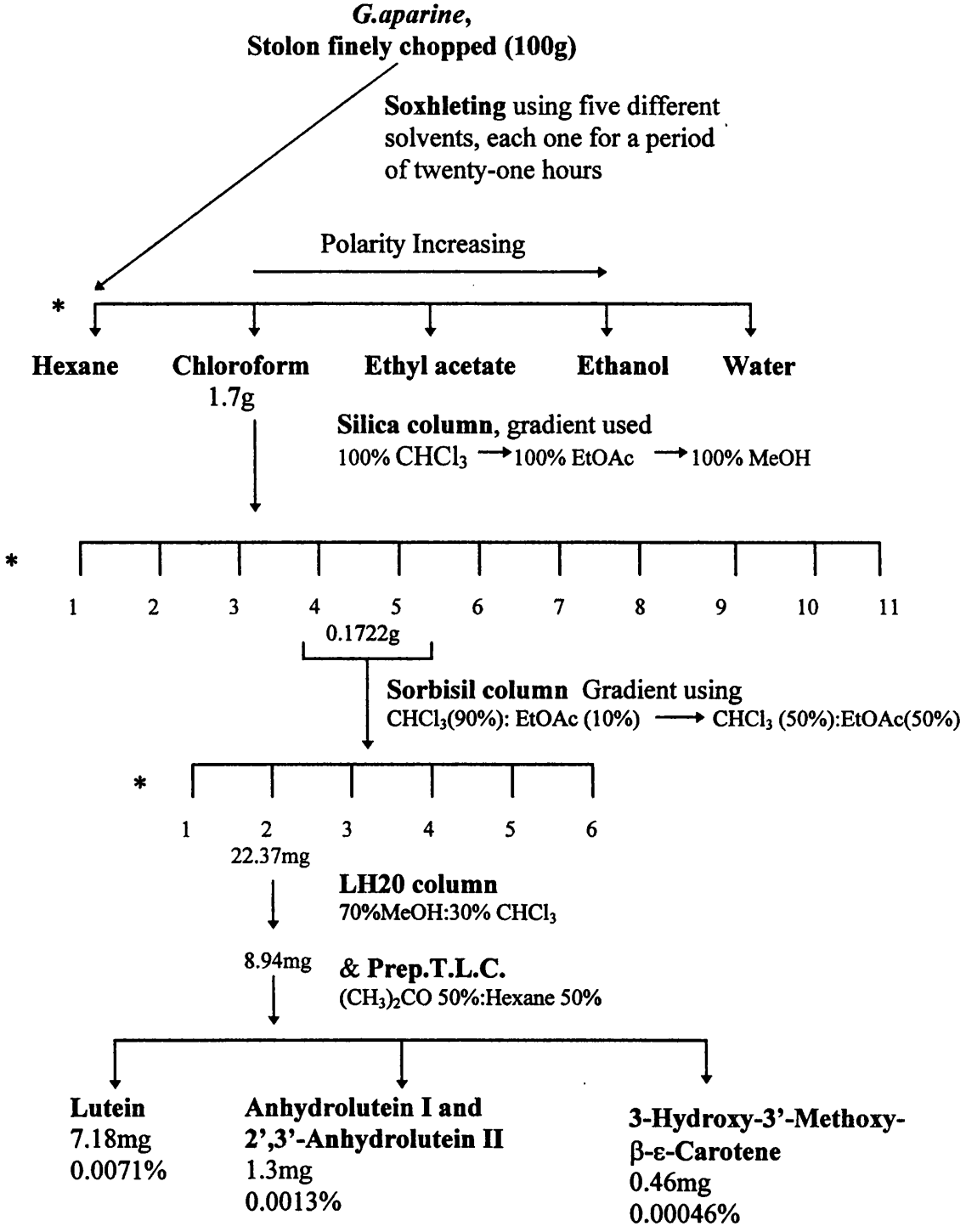
\- - Inhibitory activity in less than 50% of the mice

\- - - Inhibitory activity not recorded in any of the mice

Observations were recorded at six hours, length of time taken for TPA to induce a maximum erythema response

Fraction four was selected to undergo further separation as it exhibited strong anti-inflammatory activity. Initially preparatory silica T.L.C. (solvent ethyl acetate 5.5: hexane 5.5: methanol 1) was used to further separate fraction four. In using this method a further eight fractions were obtained and again screened using the bioassay guided technique. The results of the bioassay screening resulted in one active

Figure 3.3. **Bioassay Guided Fractionation and Isolation of Active Anti-psoriatic Compounds From *G.aparine***



* = Fractions tested using the bioassay guided technique, inhibition of chemically (TPA)induced erythema.

fraction which possessed one major and several minor compounds. Fraction three derived from the preparatory T.L.C., gave the most promising anti-inflammatory activity. Using T.L.C. the compound could be identified in normal daylight by its yellow colour and under UV (365nm) by a brown colour (R.F. 51, solvent ethyl acetate5.5: hexane 5.5: methanol 1). Upon spraying with 60% sulphuric acid the compound turned grey both in daylight and UV (365nm). A sorbisil column (40-60microns, 50cm x 1cm) was used in follow up work to further separate fraction 4 from the silica column so a larger quantity of the fraction could be separated at one time. Six fractions were obtained from the sorbisil column and fraction two identified as the most active. Fraction two corresponded to fraction 3 isolated previously using prep.T.L.C.

An LH20 column using the solvent system methanol 70% and chloroform 30% was subsequently used to purify the active compound and remove any remaining chlorophyll. However on using analytical T.L.C. (solvent systems acetone 50%: hexane 50% or ethyl acetate 70%:hexane 30%) to examine the purity of the compound using the LH20 column a further two other similar compounds were identified. The R_f values were calculated as 31 (lutein), 43 (3-hydroxy-3'-methoxy-β-ε-carotene, compound 315A) and 46 (mixture of anydrolutein I and 2',3'-anhydrolutein II) (acetone 1: hexane 1).

The three compounds were purified using preparatory T.L.C.(acetone 1: hexane1). The quantity of each compound isolated from 100g of material was as follows 7.18mg lutein (0.007%), 0.46mg 3-hydroxy-3'-methoxy-β-ε-carotene (315A) (0.00046%) and 1.3mg mixture of anydrolutein I and 2',3'-anhydrolutein II (315B) (0.0013%).

3.3.1. Identification of Lutein

Lutein, β-ε-Carotene-3, 3'-diol

Yellow crystal, R_f: 31, (acetone 1: hexane 1)

UV $\lambda_{max}^{CdCl_3}$ nm (log ε) 337 (0.184), 455 (0.512), 484 (0.429)

^{1}HNMR (400MHz,CD_3OD):δ 0.84(3H, s, H16'), 0.99(3H, s, H-17'), 1.07 (6H, s, H-16, 17), 1.36 (2H, dd, *J*=13.18, 6.81Hz, H-2'), 1.47 (2H, t, *J*=11.9, 11.9Hz, H-2), 1.62 (3H, s, H-18'), 1.73 (3H, s, H-18), 1.78 (2H, m H-2), 1.84 (2H, m H-2'), 1.91 (3H, s, H-19'), 1.96 (9H, s, H-19, 20, 20'), 2.04 (2H, dd, *J*=16.7, 9.8, H-4), 2.39 (2H, dd, *J*=16.7, 9.8Hz, H-4), 2.40 (1H, m, H-6'), 4.00 (1H, m H-3), 4.25 (1H, Br.s, H-3'), 5.43 (1H, dd, *J*=15.46, 9.80Hz, H-7'), 5.54 (1H, s, H-4'), 6.13 (2H, m, H-7, 8), 6.13 (2H, m, H-10', 8'), 6.15 (1H, m H-10), 6.25 (2H, m, H-14, 14'), 6.36 (2H, m, H-12, 12'), 6.62(1H, m, H-11'), 6.63 (2H, m, H-15, 15'), 6.64 (1H, m, H-11)

^{13}CNMR (400MHz,CD_3OD):δ 12.77 (3C, C-19, 20, 20'), 13.12 (C-19'), 21.63 (C-18), 22.88 (C-18'), 24.27(C-16'), 28.73 (C-16), 29.50 (C-17'), 30.26 (C-17), 34.04 (C-1'), 37.14 (C-1), 42.55 (C-4), 44.64 (C-2'), 48.42 (C-2), 54.96 (C-6'), 65.10 (C-3), 65.94 (C-3'), 124.47 (C-4'), 124.81 (C-11'), 124.94 (C-11), 125.59 (C-7), 126.17 (C-5), 128.74 (C-7'), 130.08 (2C, C15, 15'), 130.80 (C-10'), 131.30 (C-10), 132.58 (2C, C-14, 14'), 135.08 (C-9'), 135.70 (C-9), 136.50 (2C, C-13, 13'), 137.57 (3C, C-12, 6, 12'), 137.73 (C-5'), 137.75 (C-8'), 138.50 (C-8)

Lutein (β-ε-carotene-3, 3'-diol) is an oxygenated carotenoid known as a xanthophyll. Carotenoids are a widely distributed group of lipid soluble pigments which are C_{40} tetraterpenoids, consisting of eight isoprenoid units joined so that the arrangement of units maybe reversed at the centre of the molecule. Lutein displayed a bright yellow spot in daylight and under UV (365nm) gave a brown colour. Upon spraying with 60% sulphuric acid the compound turns grey both in daylight and UV (365nm).

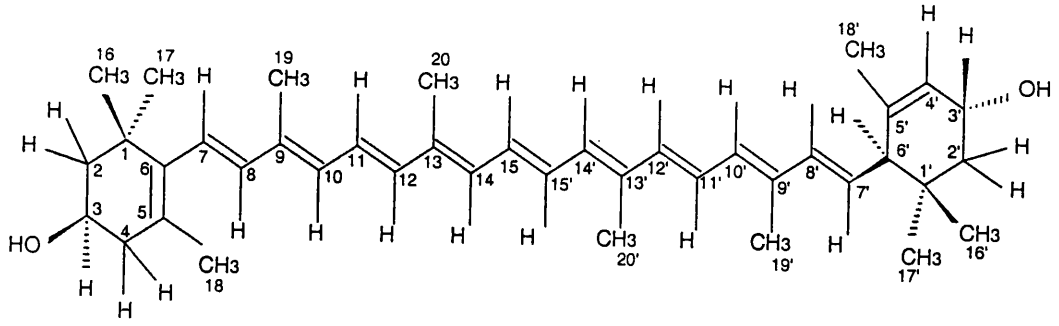
The identification of lutein was achieved using spectroscopic techniques including mass spectrometry, one and two dimensional NMR and UV analysis. FABMS, with 3-nitrobenzyl alcohol (MNOBA) and sodium (Na^+) as a matrix, afforded one prominent molecular ion at 568 (100%) corresponding to M^+ ($C_{40}H_{56}O_2$).

The ^{1}HNMR spectrum ($CDCl_3$) of lutein revealed the presence of two hydroxyl groups (Table 3.3.1., Figures 3.3.1., 3.3.1.1) at δ 4.00 and 4.25. The proton signal at δ4.25 was assigned to proton 3' and δ4.00 to proton 3. Highfield on the spectrum a total of seven shifts (all singlets) were counted all representing the methyl groups, two of which were high in intensity. These two signals were assigned to H-16 and 17 (δ 1.07) and H-19, 20 and 20' (δ 1.96). The methyl groups attached to the cyclopentane ring were found furthest down field. The protons at position 2' of lutein (Figures 3.3.1., 3.3.1.1) were identified on the ^{1}HNMR spectrum as double doublets coupling to those protons at 3' and 4'. The unprimed ring also showed four similar shifts δ2.04, 2.39, 1.47 and 1.78. The shifts at δ2.04 and 2.39 were assigned to the protons at H-4, the first shift was identified as a double doublet and the latter as a multiplet. The two shifts at δ1.47 (t) and 1.78 (m) were assigned to the protons at H-2. The proton at 6' coupling to proton 7'was found highfield at δ2.40, an indication of shielding. Proton 7' was assigned as the double doublet at δ5.43 (dd, *J*= 15.46, 9.80) coupling to both protons H-6' and 8'. The assignment of proton signals was supported by a 2D ^{1}H, ^{1}H COESY and 2D ^{1}H, ^{1}H NOESY experiments (Tables 3.3.1.1., 3.3.1.2; Figures 3.3.1.2, 3.3.1.3.).

The ^{13}C-DEPT spectrum of lutein exhibits a total of 33 signal (Table 3.3.1.3. Figure 3.3.1.4.), five of which were high in intensity and are assigned to C-19, 20 20' (δ12.77), C-15, 15' (δ130.08), C-14, 14' (δ132.58), C-13, 13' (δ136.50) and C-12, 6, 12' (δ137.57). The shifts at δ48.43, 44.56 and 42.56 were assigned to the CH_2 groups of C-2, 2' and 4 respectively. The two carbons with hydroxyls C-3 and 3' were assigned to the shifts at δ65.10 and 65.94. C-6' was found high field at δ54.97 due to shielding effects as was found with proton 6 in the ^{1}HNMR. All carbons with double bonding exhibit shifts in the region above δ124. Shifts assigned to methyl groups were found in the low frequency field region. The ^{13}CNMR data were

matched to data in the literature (Moss, 1976). The ^{13}C assignments were based on a one-bond ^{1}H, ^{13}C 2D COSY linking and all protons directly attached to ^{13}C nuclei (Table 3.3.1.4. Figure 3.3.1.5.). Although some signals were too close to each other to be separately assigned, an enhancement of the spectrum shows clearly the shift at δ124.81 corresponds to C4'. However past literature has assigned δ125.6 to C-4'.

Figure 3.3.1. Molecular Structure Of Lutein



The conjugated polyene system present in the molecule and the additional structural features give rise to the characteristic absorption spectrum of a carotenoid. Lutein exhibits an absorption spectrum with a maximum absorption at 455nm (0.512) with a shoulder peak at 484nm(0.429), a smaller peak at 337nm(0.184) was also recorded.

Table 3.3.1. ^{1}H-NMR Spectral Data of Lutein

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.1.1.

Proton	δ	Khachik et al, (1995) $CDCl_3$, 400MHz	Proton	δ	Khachik et al,(1995) $CDCl_3$, 400MH
2_{ax}	1.47	1.48	2'	1.36	1.37
2_{eq}	1.78	1.78	2'	1.84	1.84
3	4.00	4.00	3'	4.25	4.25
4_{ax}	2.04	2.05	4'	5.54	5.55
4_{eq}	2.39	2.39			
			6'	2.40	2.41
7	6.13	6.12	7'	5.43	5.43
8	6.13	6.12	8'	6.13	6.15
10	6.15	6.16	10'	6.14	6.14

Continued:

Table 3.3.1. ^{1}H-NMR Spectral Data for Lutein

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.1.1.

Proton	δ	Khachik et al, (1995) $CDCl_3$, 400MHz	Proton	δ	Khachik et al,(1995) $CDCl_3$, 400MH
11	6.64	6.64	11'	6.62	6.62
12	6.36	6.36	12'	6.35	6.36
14	6.25	6.26	14'	6.25	6.26
15	6.63	6.63	15'	6.63	6.63
OMe16	1.07	1.07	OMe 16'	0.84	0.84
OMe17	1.07	1.07	OMe 17'	0.99	0.99
OMe18	1.73	1.73	OMe 18'	1.62	1.62
OMe 19	1.96	1.96	OMe 19'	1.91	1.91
OMe 20	1.96	1.96	OMe 20'	1.96	1.96

Table 3.3.1.1.COESY ^{1}H-^{1}H NMR Spectral Data for Lutein

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.1.2.

Proton	δ		Protons - (δ)
2	1.47	-	2 (1.78), 3 (4.00)
2	1.78	-	2 (1.47), 3 (4.00)
3	4.00	-	2 (1.47), 2 (1.78), 4 (2.04), 4 (2.39)
4	2.04	-	4 (2.39), 3 (4.00)
4	2.39	-	4 (2.04), 3 (4.00)
7	6.13	-	8 (6.13)
8	6.13	-	7 (6.13)
11	6.64	-	10 (6.16)
14	6.26	-	15 (6.64)
15	6.64	-	15' (6.63)
2'	1.37	-	2' (1.84), 3' (4.25)
2'	1.84	-	2' (1.37), 3' (4.25)
3'	4.25	-	2' (1.37), 2' (1.84), 4'(5.54)
4'	5.54	-	18' (1.62), 6' (2.41), 3' (4.25)
6'	2.41	-	7' (5.43)

Continued:

Table 3.3.1.1.COESY ^1H-^1H NMR Spectral Data for Lutein

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.1.2.

Proton	δ		Protons - (δ)
7'	5.43	-	6' (2.41), 8'(6.15)
8'	6.13	-	7' (5.43)
10'	6.14	-	11' (6.62)
11'	6.62	-	10' (6.14)
14'	6.25	-	15' (6.63)
15'	6.63	-	15 (6.63)

Table 3.3.1.2. NOESY ^1H-^1H NMR Spectral Data for Lutein

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.1.3.

Proton	δ		Protons - (δ)
2	1.48	-	16 (1.07), 17 (1.07), 2 (1.78), 3 (4.00)
3	4.00	-	16 (1.07), 17 (1.073), 2 (1.47, 1.78), 18 (1.73), 4 (2.04, 2.39)
4	2.04	-	2 (1.78, 1.47), 18 (1.73), 4 (2.39)
4	2.39	-	18 (1.73), 2 (1.78, 1.47), 4 (2.04), 3 (4.00)
6	2.41	-	17' (0.99), 2' (1.36, 1.84), 18' (1.62), 7' (5.43)
7	6.13	-	19 (1.96), 18 (1.73), 17 (1.073)
8	6.13	-	19 (1.96), 18 (1.73), 17(1.073)
10	6.16	-	19 (1.96), 11 (6.64)
11	6.64	-	10 (6.15), 12 (6.36)
12	6.36	-	20 (1.96), 11 (6.64)
14	6.25	-	20 (1.96), 15 (6.63)
15	6.63	-	14 (6.25), 12 (6.36)
16	1.07	-	2 (1.48), 3 (4.00), 7 (6.13), 8 (6.13)
17	1.07	-	2 (1.48), 3 (4.00), 7 (6.13), 8 (6.13)
18	1.73	-	4 (2.04), 4 (2.39), 8 (6.13)
19	1.96	-	7 (6.13), 8 (6.13), 10 (6.15), 14(6.26), 12 (6.36), 11 (6.64), 15 (6.63)
20	1.96	-	7 (6.13), 8 (6.13), 10 (6.15), 14 (6.26), 12(6.36), 11(6.64), 15 (6.63)
2'	1.84	-	16' (0.84), 17' (0.99), 2' (1.37), 6' (2.40), 3'(4.25)
3'	4.25	-	16' (0.84), 2' (1.84), 18' (1.62), 2' (1.36, 1.84), 4' (5.54)

Continued:

Table 3.3.1.2. NOESY ^{1}H-^{1}H NMR Spectral Data for Lutein

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.1.3.

Proton	δ		Protons - (δ)
4'	5.55	-	16' (0.84), 2' (1.36, 1.84), 18'(1.62), 6' (2.40), 7' (5.43)
7'	5.43	-	16'(0.84), 18'(1.62), 19'(1.91), 6'(2.40), 8' (6.13)
8'	6.13	-	17' (0.99), 18' (1.62), 7' (5.43), 11' (6.62)
10'	6.13	-	17' (0.99), 18' (1.62), 7' (5.43), 11' (6.62)
11'	6.62	-	19' (1.91), 20'(1.96), 10'(6.14), 12' (6.35)
12'	6.35	-	20' (1.96), 11'(6.62)
14'	6.25	-	20' (1.96), 15' (6.63)
15'	6.63	-	14' (6.25), 12' (6.35)
16'	0.84	-	2' (1.84), 17' (0.99), 2' (1.36), 6' (2.40), 7' (5.43)
17'	0.99	-	2' (1.84), 16'(0.848), 2' (1.36), 6' (2.40)
18'	1.62	-	4' (5.54), 7' (5.43), 10' (6.14), 8' (6.13)
19'	1.91	-	7' (5.43), 10'(6.14), 11'(6.62)
20'	1.96	-	10' (6.14), 8' (6.13), 14'(6.25), 12' (6.35), 11' (6.62), 15' (6.63)

Table 3.3.1.3.^{13}C-NMR (DEPT) Spectral Data for Lutein

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.1.4.

Carbon	δ	Moss (1976)	Carbon	δ	Moss (1976)
1	37.14	37.1	1'	34.04	34.0
2	48.42	48.4	2'	44.64	44.7
3	65.10	65.1	3'	65.94	65.9
4	42.55	42.5	4'	124.47	125.6 *[1]
5	126.17	126.2	5'	137.73	137.8
6	137.57	137.6	6'	54.96	55.0
7	125.59	125.6	7'	128.74	128.6
8	138.50	138.5	8'	137.75	137.8
9	135.70	135.6	9'	135.08	135.0
10	131.30	131.3	10'	130.80	130.8

*[1] Values may be interchanged

Continued:

Table 3.3.1.3.^{13}C-NMR (DEPT) Spectral Data for Lutein

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.1.4.

Carbon	δ	Moss (1976)	Carbon	δ	Moss (1976)
11	124.94	124.9	11'	124.81	124.5 *[1]
12	137.57	137.6	12'	137.57	137.6
13	136.50	136.5	13'	136.50	136.5
14	132.58	132.6	14'	132.58	132.6
15	130.08	130.0	15'	130.08	130.0
16	28.73	28.7	16'	24.27	24.3
17	30.26	30.2	17'	29.50	29.5
18	21.63	21.6	18'	22.88	22.8
19	12.77	12.7	19'	13.12	13.2
20	12.77	12.7	20'	12.77	12.7

*[1] Values may be interchanged

Table 3.3.1.4. ^{13}C-^{1}H One Bond Correlation NMR Data for Lutein

$CDCl_3$, 400MHZ, TMS=0.0000, Figure 3.3.1.5.

Carbon	δ		Proton	δ
C-2	48.42	-	H-2	1.47 & 1.78
C-3	65.10	-	H-3	4.00
C-4	42.55	-	H-4	2.04 & 2.39
C-7	125.59	-	H-7	6.13
C-10	131.30	-	H-10	6.15
C-11	124.94	-	H-11	6.64
C-12	137.57	-	H-12	6.36
C-14	132.58	-	H-14	6.25
C-15	130.08	-	H-15	6.63
C-16	28.73	-	H-16	1.07
C-17	30.26	-	H-17	1.07
C-18	21.63	-	H-18	1.73
C-19	12.77	-	H-19	1.96
C-20	12.77	-	H-20	1.96
C-2'	44.64	-	H-2'	1.36 & 1.84

Continued:

Table 3.3.1.4. **^{13}C-^{1}H One Bond Correlation NMR Data for Lutein**

$CDCl_3$, 400MHZ, TMS=0.0000, Figure 3.3.1.5.

Carbon	δ		Proton	δ
C-3'	65.94	-	H-3'	4.25
C-4'	124.80	-	H-4'	5.54
C-6'	54.96	-	H-6'	2.40
C-7'	128.74	-	H-7'	5.43
C-8'	137.57	-	H-8'	6.13
C-10'	130.80	-	H-10'	6.14
C-11'	124.81	-	H-11'	6.62
C-12'	137.57	-	H-12'	6.35
C-14'	132.58	-	H-14'	6.25
C-15'	130.08	-	H-15'	6.63
C-16'	24.27	-	H-16'	0.84
C-17'	29.50	-	H-17'	0.99
C-18'	22.88	-	H-18'	1.62
C-19'	13.12	-	H-19'	1.91
C-20'	12.77	-	H-20'	1.96

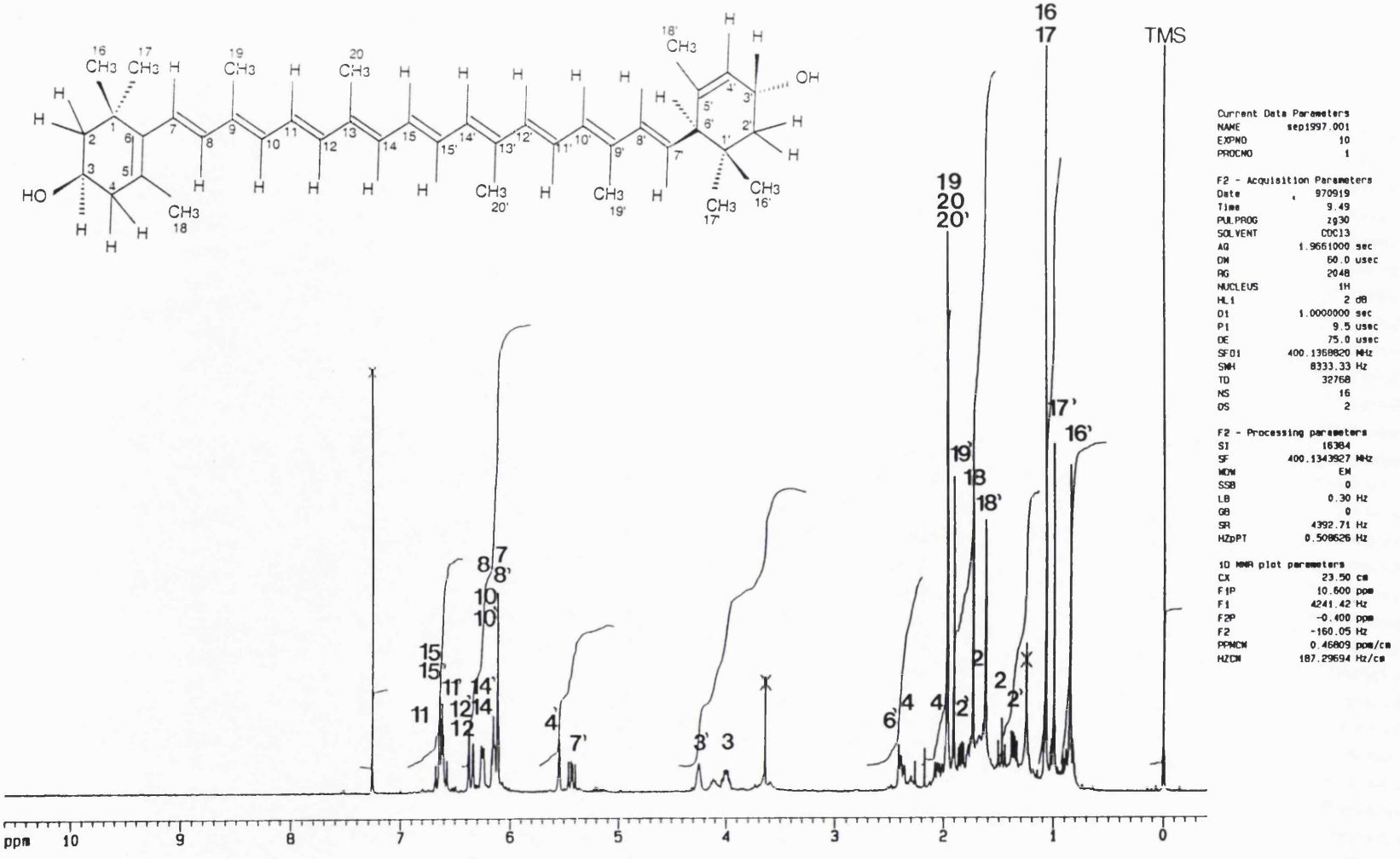


Figure 3.3.1.1. **^{1}H-NMR Spectrum (400MHz,$CDCl_3$) of Lutein, β-ε-carotene-3, 3'-diol**

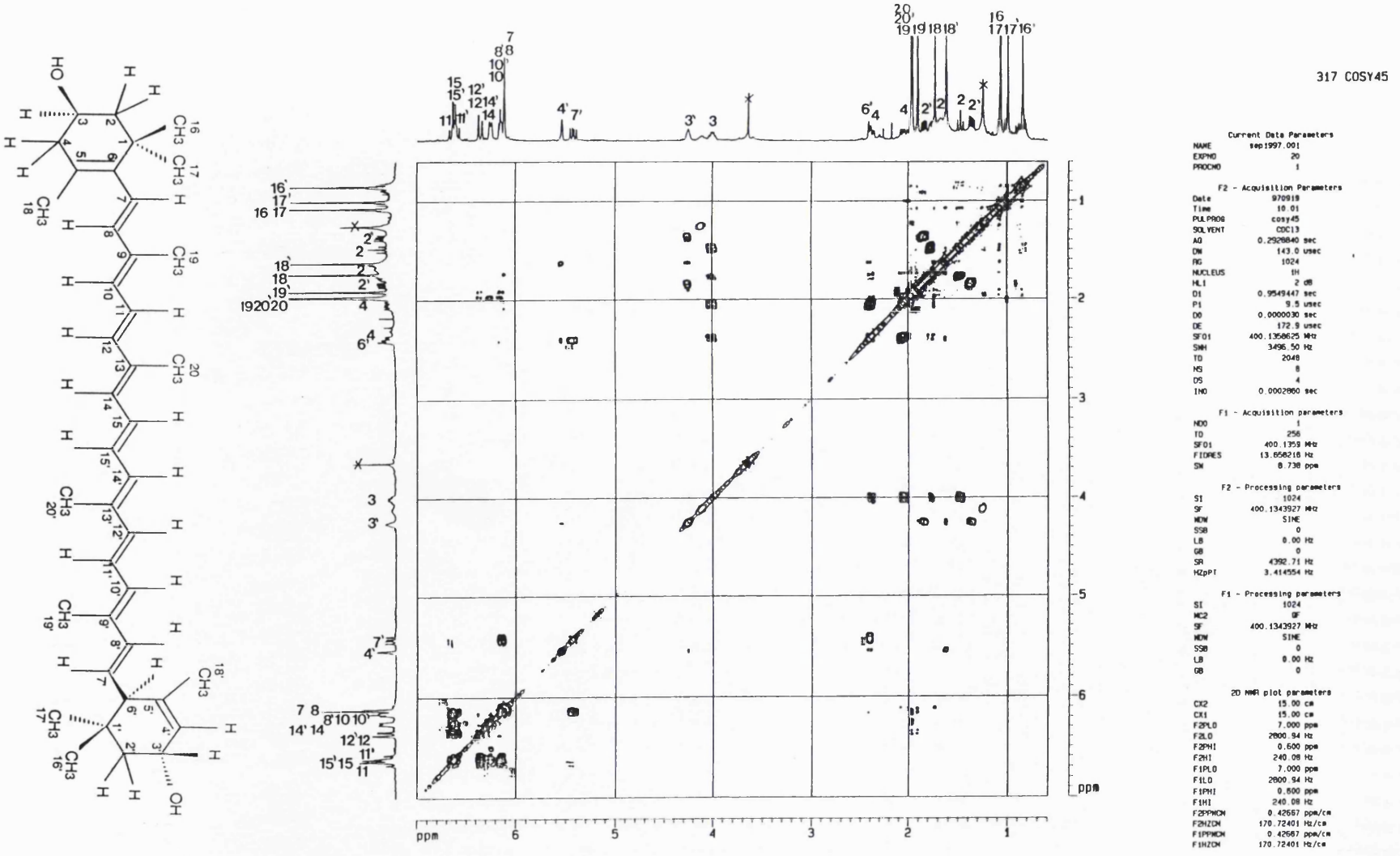


Figure 3.3.1.2. COESY ^{1}H-^{1}H-NMR Spectrum (400MHz,$CDCl_3$) of Lutein, β-ε-carotene-3, 3'-diol

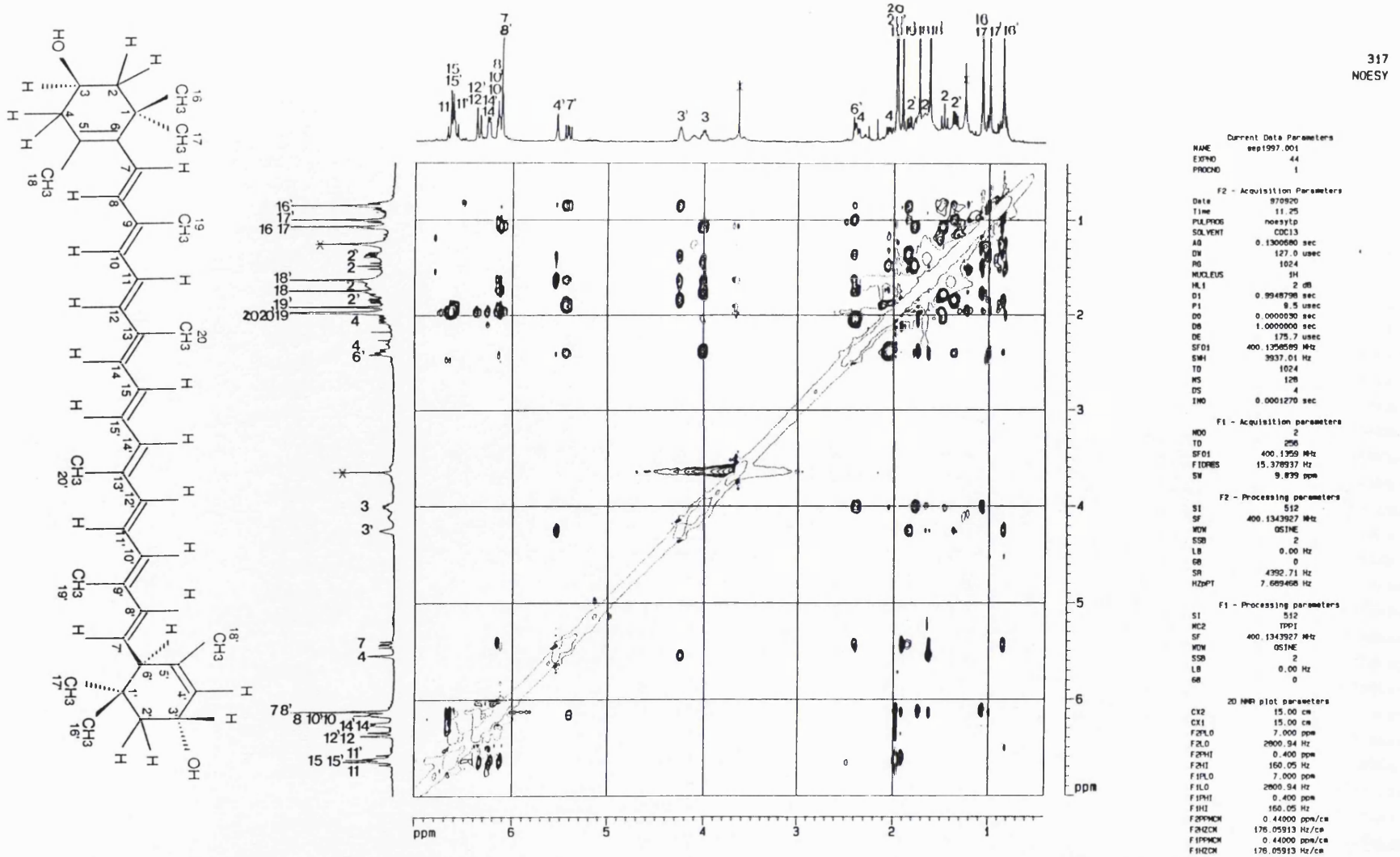


Figure 3.3.1.3. NOESY ^{1}H-^{1}H-NMR Spectrum (400MHz,$CDCl_3$) of Lutein, β-ε-carotene-3, 3'-diol

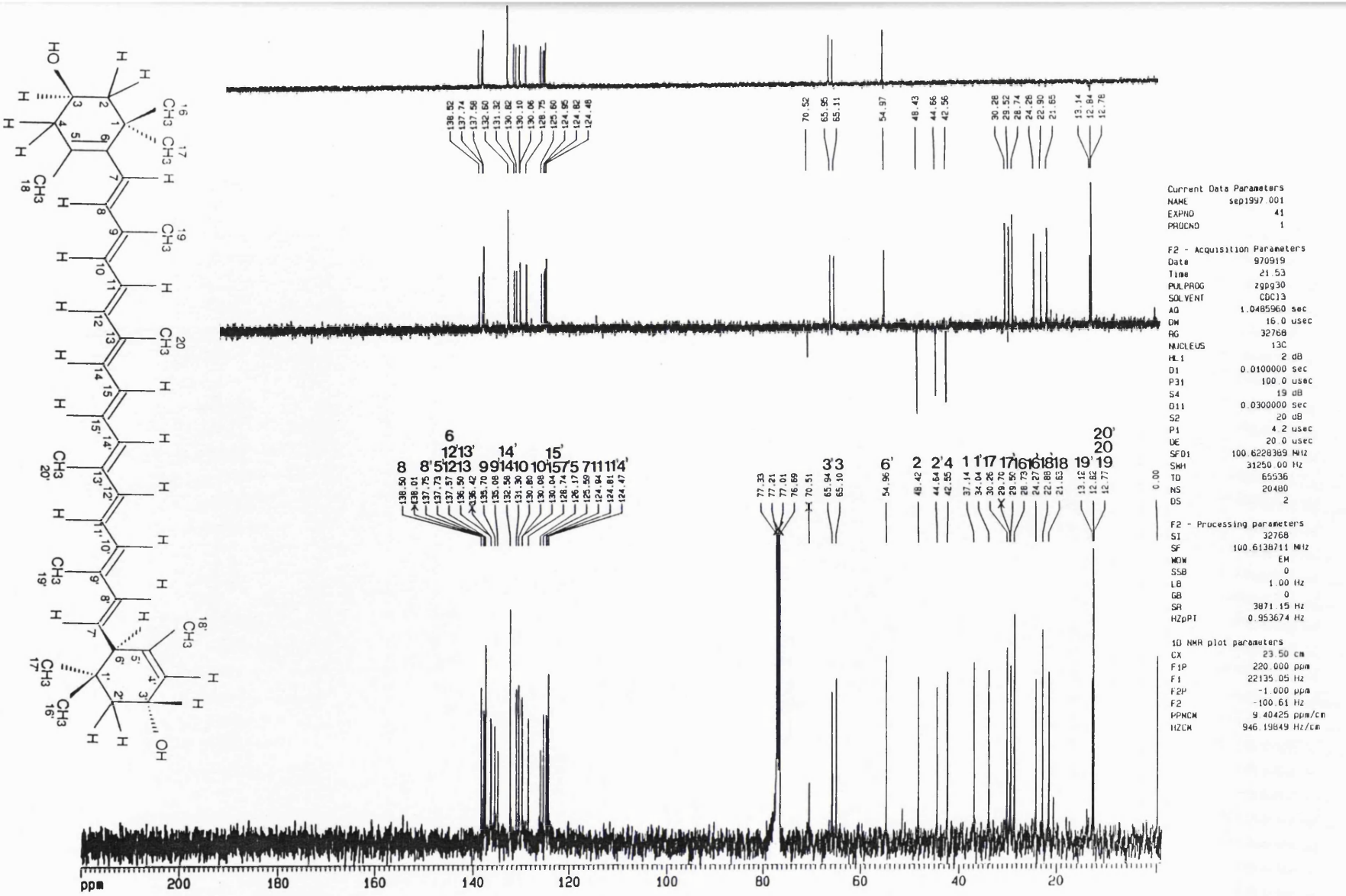


Figure 3.3.1.4. **^{13}C-NMR Spectrum (400MHz,$CDCl_3$) of Lutein, β-ε-carotene-3, 3'-diol**

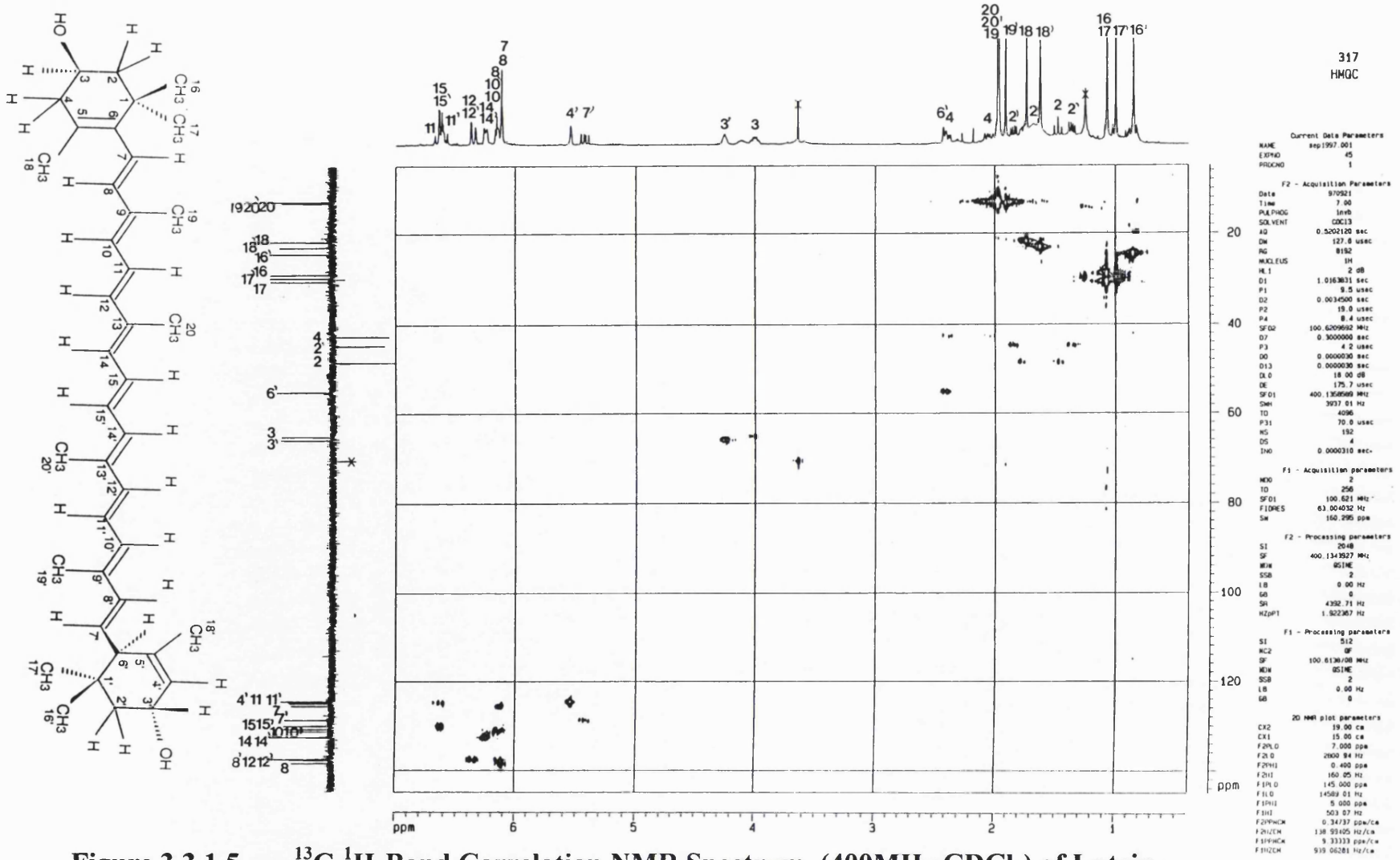


Figure 3.3.1.5. ^{13}C-^{1}H-Bond Correlation NMR Spectrum (400MHz,$CDCl_3$) of Lutein, β-ε-carotene-3, 3'-diol

3.3.2. Identification of anhydrolutein I and 2', 3'anhydrolutein II

Anhydrolutein I (3-hydroxy-3',4'-didehydro-β,γ-carotene) and

2', 3'-Anhydrolutein II (3-hydroxy-2',3'-didehydro-β-ε-carotene)

Yellow crystal, R_f , 46 (acetone 50: hexane 50)

UV $\lambda_{max}^{CdCl_3}$ nm (log ε) 273 (0.323), 339 (0.3380, 451 (0.528), 480 (0.412)

Anhydrolutein I, (3-hydroxy-3',4'-didehydro-β,γ-carotene)

^{1}HNMR (400MHz, CD_3Od): δ 0.87 (3H, s, H-16'), 0.90(3H, s, H-17'), 1.07 (3H, s, H-16, 17), 1.36 (1H, m, H-3), 1.47 (2H, t, *J*=11.92, 11.92Hz, H-2), 1.73 (3H, s, H-18), 1.77 (2H, m, H-20), 1.91 (2H, m, H-2'), 1.92 (3H, s, H-19'), 1.96 (9H, s, H-20, 19, 20'), 2.04 (2H, m, H-4), 2.20(1H, d, *J*=9.2), 2.39 (2H, m, H-4), 2.64 (1H,d, *J*=9.24Hz, H-6'), 4.00 (1H, m, H-3), 4.81 (2H,s, Ha-18'), 4.88 (2H, s, Hb-18'), 5.64 (1H, m, H-7'), 5.71 (1H, m H-3'), 6.09 (1H, m, H-7), 6.15 (1H, m, H-4'), 6.13 (1H, m, H-10'), 6.14 (1H, m, H-8), 6.15 (1H, m, H-10), 6.16 (1H, m, H-8'), 6.25 (2H, m, H-14, 14'), 6.36 (1H, d *J*= 14.9Hz, H-12), 6.33 (1H, m, H-12'), 6.63 (2H, m, H-15, 15'), 6.64(2H, m, H-11, 11')

2', 3'-Anhydrolutein II, (3-hydroxy-2',3'-didehydro-β-ε-carotene)

^{1}HNMR (400MHz, CD_3Od): δ 0.936 (3H, s, H-16'), 1.00(3H, s, H-17'), 1.073 (3H, s, H-16, 17), 1.36 (1H, m, H-3), 1.478 (2H, t, *J*=11.92, 11.92Hz, H-2), 1.71 (3H, s, H-18'), 1.73 (3H, s, H-18),1.77 (2H, m, H-2), 1.88 (3H, s, H-19'), 1.968 (9H, s, H-20, 19, 20'), 2.045 (2H, m, H-4), 2.39 (2H, m, H-4), 2.64 (1H, d,*J*=9.96, H-6'), 4.00 (1H, m, H-3), 5.32 (2H, d, *J*=9.4, H-2'), 5.60 (1H, m, H-4'), 5.64 (1H, m, H-7'), 5.71 (1H, m H-3'), 6.09 (1H, m, H-7), 6.13 (1H, m, H-10'), 6.14 (1H, m, H-8), 6.15 (1H, m, H-10), 6.16 (1H, m, H-8'), 6.25 (2H, m, H-14, 14'), 6.36 (1H, d *J*= 14.9Hz, H-12), 6.33 (1H, m, H-12'), 6.63 (2H, m, H-15, 15'), 6.64(2H, m, H-11, 11')

The ^{1}HNMR spectrum of 315B indicated the presence of two compounds anhydrolutein I and 2', 3' anhydrolutein II. Analytical HPLC was used to try and separate and isolate these two compounds incorporating the method described by Khachick *et al.*(1995). A C18 reversed phase column using an isocratic mixture of

acetonitrile (85%), methanol (10%) and hexane dichloromethane (1:1)(5%)(containing 0.1%, N,N-Diisopropylethylamine) at time 0 minutes was followed by a linear gradient at time 10 minutes and completed at time 40 minutes. The final composition of the gradient mixture was acetonitrile (45%), methanol (10%), hexane-dichloromethane (1:1)(45%). The chromatogram was monitored at 250, 300, 350, 400, 450 and 500nm using the photodiode array detector to determine any separation of the two compounds and the presence of impurities. A 20µl sample was injected onto the column at time 0 minutes. Four peaks were recorded at time 4.99minutes (0.05AU; 330nm), 6.24minutes (0.02AU; 380, 400 and 430nm), 7.05minutes (0.02AU; 270, 400, 410, 440nm) and 7.9minutes (2AU; 440 and 482nm). The major peak at 7.90 minutes was attributed to mixture 315B, whilst the other peaks were either the solvent front or minor impurities. Variations in the method including, using different flow rates, different ratios of solvent and the replacement of N,N-Diisopropylethylamine with triethyalamine proved unsuccessful in separating the two compounds.

Mixture 315B gave very similar chemical responses to lutein. On TLC 315B displayed a bright yellow spot and under UV (365nm) appeared brown in colour. Spraying with 60% sulphuric acid the compound went grey both in daylight and under UV (365nm).

FABMS with 3-nitrobenzyl alcohol (MNOBA) and sodium (Na^+) as a matrix afforded one prominent ion at *m/z* 550 corresponding to $C_{40}H_{54}O$.

The separation of these two compounds using chromatographic techniques was unsuccessful, however the ^{1}HNMR data were matched to two compounds within the published literature (Khachik, 1995), (Table 3.3.2.). These two compounds have identical *m/z* (550) and UV spectra making initial identification that there were two compounds present impossible. The two compounds were identified as anhydrolutein I and 2', 3'-anhydrolutein II. The structure of these two compounds and thus ^{1}HNMR spectra is very similar to lutein, apart from the primed ring (figures 3.3.2, 3.3.2.1.). The methyl groups attached to the primed ring and H-19' exhibit different shifts within the two compounds. The methyl groups, H-16', 17', 18'and

19', of anhydrolutein II exhibit shifts at δ0.937, 1.006, 1.718 and 1.885, whilst the methyl groups, H-16', 17', and 19' of anhydrolutein I exhibit shifts at δ0.876, 0.900, and 1.919. The methyl group at H-18' is replaced by a methylene (CH_2) and corresponding shifts were found at δ4.81 (Ha) and 4.88 (Hb). Other signals indicating the presence of these two compounds were at δ2.65 (1H, d, *J*=9.24, H-6', anhydrolutein I), δ2.21 (1H, d, *J*=9.96, H-6', anhydrolutein II), δ6.15 (1H, m, H-4',anhydrolutein I) and δ5.60 (1H, m, H-4', anhydrolutein II). The shift at δ5.324 (1H, d, *J*=9.4, H-2', anhydrolutein II) corresponds to a single proton at H-2' of anhydrolutein II coupling to H-3'. H-2' of anhydrolutein I corresponds for two protons with shifts at δ1.91 and 2.01. The ^{1}HNMR of 315B clearly indicates the presence of these two compounds. The ratio of the two compounds within the mixture is 5 (anhydrolutein I): 4 (anhydrolutein II).

The UV absorption spectrum of anhydrolutein I and II were almost identical. 315B ($CDCl_3$) gave an absorption spectrum of 273 (0.323), 339 (0.338), 451 (0.528) and 480nm (0.412). In the published literature the absorption spectrum of anhydrolutein I is as follows ($CDCl_3$), 273, 337, 454, 484; (EtOH) 332, 446, 474; (Hexane) 266, 332, 421, 444, 473 and anhydrolutein II ($CDCl_3$) 273, 339, 455, 484; (EtOH) 334, 447, 475 and (Hexane) 266, 332, 421, 444, 473.

Table 3.3.2. ^{1}H-NMR Spectral Data for 315B

(Anhydrolutein I and 2', 3'-Anhydrolutein II)

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.2.2.

Proton: Anhydro-lutein I	Proton: Anhydro-lutein II	δ 315B	δ Anhydrolutein I (Khachik *et al.*1995)	δ Anhydrolutein II (Khachik *et al.*1995)
2	2	1.47(t,*J*=11.9, 11.9)	1.48(t,*J*=12, 12)	1.48(t,*J*=12, 12)
2	2	1.77(m)	1.77(ddd,*J*=12,3.5,2)	1.77(ddd,*J*=12,3.5,2)
3	3	1.36(d,*J*=6.96)	1.33(d,*J*=4.9)	1.34(d, *J*=4.6)
3	3	4.00(m, br)	4.000(m, br)	4.00(m, br)
4	4	2.04(dd,*J*=9.6,16.9)	2.05(dd,br,*J*=9.7,16.5)	2.05(dd,br,*J*=9.7,16.5)
4	4	2.39(m)	2.39(ddd,*J*=16.5,6,1.5)	2.39(ddd,*J*=16.5,6,1.5)
7	7	6.09(m)	6.09(d,*J*=16.12)	6.09(d,*J*=16.6)
8	8	6.14(m)	6.14(d,*J*=16)	6.13(dd,*J*=16)
10	10	6.15(m)	6.15(d,*J*=11.5)	6.15(d,*J*=10.7)
11	11	6.64	6.64(dd,*J*=11.2, 14.5)	6.64(dd,*J*=11.5,14.7)
12	12	6.36(d,*J*=14.9)	6.36(d,*J*=14.9)	6.36(d,*J*=14.8)
14	14	6.25(m,br)	6.25(m,br)	6.25(m,br)
15	15	6.63(m)	6.63(m)	6.63(m)
16	16	1.07(s)	1.07(s)	1.07(s)
17	17	1.07(s)	1.07(s)	1.07(s)
18	18	1.73(s)	1.73(s)	1.73(s)
19	19	1.96(s)	1.96(s)	1.96(s)
20	20	1.96(s)	1.96(s)	1.96(s)
2'		1.91(m)	1.91(br,*J*=17)	
	2'	5.32(d,*J*=9.4)		5.32(d,*J*=9.6)
3'	3'	5.71(m)	5.71(d,br,*J*=9.7,4)	5.75(dd,*J*=9.6,5.2)
	4'	5.60(m)		5.60(m,br)
4'		6.15(m)	6.15(d,*J*=9.5)	
6'		2.20(d,*J*=9.9)		2.21(d,*J*=9.8)
	6'	2.64(d,*J*=9.2)	2.65(d,*J*=9.2)	
7'	7'	5.64(m)	5.64(dd,*J*=15.5, 9.2)	5.57(dd,*J*=9.7, 15.4)
8'	8'	6.16(m)	6.16(d,*J*=15.5)	6.14(d,*J*=15.5)
10'	10'	6.13(m)	6.13(d,*J*=12)	6.12(d,*J*=11.5)

Proton: Anhydro-lutein I	Proton: Anhydro-lutein II	δ 315B	δ Anhydrolutein I (Khachik *et al.* 1995)	δ Anhydrolutein II (Khachik *et al.* 1995)
11'	11'	6.64(m)	6.64(dd,*J*=11.5,15)	6.64(dd,*J*=11.5,15)
12'	12'	6.33(d,*J*=15.9)	6.34(d,*J*=14.9)	6.34(d,*J*=14.7)
14'	14'	6.25(m)	6.25(m)	6.24(m)
15'	15'	6.63(m)	6.63(m)	6.63(m)
16'		0.87(s)	0.87(s)	
	16'	0.93(s)		0.93(s)
17'		0.90(s)	0.90(s)	
	17'	1.00(s)		1.00(s)
	18'	1.71(s)		1.17(s)
18'		4.81	4.81(H_a)	
18'		4.88	4.88(H_b)	
	19'	1.88(s)		1.88(s)
19'		1.92(s)	1.91(s)	
20'	20'	1.96(s)	1.96(s)	1.96(s)

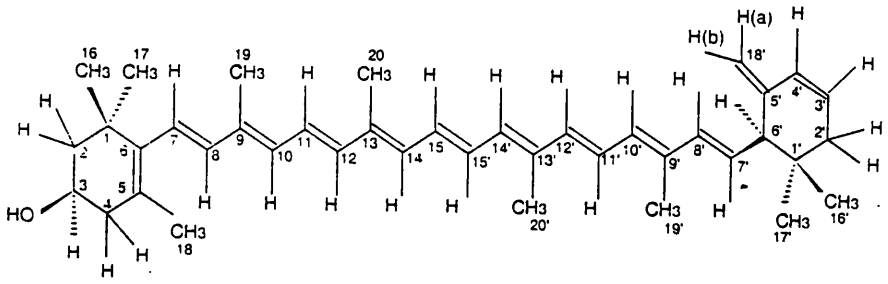


Figure 3.3.2. **Molecular Structure of Anhydrolutein I**

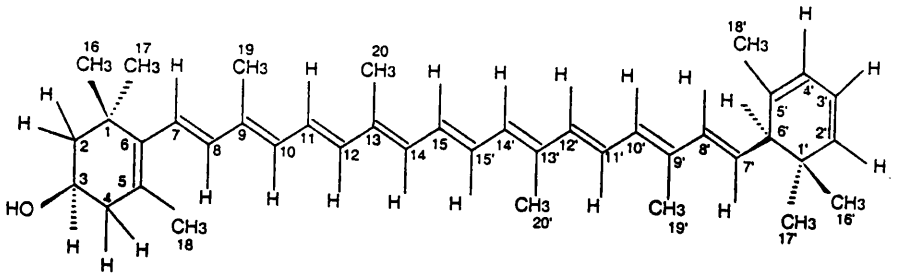


Figure 3.3.2.1. **Molecular Structure of 2',3'-Anhydrolutein II**

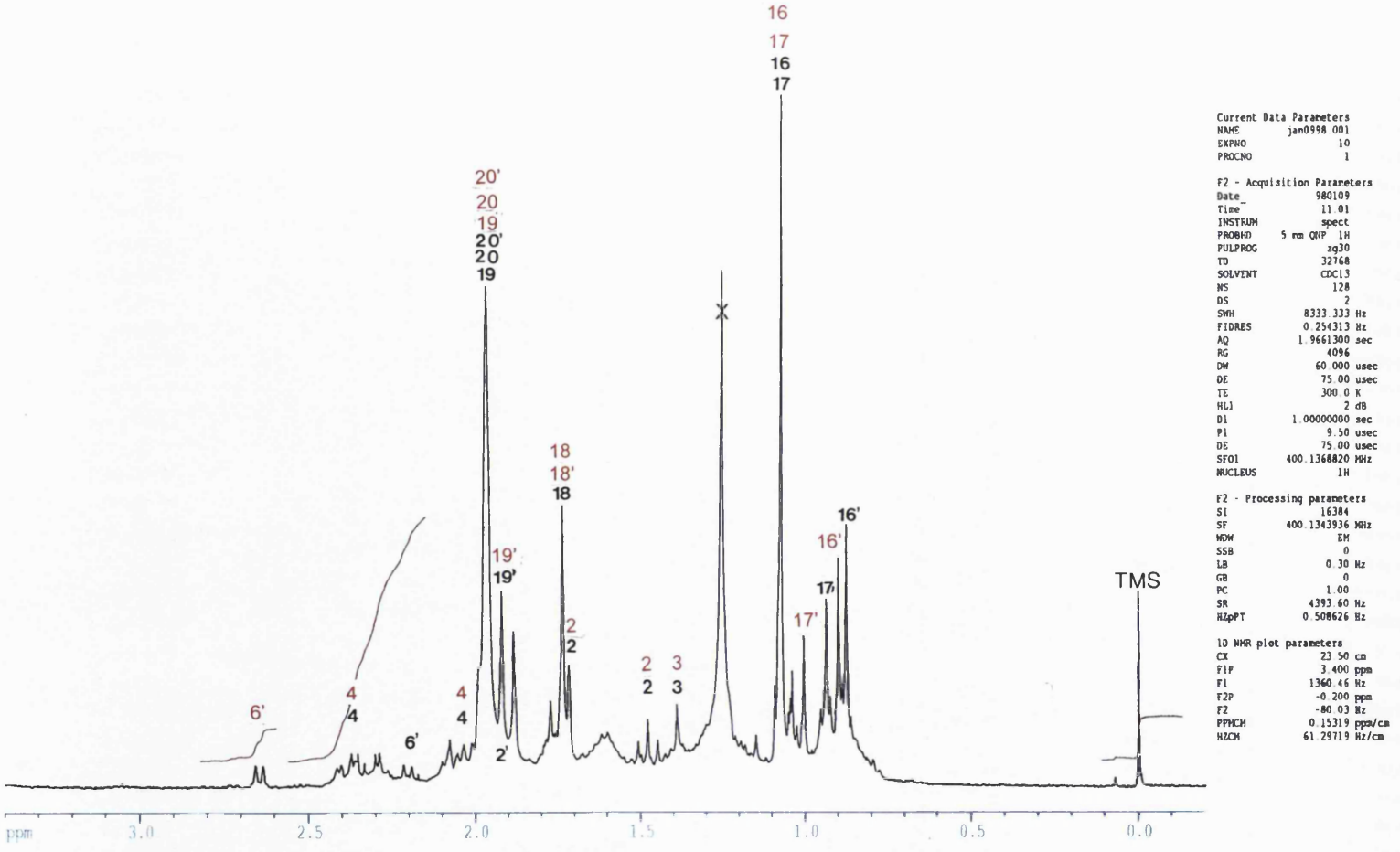


Figure 3.3.2.2. a) ^{1}H-NMR Spectrum (400MHz, $CDCl_3$) of Anhydrolutein I and 2', 3'-Anhydrolutein II
Anhydrolutein I = Black Numbers, 2,3'-Anhydrolutein II = Red Numbers

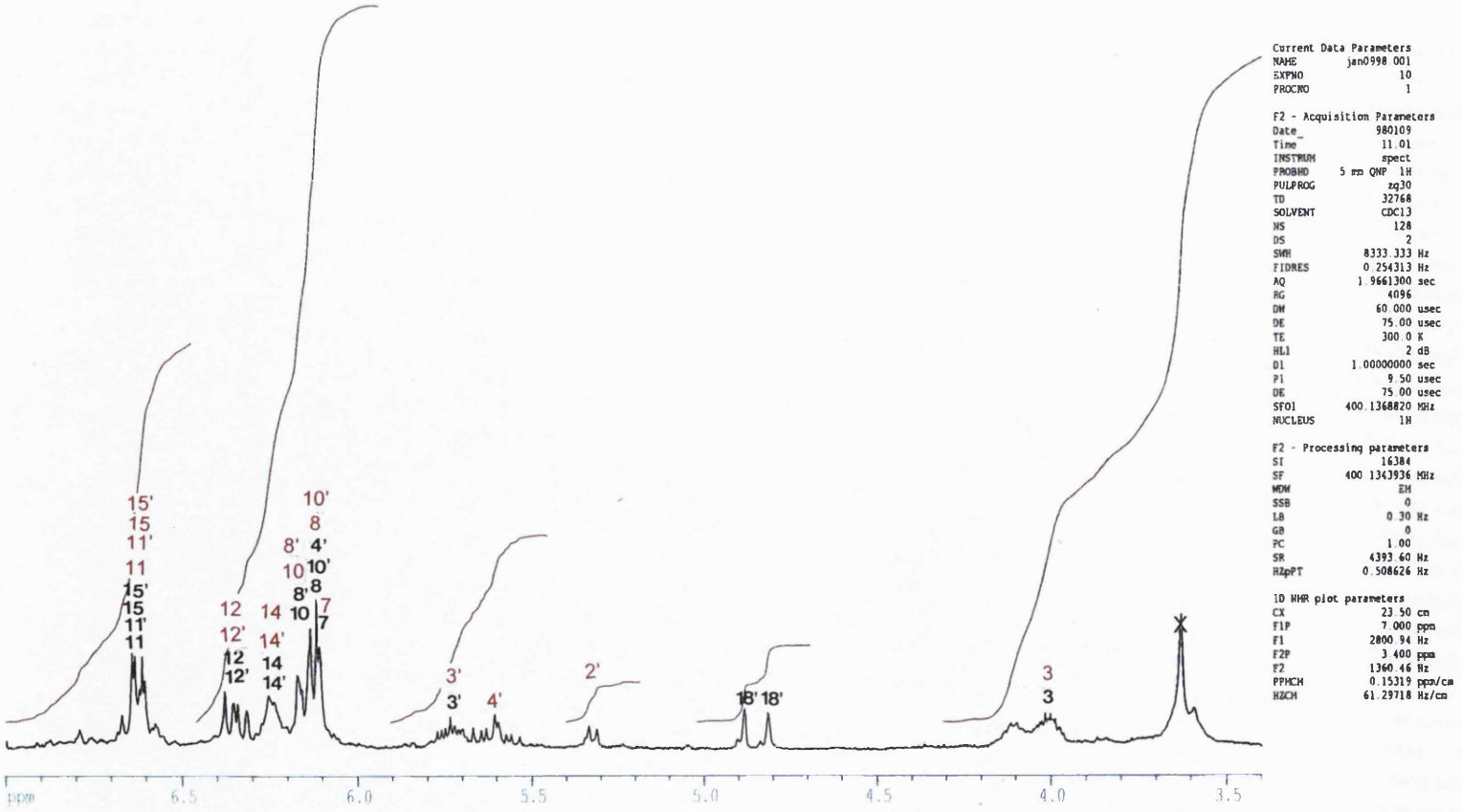


Figure 3.3.2.2. **b) ^{1}H-NMR Spectrum (400MHz, $CDCl_3$) of Anhydrolutein I and 2', 3'-Anhydrolutein II**
Anhydrolutein I = Black Numbers, 2,3'-Anhydrolutein II = Red Numbers

3.3.3. Identification of 3-Hydroxy-3'-Methoxy-β-ε-Carotene (Compound 315A)

3-Hydroxy-3'-Methoxy-β-ε-Carotene (Compound 315A)

Yellow crystal, R_f 43, (acetone 1: hexane 1)

UV $\lambda_{max}^{CdCl_3}$ nm (log ε) 337(0.298), 340(0.263), 452 (1.099), 482 (0.930)

^{1}HNMR (400MHz, CD_3Od): δ 0.834 (3H, s, H-16'), 0.999 (3H, s, H-17'), 1.074 (6H, s, H-16,17), 1.3983 (2H, m, H-2'), 1.479 (2H, m, H-2), 1.620 (3H, s, H-18'), 1.736 (3H, s, H-18), 1.780 (2H, m, H-2), 1.911 (3H, s, C-19'), 1.966 (12H, s, H-19,20,20'), 2.044 (2H, m, H-4), 2.390 (2H, m, H-4), 2.407 (1H, m H-6'), 3.645 (3H, s, H-21), 3.79 (1H, Br.S, H-3'), 4.00 (1H, m H-3), 5.44 (1H, dd, *J*=15.7, 10, H-7'), 5.604 (1H, s, H-4'), 6.13 (2H, m, H-7,8), 6.137 (2H, m, H-10', 8'), 6.150 (1H, m, H-10), 6.252 (2H, m, H-14, 14'), 6.361 (2H, m, H-12, 12'), 6.620 (1H, m, H-11'), 6.630 (2H, m, H-15, 15'), 6.640 (1H, m, H-11)

315A responded similarly to those carotenoids already identified. Using analytical TLC the compound displays a yellow spot, which under UV(365nm) appears brown in colour. Upon spraying with 60% H_2SO_4 the spot turns grey both in daylight and UV (365nm). The Marquart test results in two layers forming, the upper layer turning blue in colour. The UV absorption spectrum of 315A was very similar to lutein giving a maximum absorbency peak at 452nm (1.099) other peaks were recorded at 273 (0.298), 482(0.930) and 340nm (0.263).

FABMS with 3-nitrobenzyl alcohol (MNOBA) with sodium (Na^+) as a matrix afforded one prominent ion $(M+1)^+$ ion at 583 (100%). The most likely molecular formula for compound 315A, which is closely related to lutein is $C_{41}H_{58}O_2$.

The ^{1}HNMR spectrum ($CDCl_3$) of 315A was very similar to lutein (Table 3.3.3., Figures 3.3.3.1.). Due to insufficient quantities of 315A some of the signals appear quite weak, however the spectrum does clearly show some similarities to lutein. Identical shifts were found on the spectrum of 315A corresponding to the methyl groups and the protons from the polyene system of lutein. The shifts which correspond to the unprimed ring of lutein were also identified in the spectrum of

315A; the major structural changes from lutein were identified on the primed ring. The major differences in the spectra were found with H-3' and H-2'. The shift of the signal for H-3' appears higher field at δ3.79 suggesting an increase in shielding. The shift at δ5.54 in lutein (H-4') remains assigned to H-4' in 315A (δ5.60). The most likely structure for 315A is that of the 3'-methoxy analogue of lutein, namely 3-hydroxy-3'-methoxy-β-ε-carotene (Figure 3.3.3.). The shift of H-3' is moved highfield to δ3.79. The shift at δ3.64 could therefore be assigned to OCH_3 (referred to as CH_3-21) as established from the ^{1}HNMR tables. 3-Hydroxy-3'-methoxy-β-ε-carotene has been reported previously as being prepared by methylation of β-ε-carotene-3-ol (Hertzberg *et al.*, 1985; Jensen & Hertzberg, 1966). Their ^{1}HNMRdata were incomplete (see Table 3.3.3.) and they failed to report the presence of a signal for the C-3' methoxyl substituent (Hertzberg *et al.*, 1985). 3-Hydroxy-3'-methoxy-β-ε-carotene has not been previously reported as a natural product and it is therefore a novel natural product.

Figure 3.3.3. **Molecular Structure of Compound 3-Hydroxy-3'-Methoxy-β-ε-Carotene (315A)**

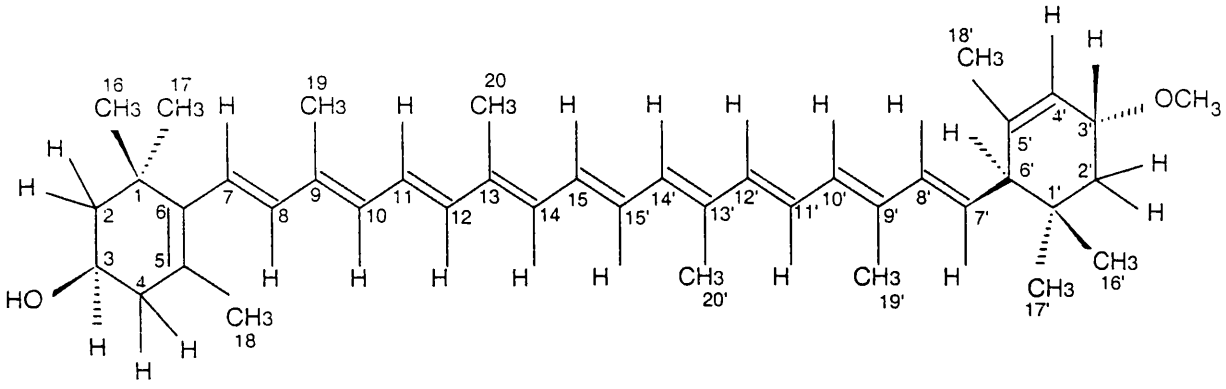


Table 3.3.3. ^{1}H-NMR Spectral Data for 3-Hydroxy-3'-Methoxy-β-ε-Carotene (315A)

$CDCl_3$, 400MHZ, TMS=0.000099m, Figure 3.3.3.1.

Proton	δ β-ε-Carotene-3-ol,3'-methoxy (315A)	δ Lutein	δ 3-Hydroxy-3'Methoxy-β-ε-Carotene (Hertzberg *et al*, 1985)
2	1.47(t, *J*=11.92,11.92)	1.47(t,*J*=11.92, 11.92)	
2	1.78(m)	1.78(m)	
3	4.00(m)	4.00(m)	
4	2.04(m)	2.04(dd, *J*= 16.7, 9.8)	
4	2.39(m)	2.39(m)	
7	6.13(m)	6.13(d, *J*=9.5)	
8	6.13(m)	6.13(d, *J*= 9.5)	
10	6.15(m)	6.15(d, *J*=10.98)	
11	6.64(m)	6.64(m)	
12	6.36(m)	6.36(d, *J*=15.12)	
14	6.25(m)	6.25(d, *J*= 6.64)	
15	6.63(m)	6.63(m)	
16	1.07(s)	1.07(s)	1.07(s)
17	1.07(s)	1.07(s)	1.07(s)
18	1.73(s)	1.73(s)	1.73(s)
19	1.96(s)	1.96(s)	1.97(s)
20	1.96(s)	1.96(s)	1.97(s)
2'	1.39(m)	1.36(dd, *J*=13.8, 6.81)	
2'		1.84(dd,*J*=15.92, 13.12)	
3'	3.79(s)	4.25(s)	
4'	5.60(s)	5.54(s)	
6'	2.40(m)	2.40(d, *J*=9.32)	
7'	5.44(dd, *J*=10,15.7)	5.43(dd, *J*=15.46, 9.80)	
8'	6.13(m)	6.13(d, *J*=12.8)	

Proton	δ β-ε-carotene-3-ol,3'-methoxy (315A)	δ Lutein	δ 3-Hydroxy-3'Methoxy-β-ε-Carotene (Hertzberg *et al*, 1985)
10'	6.13(m)	6.13(d,*J*=12.8)	
11'	6.62(m)	6.62(m)	
12'	6.36(m)	6.35(d, *J*=14.88)	
14'	6.25(m)	6.25(m)	
15'	6.63(m)	6.63(m)	
16'	0.83(s)	0.84(s)	0.84(s)
17'	0.99(s)	0.99(s)	0.94(s)
18'	1.62(s)	1.62(s)	1.642(s)
19'	1.91(s)	1.91(s)	1.92(s)
20'	1.96(s)	1.96(s)	1.97(s)
21'	3.64(s)		

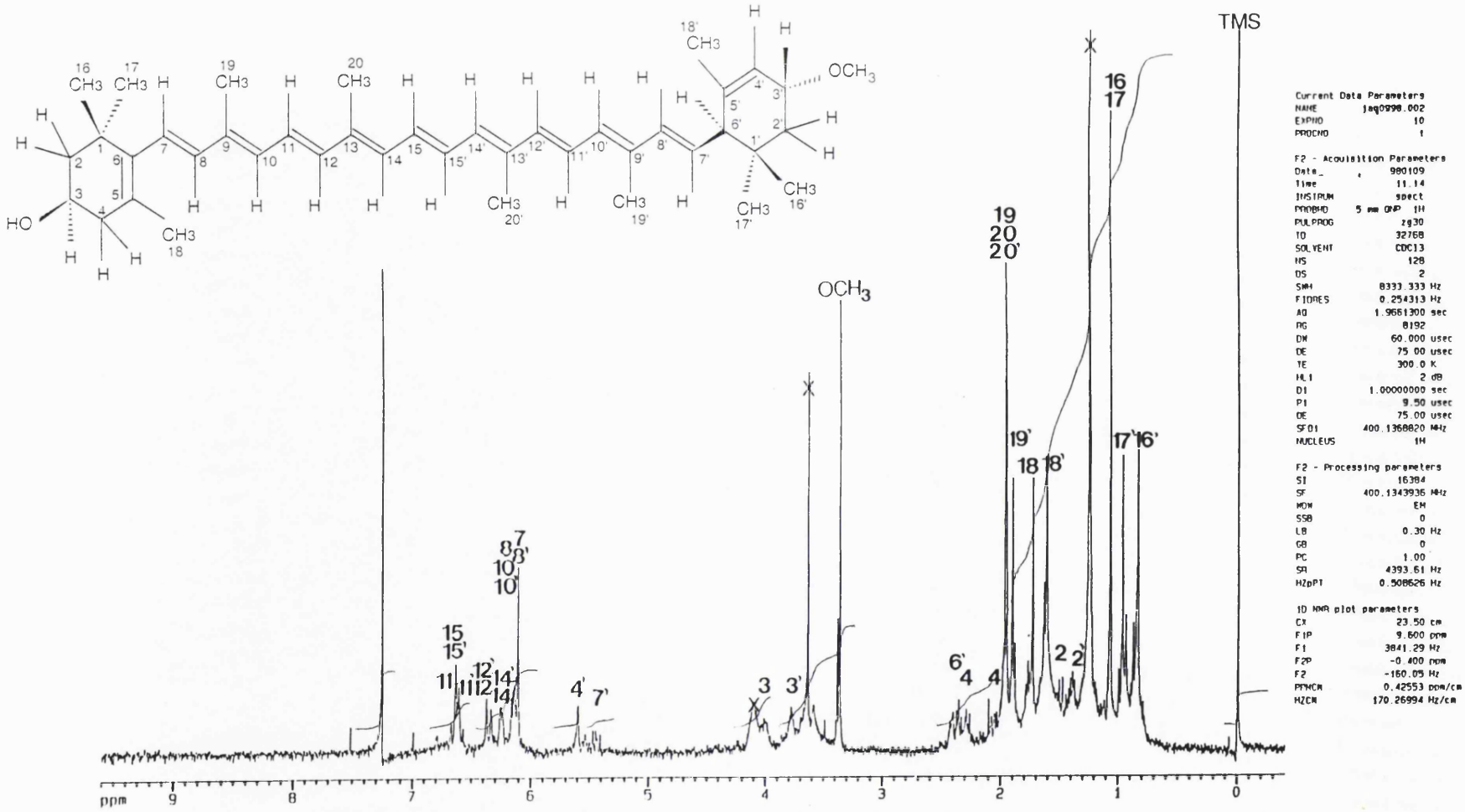


Figure 3.3.3.1. **^{1}H-NMR Spectrum (400MHz,$CDCl_3$) of Compound 315A, 3-hydroxy-3'-methoxy-β,ε-carotene**

3.3.4. Inhibition of chemically induced erythema on the mouse ear by isolated *G.aparine* compounds

Inhibition of chemically induced erythema on the mouse ear was conducted as two different assays as before, whereby the application time of TPA differed. Six mice were used per test group and the erythema response calculated as the percentage of animals giving an inhibitory response.

The IC50 response for each compound was calculated at six hours after the application, the time point at which TPA produces maximum erythema response. When TPA was applied after lutein, the IC50 was estimated between 1-5μg/ear (Table 3.3.4.), at doses of 5, 10 and 20μg/ear more than 50% of the mice exhibited an inhibitory response to TPA induced erythema, whilst at 50 and 100μg/ear there was a total inhibition to erythema. At 0.5 and 0.1μg/ear all animals responded with erythema therefore giving a negative response. The application of TPA before lutein increased the percentage of animals giving a 100% response to erythema; the time taken to reach a 100% response also decreased (Table 3.3.4.1.). The IC50 was now estimated between 5 and 10μg/ear. At 5μg/ear a 100% erythema response to TPA was recorded and at 10μg/ear 50% and more of the animals exhibited an inhibitory response.

Compound 315B, established to be a mixture of anhydrolutein I and 2', 3'-anhydrolutein II, was estimated to have an IC50 between 1 and 5μg/ear when TPA was applied after 315B (Table 3.3.4.). Total inhibition to erythema was observed in those mice treated with 5μg/ear and above, at four hours after the application of TPA. Only one group of mice was recorded as having a 100% negative inhibitory response to erythema, those treated with 0.5μg/ear at six hours. Application of TPA before the test compound had little affect on the inhibitory response to erythema, the IC50 remained between 5 and 10μg/ear. All animals treated with 10μg/ear or more inhibited erythema completely at six hours (Tables 3.3.4.1.). Those treated with 1μg/ear or less gave a 100% negative response at six hours after application.

Dose µg/ear	Lutein			Anhydrolutein I & 2',3'-Anhydrolutein II (315B)			3-Hydroxy-3'-Methoxy-β-ε-Carotene (315A)			Betacarotene (Sigma) - Control			Indomethacin (Sigma) - Control			Dithranol (Sigma) - Control		
	3hr	4hr	6hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr
100	+	+	+	+	+	+				+	+	+	+	+	+	+	-	-
50	+	+	+	+	+	+				+	+	+	+	+	+	+	-	-
20	+	+	-	+	+	- -	+	-	- -	-	+	- - -	+	+	+	+	-	- -
10	+	-	-	+	+	-	+	+	- -	+	+	- - -	+	-	+	+	+	-
5	+	-	-	+	+	-	+	-	- -	- -	- -	- - -	+	- -	- -	-	- -	- -
1	+	-	- -	+	-	- -	+	-	- - -	-	- -	- - -	+	+	-	+	-	- -
0.5	-	- -	- - -	-	- -	- - -	-	- -	- - -	- -	- -	- - -	+	- -	- - -	- -	- -	- - -
0.1	- -	- - -	- - -	-	- -	- -	- -	-	- - -	-	- -	- - -	+	- -	- - -	- -	- -	- - -

Number of mice per group was six

\+ Inhibition of erythema present in all mice

\- Inhibition of erythema in +50% of the mice

\- - Inhibition of erythema present in less than 50% of mice

\- - - No inhibition of erythema

Observations recorded at 3, 4 and 6 hours

Table 3.3.4. **Inhibition of Chemically Induced Erythema on the Mouse (CD1) Ear by *G.aparine* Compounds Applied 20 Minutes Prior to TPA**

Dose µg/ear	Lutein			Anhydrolutein I & 2',3'-Anhydrolutein II (315B)			3-Hydroxy-3'-Methoxy-β-ε-Carotene (315A)			Betacarotene (Sigma) - Control			Indomethacin (Sigma) - Control			Dithranol (Sigma) - Control		
	3hr	4hr	6hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr	3 hr	4 hr	6 hr
100	+	+	+	+	+	+				+	+	+	+	+	+	-	+	- - -
50	+	+	+	+	+	+				+	-	-	+	+	+	+	+	- -
20	+	+	-	+	+	+	+	-	- -	-	- -	- - -	+	+	+	+	- -	- - -
10	+	+	-	+	+	+	+	-	- -	-	-	- - -	+	-	+	- -	- -	-
5	-	- -	- - -	+	+	- -	+	-	- - -	-	- -	- - -	+	- -	- -	-	- -	- - -
1	-	- - -	- - -	+	-	- - -	- - -	- - -	- - -	-	- - -	- - -	+	+	-	- -	- - -	- -
0.5	- -	- - -	- - -	+	- -	- - -	- -	- -	- - -	- -	- -	- - -	+	- -	- - -	-	- -	- - -
0.1	- -	- - -	- - -	-	- -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+	- -	- - -	- -	- - -	- - -

Number of mice per group was six

\+ Inhibition of erythema present in all mice

\- Inhibition of erythema in +50% of the mice

\- - Inhibition of erythema present in less than 50% of mice

\- - - No inhibition of erythema

Observations recorded at 3, 4 and 6 hours

Table 3.3.4.1. **Inhibition of Chemically Induced Erythema on the Mouse (CD1) Ear by *G.aparine* Compounds Applied 20 Minutes After TPA**

3-Hydroxy-3'-methoxy-β-ε-carotene (compound 315A) could not be tested at doses above 20μg/ear due to insufficient quantities of the compound. However it was established that the IC50 (at six hours) against TPA induced erythema whether applied twenty minutes before or after the compound was above 20μg/ear. In those animals treated with TPA after the application of the test compound at 1μg/ear or less, a 100% negative response was recorded. When TPA was applied after the test compound a 100% negative response was observed in all animals treated with 5μg/ear or less.

The IC50 for β-carotene when applied before TPA was established between 20 and 50μg/ear, at concentrations lower than this a 100% negative response was recorded. A negative inhibitory response was also observed at three hours for those animals treated with 5μg/ear or less (tables 3.3.4., 3.3.4.1.). Application of TPA prior to β-carotene enhanced the number of animals showing an erythema response. The IC50 was estimated between 50 and 100μg/ear, again a 100% negative response was observed in animals treated with 20μg/ear or less. A 100% irritant response was observed from three hours onwards in those animals treated with 0.1μg/ear.

The two compounds indomethacin and dithranol used as positive controls are included on Tables 3.3.4. and 3.3.4.1. for comparison.

3.3.5. Inhibition of TPA and ADP induced blood platelet aggregation by isolated *G.aparine* compounds

The percentage of inhibition to platelet aggregation was dependent on whether aggregation was induced by TPA or ADP (Tables 3.3.5., 3.3.5.1.). Colchicine and aspirin were used as positive controls and β-carotene was used for comparison.

Lutein expressed significant inhibitory activity against ADP induced platelet aggregation. At 200 and 100μg/500μl PRP platelet aggregation was inhibited by

Dose µg/500µlPRP	Lutein		Anhydrolutein I & 2',3'-Anhydrolutein II (315B)		3-Hydroxy-3'-Methoxy-β-ε-Carotene (315A)		Beta-carotene		Colchicine		Aspirin	
	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.
200	46.3**	0.12	86.9**	0.10			72.7**	0.45	8.2*	0.20	26.6**	0.00
100	34.1**	0.17	51.9**	0.15	0.0	0.10	33.3**	0.35	4.3*	0.12	19.3**	0.25
50	26.0**	0.00	33.1**	0.15	0.0	0.20	31.3**	0.06	3.5	0.26	18.1**	0.06
20	18.0**	0.00	26.3**	0.15	-2.6	0.10	21.2**	0.15	8.2*	0.36	16.3**	0.00
10	20.0**	0.06	20.6*	0.12	-7.0	0.06	12.7*	0.06	3.5	0.10	20.0**	0.10
5	23.0**	0.32	19.4*	0.10	-7.0	0.31	14.7*	0.06	5.9*	0.10	15.0**	0.26
1	20.1**	0.00	11.9*	0.00	-4.4	0.21	7.7	0.61	5.9*	0.00	12.1**	0.10
0.5	23.2**	0.35	16.3*	0.23	-17.5	0.23	3.0	0.06	2.7	0.23	13.5**	0.15
0.1	18.9*	0.06	11.9*	0.10	-23.7	0.10	1.0	0.15	0.4	0.06	17.5**	0.06

Number of measurements per group was three
One-sided Paired T-test
* 5% level of significance
** 1% level of significance

Table 3.3.5. **Inhibition of ADP Induced Blood Platelet Aggregation By *G.aparine* Compounds**

Dose µg/500µlPRP	Lutein		Anhydrolutein I & 2',3'-Anhydrolutein II (315B)		3-Hydroxy-3'-Methoxy-β-ε-Carotene (315A)		Beta-carotene		Colchicine		Aspirin	
	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.	% Inhi.	S.D.
200	43.3**	0.06	82.9**	0.20			86.7**	0.06	37.7**	0.06	-0.1	0.00
100	27.6**	0.21	38.6**	0.12	31.0**	0.10	12.5	0.50	24.6*	0.12	0.8	0.00
50	13.4*	0.23	25.0**	0.10	26.4**	0.15	14.2	0.45	13.0	0.17	-0.1	0.60
20	3.0	0.06	10.0*	0.20	0.0	0.26	16.7**	0.06	-4.3	0.10	-0.1	0.00
10	3.7	0.17	7.1*	0.12	1.1	0.23	-5.8	0.06	-2.9	0.15	-2.0	0.00
5	3.4	0.00	7.1	0.06	1.1	0.12	-6.7	0.06	-5.8	0.06	-2.0	0.12
1	-0.7	0.00	3.6	0.10	-1.1	0.15	-8.3	0.06	-10.1	0.06	-3.0	0.00
0.5	0.7	0.06	2.1	0.06	2.3	0.15	-6.2	0.10	-5.8	0.06	-2.5	0.06
0.1	-0.7	0.00	-2.1	0.06	-1.1	0.21	-5.2	0.06	-13.0	0.26	-1.1	0.10

Number of measurements per group was three
One-sided Paired T-test
* 5% level of significance
** 1% level of significance

Table 3.3.5.1. **Inhibition of TPA Induced Blood Platelet Aggregation By *G.aparine* Compounds**

46% (P<1%) and 34% (P<1%) respectively. The inhibitory activity to ADP induced aggregation remained significantly high for all doses tested from 0.1μg/500μl PRP and upwards, the inhibitory activity always remaining above 18.9% (P<5%). Lutein expressed significant inhibitory activity against TPA induced aggregation at 200μg/500μl PRP, however at 100μg/500μl PRP this decreased to 27% (P,1%) and at 50μg/500μl PRP to 13%. Lutein tested at doses lower than 50μg/500μl PRP did not express inhibitory activity against TPA induced aggregation (Figures 3.3.5., 3.3.5.1.).

Compound 315B, the mixture of anhydrolutein I and 2',3'anhydrolutein II, expressed similar inhibitory response to both forms of platelet aggregation as lutein. At 200μg/500μl PRP 315B inhibited ADP induced aggregation by 86% and TPA induced aggregation by 82%;both were recorded as being highly significant from the control (P<1%) (Table 3.3.5., Figure 3.3.5.1). At 100μg/500μl PRP the inhibitory response to ADP induced aggregation decreased to 51%, inhibitory activity was still evident at 0.1μg/500μl PRP (11.9%). Inhibition to TPA induced aggregation remained significant at doses of 50μg/500μl PRP and above; below this level significant inhibitory activity was not recorded.

3-Hydroxy-3'-methoxy-β-ε-carotene (compound 315A) exerted anti-platelet activity against TPA induced aggregation only. At 50 and 100μg/500μl PRP TPA induced platelet aggregation was inhibited by 26 and 31% only; both were recorded as significant from the control (P<1%)(Table 3.3.5.1. Figure 3.3.5.1.). At doses lower than this no inhibitory effect was recorded. No inhibitory activity was recorded against ADP induced aggregation at the doses of 100μg/500μl PRP and below which were tested (Table 3.3.5., Figure 3.3.5.). Platelet aggregation was actually enhanced at concentrations of 0.5 and 0.1μg/500μl PRP, by 17 and 23% respectively.

β-Carotene inhibited both ADP and TPA induced aggregation significantly at the dosage of 200μg/500μl PRP. ADP induced aggregation was inhibited by 72% and TPA induced aggregation by 86% (P<1%). At 100μg/500μl PRP the level of inhibition for both ADP and TPA induced aggregation reduced considerably to 33

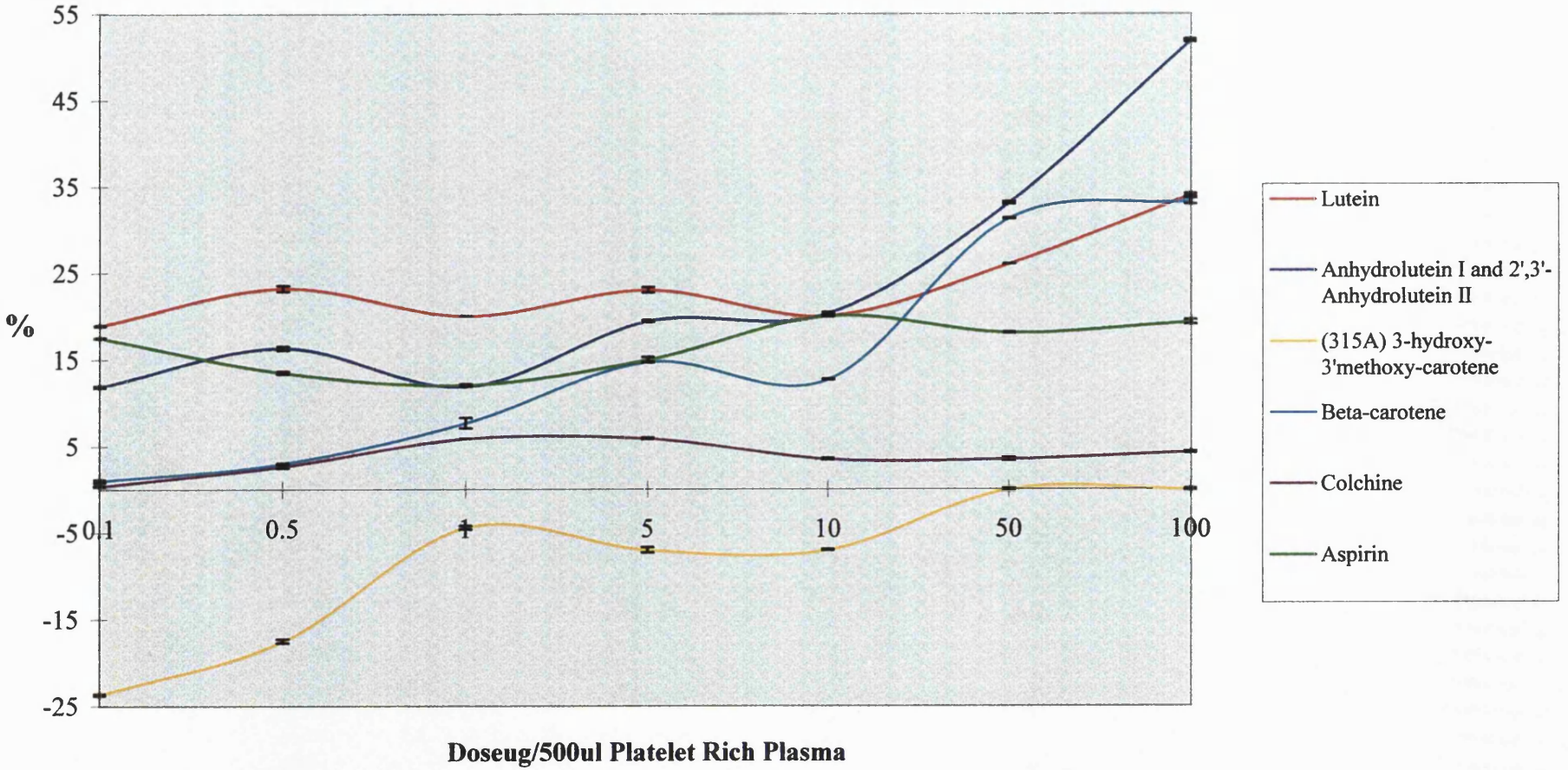


Figure 3.3.5. **Inhibition of ADP Induced Platelet Aggregation By *G.aparine* Compounds**

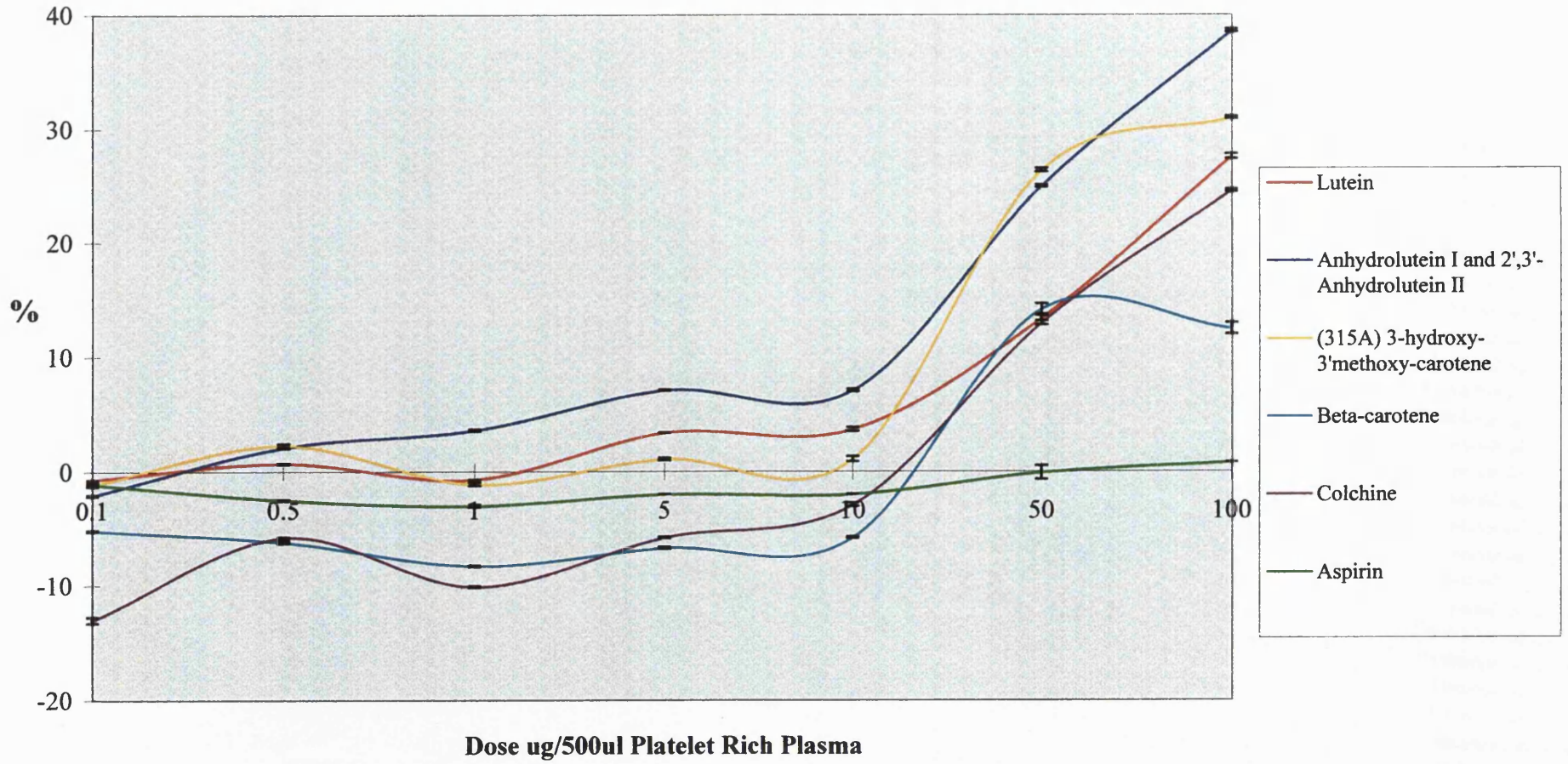


Figure 3.3.5.1. **Inhibition Of TPA Induced Platelet Aggregation By *G.aparine* Compounds**

and 12% respectively. At 10μg/500μl PRP and lower inhibition against TPA induced aggregation ceased and β-carotene enhanced the aggregation between 5 and 8% for all doses tested. ADP aggregation was recorded as being significantly inhibited at doses of 5μg/500μl PRP and more; at concentrations lower than this no inhibitory activity was recorded.

3.3.6. Inhibition of Swiss 3T3 fibroblast cell proliferation using *G.aparine* compounds

A dose response study using the isolated compounds from *G.aparine*, the crude chloroform extract and β-carotene was established for cytotoxic effects on Swiss 3T3 mouse fibroblast proliferation. β-Carotene was used as a positive as its cytotoxic effects were already published in the literature.

The crude chloroform extract form *G.aparine* produced the most significant cytotoxic effects on normal cell growth; at 20μg an 85% inhibitory response was expressed (P<1%)(Table 3.3.6., Figure 3.3.6.). The chloroform extract exhibited a normal dose response curve, at 10μg the percentage inhibition was 62 and at 0.1μg 10%. However below a dose of 0.1μg the effects of the chloroform extract were no longer inhibitory, but enhanced the proliferation of cells.

The effects of lutein on cell growth were parallel to the chloroform extract although the effects were not as significant. At 20μg the percentage inhibition was recorded as 73%. A dose dependent relationship was observed until 0.1μg where by the proliferation of cells was enhanced by 9%. When dosed with 0.01μg the percentage of enhancement was measured as significant from the control (P<1%) (Table 3.3.6, Figure 3.3.6.). The mixture, 315B, inhibited the proliferation of fibroblasts at 20μg by 47% (P<1%), at 10μg the percentage of inhibition remained at 45%. An inhibitory response was exhibited for all doses at 0.01μg (6.5%) and above.

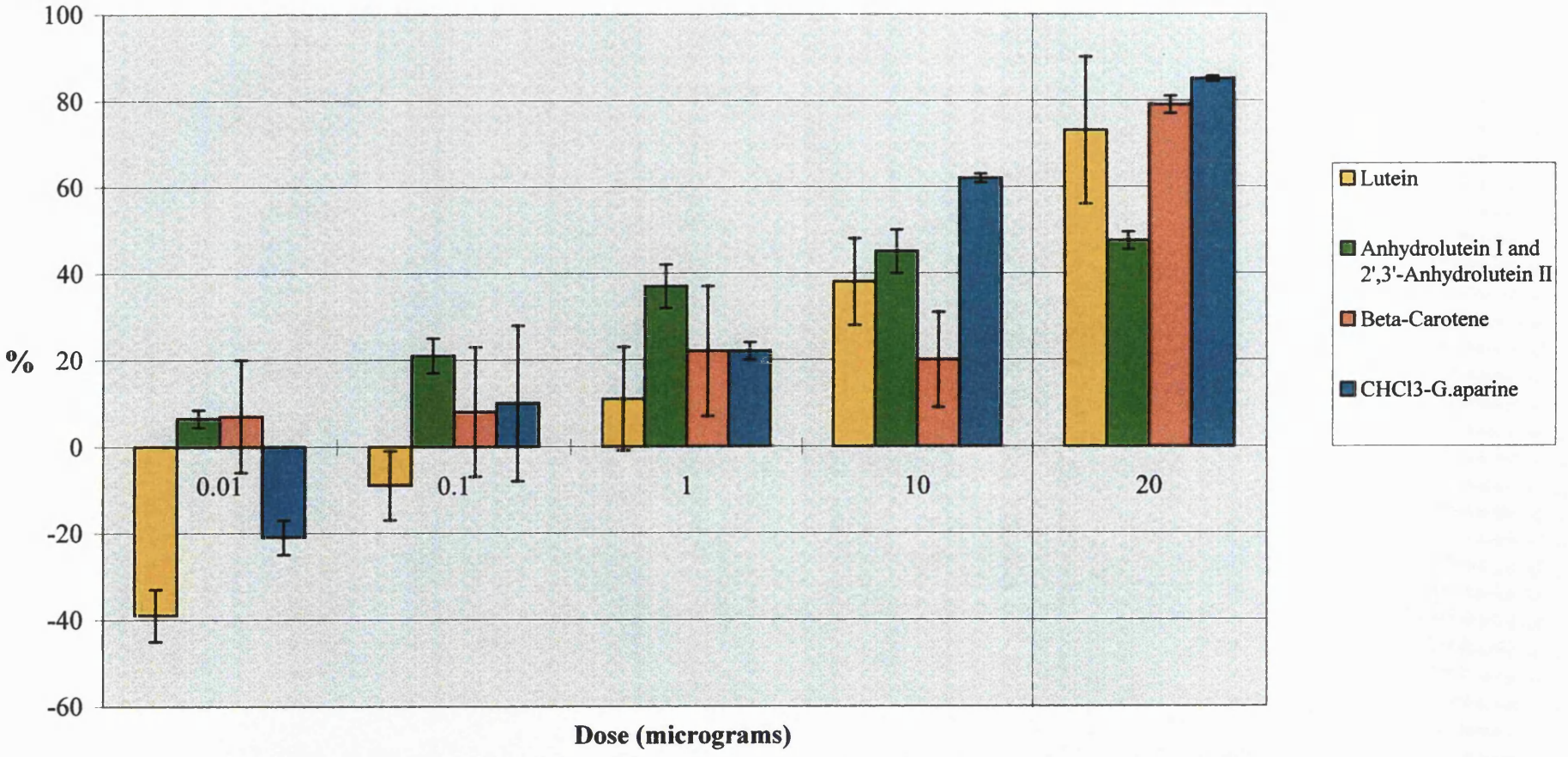


Figure 3.3.6. **Inhibition of Swiss 3T3 Fibroblast Proliferation By *G.aparine* Compounds**

Dose µg/1ml	Lutein		315B (anhydroluteinI & 2',3'-anhydrolutein II)		β-carotene		Chloroform *G.aparine*	
	%	S.E.M.	%	S.E.M.	%	S.E.M.	%	S.E.M.
0.01	-39 **	6	6.5	2.5	7	13	-24 *	4
0.1	-9	8	21**	4	8	15	10	18
1	11	12	37**	5	22 *	15	22	2
10	38 **	10	45**	5	20	11	62 **	1
20	73 **	17	47**	2	79 **	2	85 **	0.6

* 5% level of significance
** 1% level of significance
S.E.M. Standard error of mean

Table 3.3.6. Inhibition of Swiss 3T3 fibroblast cell proliferation by *G.aparine* compounds

β-Carotene was used as a positive control and the cytotoxic effects recorded were similar to those carotenoids extracted from *G.aparine*. At a dose of 20µg the percentage inhibition was recorded as 79% (P<1%). However at 10µg this reduced considerably to 20% and was calculated as no longer significant from the control.

3.3.6.1 Inhibition of TPA induced Swiss 3T3 fibroblast cell proliferation when *G.aparine* compounds and TPA added at same time point

Lutein and 315B (anhydrolutein I & 2',3'-anhydrolutein II) exhibited strong anti-proliferative effects on TPA induced proliferation at 20µg, both of which were significant from the control (65%, lutein; 55% 315B(P<1%)) (Table 3.3.6.1., Figure 3.3.6.1.). At 10µg lutein still exhibited a 68% inhibition against the TPA induced proliferation whilst the percentage of inhibition for 315B decreased to 40% (P<1%). The percentage inhibition for both lutein and 315B at doses between 0.01 and 1µg

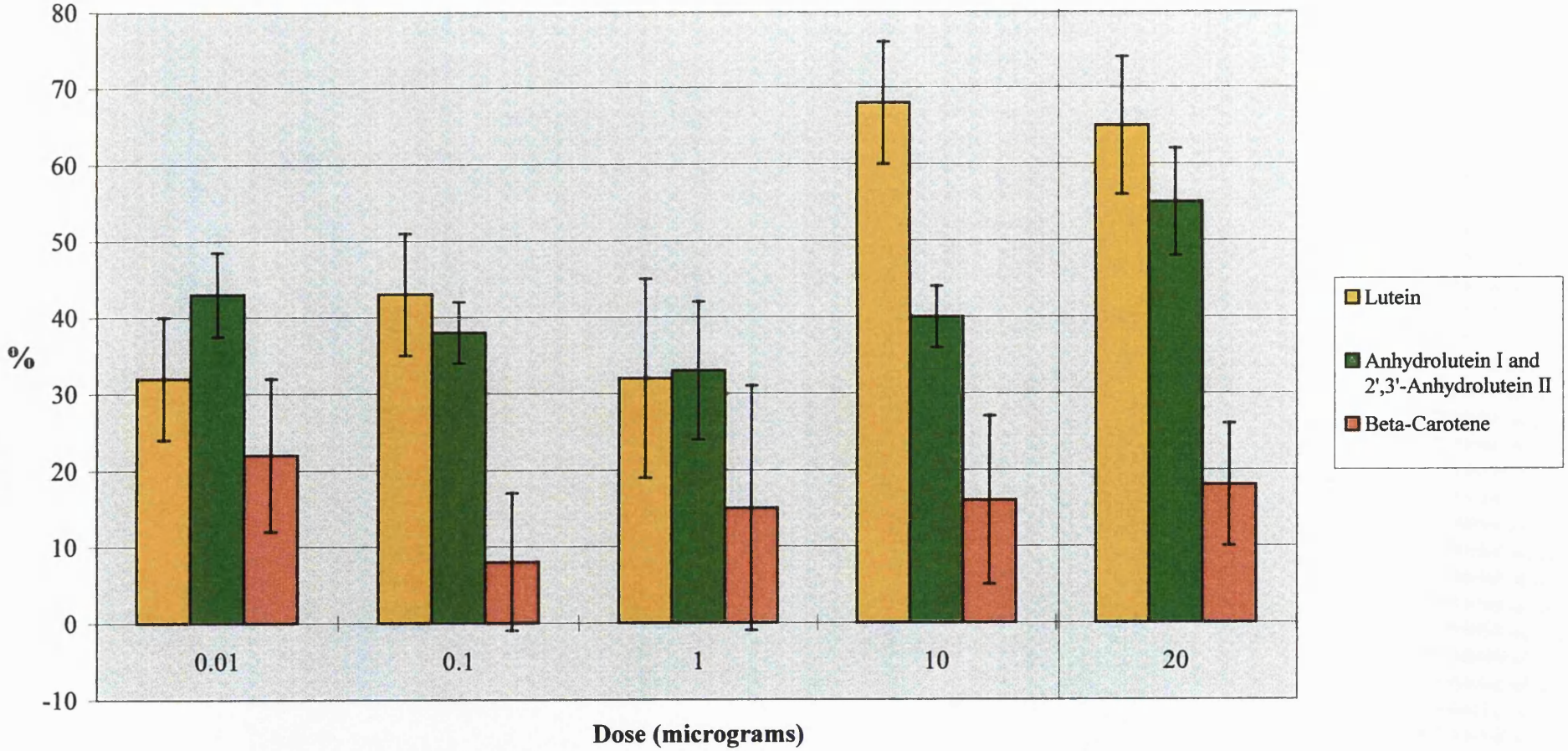


Figure 3.3.6.1. **Inhibition of TPA Induced Swiss 3T3 Fibroblasts Cell Proliferation When TPA and *G.aparine* Compounds Added at the Same Time Point**

remained above 30% and were recorded as being significant from the control (P<1%).

Dose µg/1ml	Lutein		315B (anhydrolutein I & 2',3'-anhydrolutein II)		β-carotene	
	%	S.E.M.	%	S.E.M.	%	S.E.M.
0.01	32 *	8	43 **	5	22 *	15
0.1	43 **	8	38 **	4	8	9
1	32 *	15	33 *	9	15	16
10	68 **	8	40 **	4	15	11
20	65 **	9	55 **	7	18*	8

* 5% level of significance
** 1% level of significance
S.E.M. Standard error of mean

Table 3.3.6.1. Inhibition of TPA induced Swiss 3T3 cell proliferation when *G.aparine* compounds and TPA are added at the same time point

β-carotene did not inhibit the TPA induced proliferation of the cells at significant levels. Inhibition to proliferation was recorded between 8 and 22% for all doses tested, however the standard error of the mean at some doses was quite high.

3.3.6.2. Inhibition of TPA induced Swiss 3T3 fibroblast cell proliferation when *G.aparine* compounds added at 24 hours prior to TPA

The mixture 315B (anhydrolutein I & 2',3'-anhydrolutein II)(20µg) when added 24 hours prior to the application of TPA gave the strongest inhibition to the chemically induced proliferation of cells; a 65% inhibitory response was recorded.

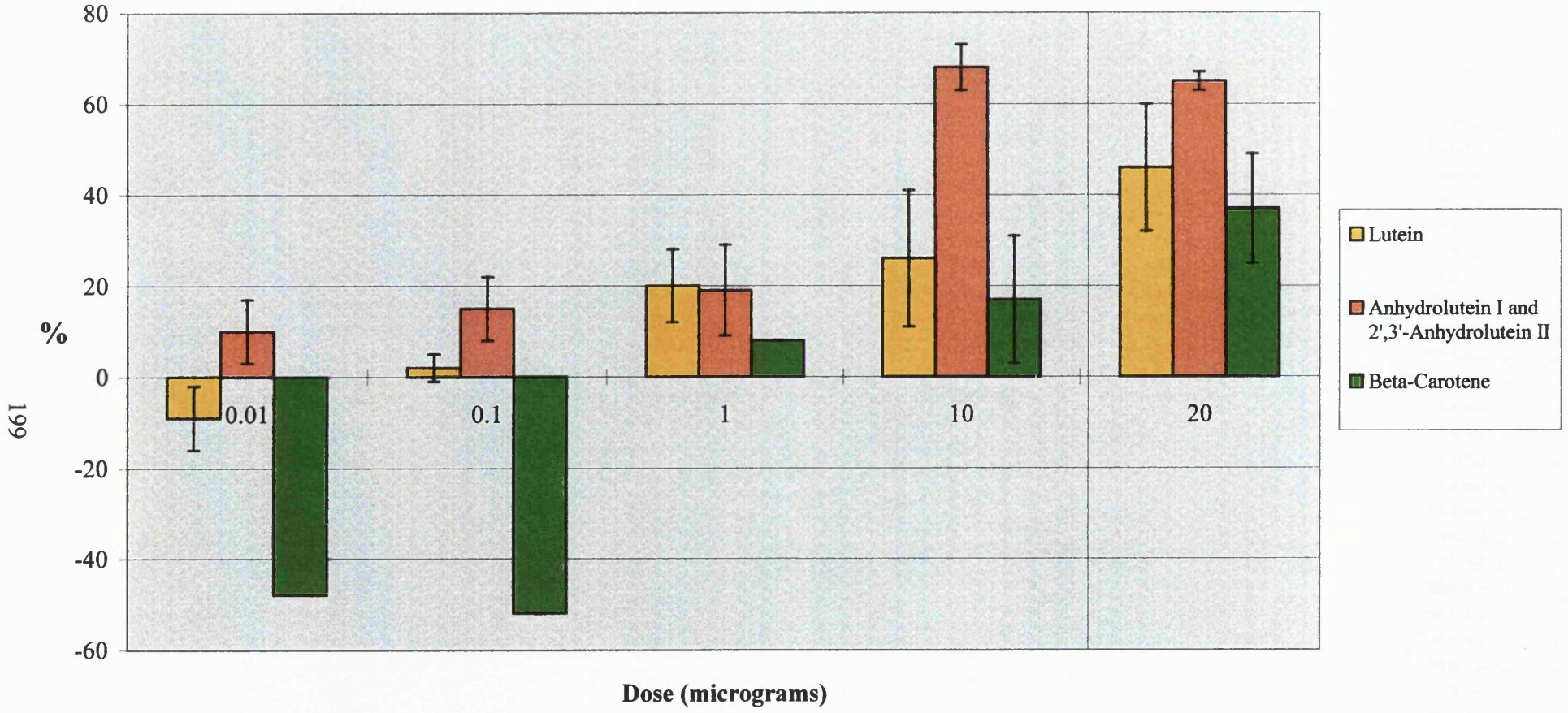


Figure 3.3.6.2. **Inhibition of TPA Induced Swiss 3T3 Fibroblast Cell Proliferation When *G.aparine* Compounds Added 24 Hours Prior to TPA**

Lutein exhibited a 46% inhibition at 20μg/1ml and β-carotene 37% (P<1%). At 10μg 315B remained active at 68% and the activity of lutein and β-carotene dropped to 26 and 17% respectively. However at 0.1μg β-carotene enhanced the proliferation to 52% which was calculated as being significant from the control (P<1%). At 0.01μg the percentage of enhancement remained at 48%. Lutein and 315B still gave an inhibitory response to TPA induced proliferation of fibroblasts at the lower doses apart from at 0.01μg where lutein enhanced the proliferation of cells slightly by 9% (Table 3.3.6.2, Figure 3.3.6.2.).

Dose μg/1ml	Lutein		315B (anhydrolutein I & 2',3'-anhydrolutein II)		β-carotene	
	%	S.E.M.	%	S.E.M.	%	S.E.M.
0.01	-9	7	10	7	-48**	0.07
0.1	2	3	15	7	-52**	0.08
1	20	8	19	10	8	14
10	26	15	68**	5	17	12
20	46**	14	65**	2	37*	0

* 5% level of significance
** 1% level of significance
S.E.M. Standard error of mean

Table 3.3.6.2. Inhibition of TPA induced Swiss 3T3 cell proliferation when *G.aparine* compounds are added 24 hours prior to TPA

3.3.7. Inhibition of UVB physically induced erythema using *G.aparine* compounds

The chloroform extract of *G.aparine*, lutein and β-carotene were evaluated for their activity against UVB induced dermatitis.

The average number of cells and thickness of the control sections measured on animals treated with the chloroform extract of *G.aparine* were 5.86μm and 1.44 respectively. On exposure to UVB light the thickness and number of cell layers

increased by 111% in both cases (table 3.3.7., figure 3.3.7. & 3.3.7.1.). The average thickness of the epidermal layer was measured as 12.30μm (P<1%) and the number of cells counted as 3.06 (P<1%). On application of the *G.aparine* chloroform extract to areas of UVB exposed skin the percentage of increase in the thickness of the epidermal layer was 63% (P<1%), a 43% inhibitory response. The number of cell layers counted was 1.80 a 25% increase as compared to the control but a 77% inhibitory response to UVB induced inflammatory response. However application of *G.aparine* chloroform extract only without exposure to UVB light did induce a thickening of the epidermal layer (8.90μm, 53%, P<1%) with a corresponding increase in the number of epidermal cell layers (24%, P<5%) (figure 3.3.7.2.)

The control sections of rats treated with the isolated compound lutein were measured as having 1.82 epidermal layers and being 8.82μm in thickness. On exposure to UVB light the number of cell layers increased to 3.54 a 97% increase (P<1%) and the thickness correspondingly increased by 92% to 16.94μm (P<1%) (Table 3.3.7., Figure 3.3.7.1.). When treated with lutein approximately a 50% inhibitory response was exhibited for both the epidermal thickening and the number of cell layers; the thickness of the epidermal layer was measured as 13.6μm (50% increase compared to control, but 46% inhibition compared to UVB irradiated skin) and the number of cell layers counted as 2.62 (52% inhibition). However in both cases they were measured as significant from the control (P<1%). Application of lutein to non-exposed UVB skin induced a 20% thickening and 25% increase in the number of cell layers, However neither were found to be significant from the control (figure 3.3.7.3.)

The control epidermal sections of animals treated with β-carotene were measured as having an epidermal layer thickness of 7.02μm and 1.62 cell layers. Sections treated with UVB radiation showed an 87% increase in epidermal thickness (13.05μm, P<1%) and an 82% increase in the number of cells (3.02, P<1%). Application of β-carotene did not inhibit the epidermal response to UVB radiation to an insignificant level from the control (P<1%). The thickness of the epidermal layer was measured as 11μm (33% inhibition) and the number of the cell layers as 2.32 (51% inhibition). Application of β-carotene to untreated skin did increase the epidermal layer thickness

	Control		U.V.		U.V. + Compound		Compound Only	
	Thickness (μm)	Number Cells	Thickness (μm)	Number Cells	Thickness (μm)	Number Cells	Thickness (μm)	Number Cells
***G.aparine*, Chloroform Extract**								
Average	5.86	1.44	12.30	3.06	9.56	1.80	8.90	1.80
S.E.M.	0.46	0.15	1.82	0.46	1.05	0.35	0.99	0.31
% Difference from control			111 **	112 **	63 **	25	53 **	24
% Inhibition from UVB					43 **	77 **		
***G.aparine* - Lutein**								
Average	8.82	1.82	16.94	3.54	13.16	2.62	10.72	2.32
S.E.M.	1.40	0.38	3.75	0.74	2.42	0.37	2.81	0.75
% Difference from control			92 **	97 **	50 **	46 **	20 *	25 *
% Inhibition from UVB					46 **	52 **		
Beta-Carotene								
Average	7.02	1.62	13.05	3.02	11.00	2.32	9.87	1.87
S.E.M.	0.66	0.19	1.42	0.49	1.05	0.51	1.36	0.33
% Difference from control			87 **	82 **	58 **	40 *	37 *	14.4
% Inhibition from UVB					33	51 *		

One sided Paired T-test

*** = 5% significance from control/UVB, ** = 1% significance from control/UVB**

Table 3.3.7. **Inhibition of Physically Induced Erythema By *G.aparine* Compounds**

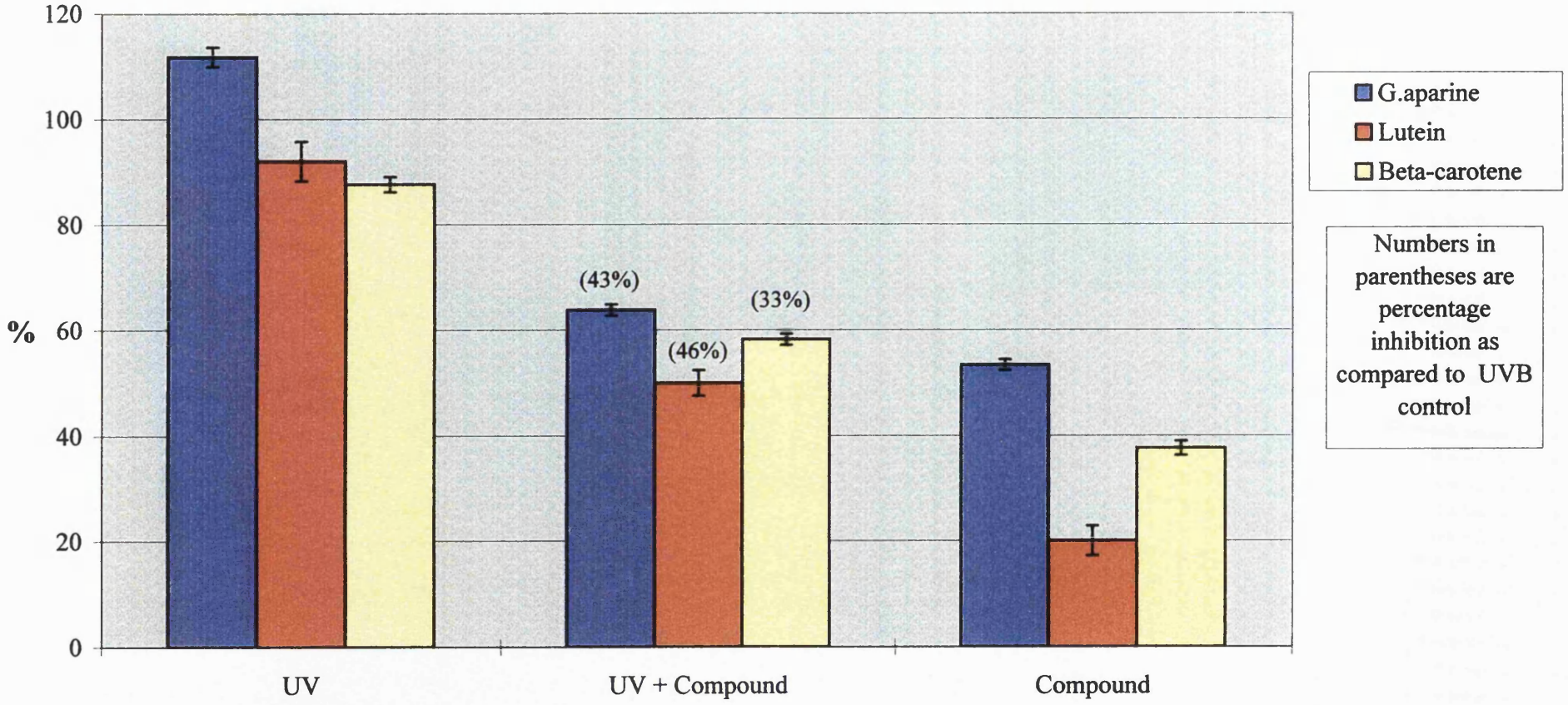


Figure 3.3.7. **Effects of *G.aparine* Compounds on UVB Induced Wistar Rat Epidermal Skin Thickening**

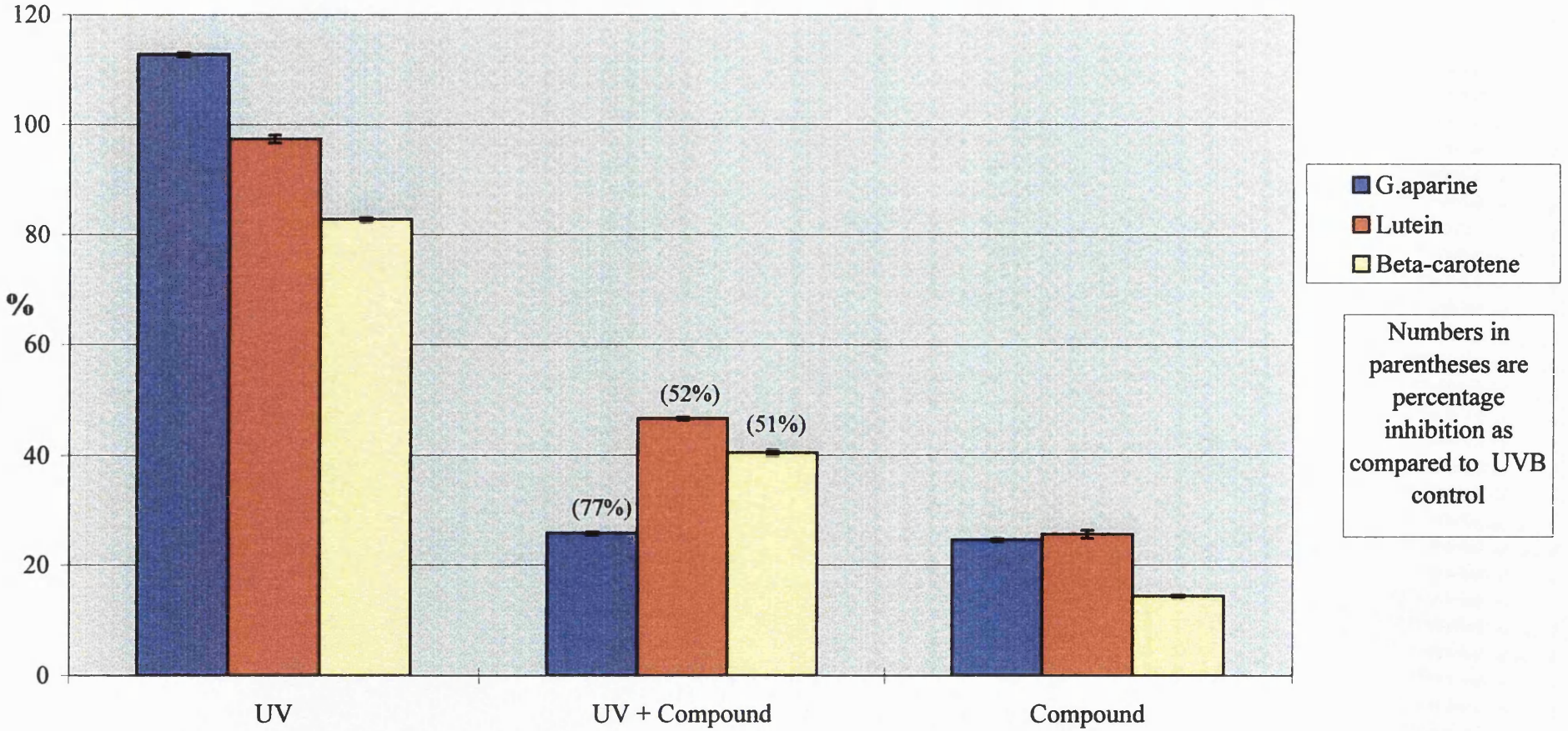
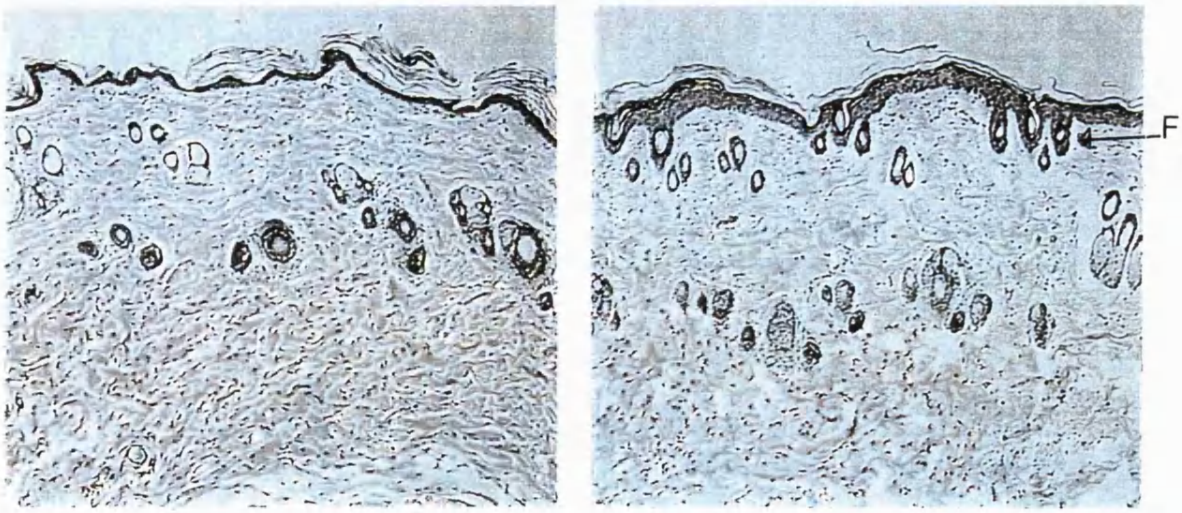


Figure 3.3.7.1 **Effects of *G.aparine* Compounds on UVB Induced Wistar Rat Epidermal Cell Proliferation**

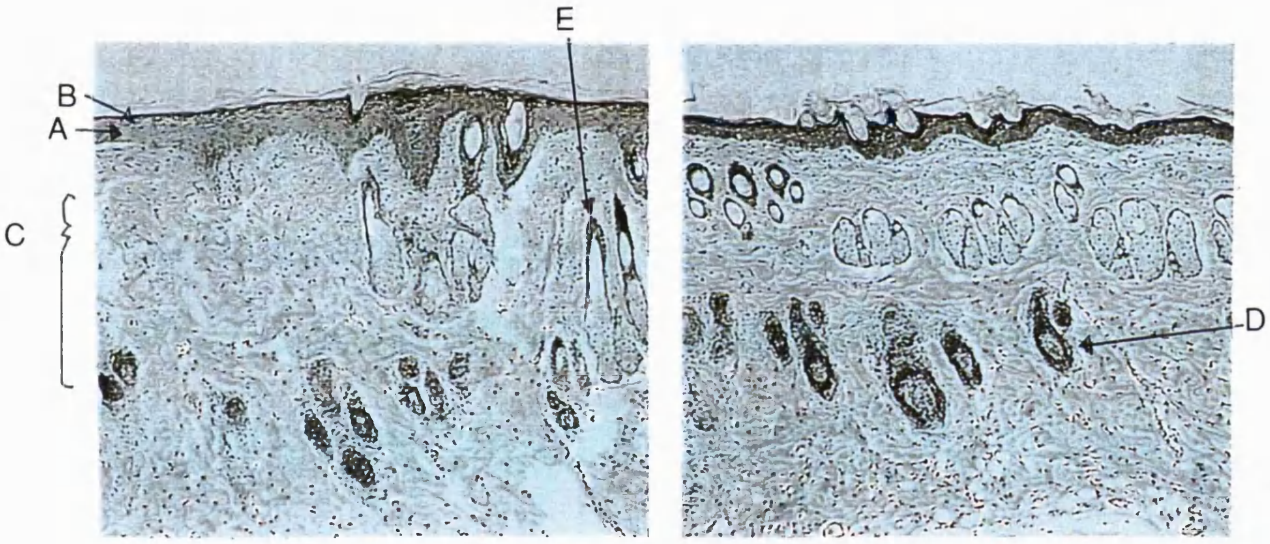
to a level significant from the control (9.87μm, 37% increase, P<1%), however the number of epidermal cell layers increased only by 14.4% to 1.87μm (figure 3.3.7.4.).

Histologically the effects of UVB can be clearly seen in figures 3.3.7.2.c., 3.3.7.3.c. and 3.3.7.4.c. The most noticeable feature was the increased proliferation of epidermal cells and consequently the increased epidermal thickness. However an increase in epidermal cell proliferation and cell thickness can be observed in skin samples which have not been exposed to UVB, but to which test compounds have been applied (figures 3.3.7.2.b. and 3.3.7.3.b.). The development of microabscesses in UVB irradiated skin does not appear to be inhibited with the application of either the *G.aparine* $CHCl_3$ extract, lutein or β-carotene (figures 3.3.7.2.d., 3.3.7.3.d. and 3.3.7.4.d).



a) Control

b) *G.aparine*, Chloroform Extract



c) UVB

d) UVB & *G.aparine*, Chloroform Extract

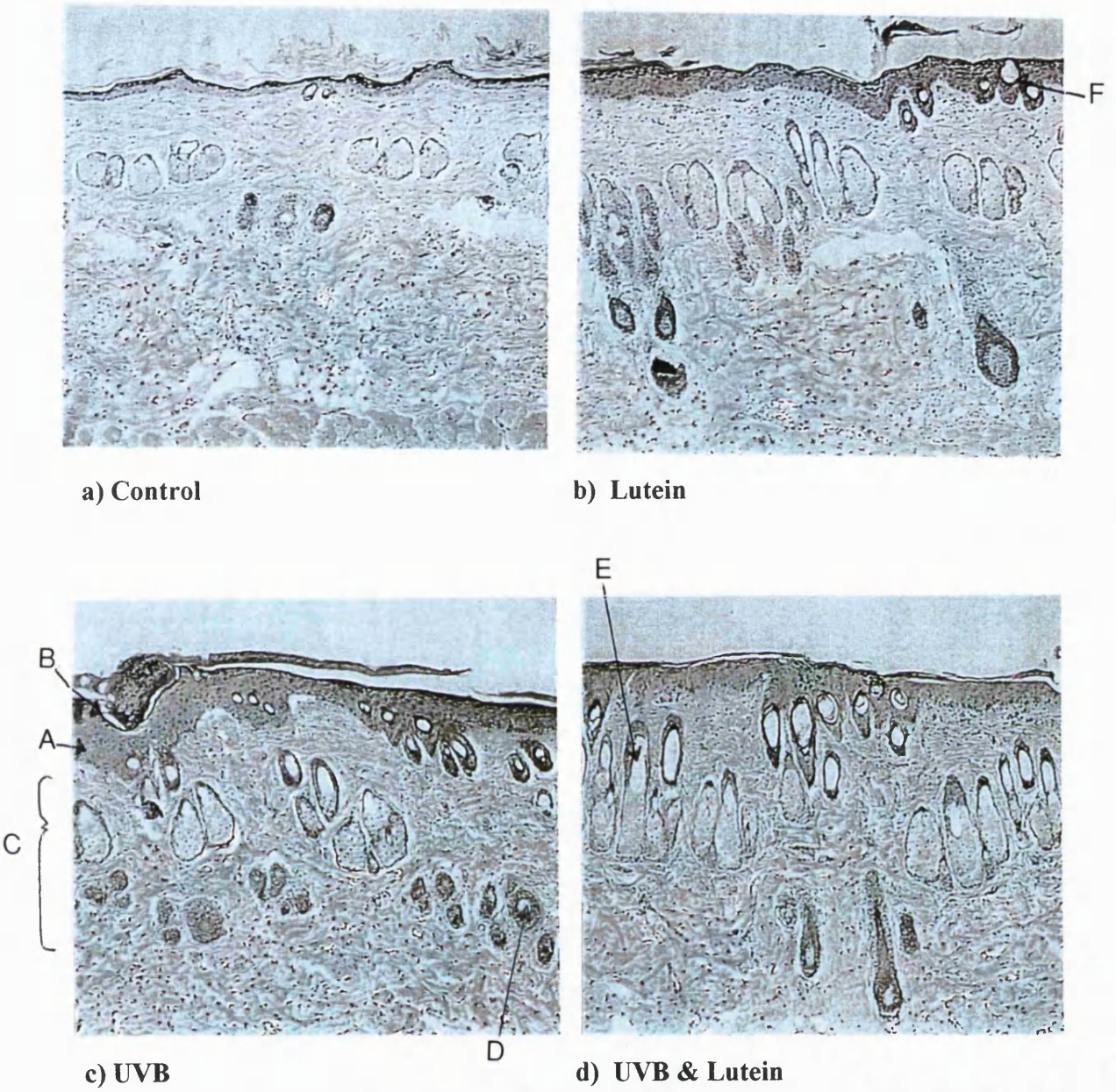
Stain: Haematoxylin and Eosin

Magnification x4

Key

A=Epidermis
B=Stratum Corneum
C=Dermis
D=Tortuous Capillaries
E=Hair Follicles
F=Microabscess

Figure 3.3.7.2. Histological Appearance of Male Wistar Rat Skin Five Days After Treatment With UVB Irradiation and *G.aparine*, Chloroform Extract



a) Control **b) Lutein**

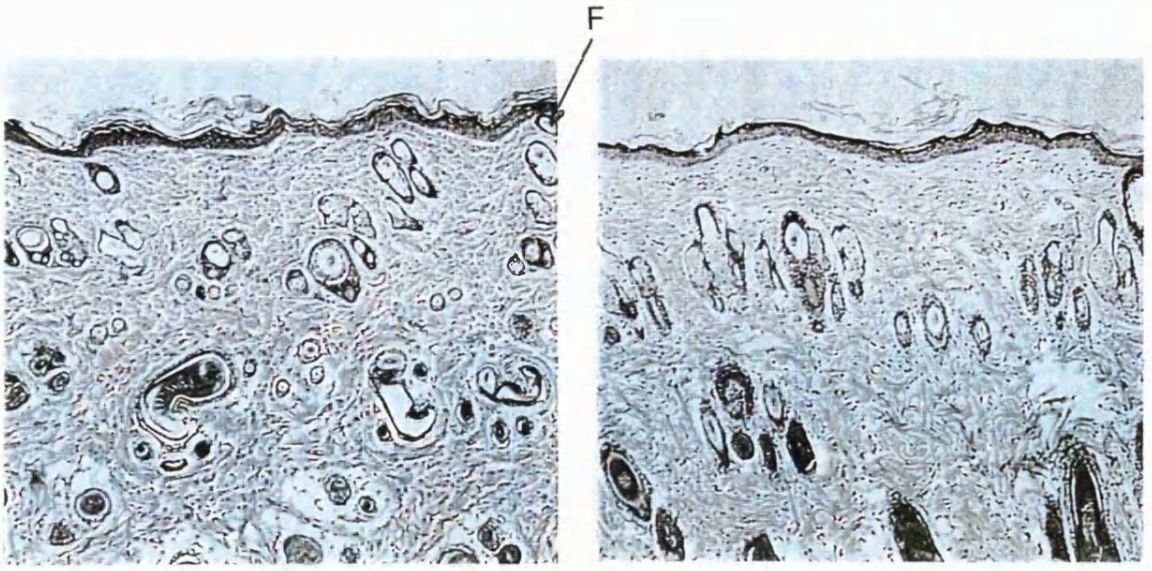
c) UVB **d) UVB & Lutein**

Stain: Haematoxylin and Eosin Magnification x4

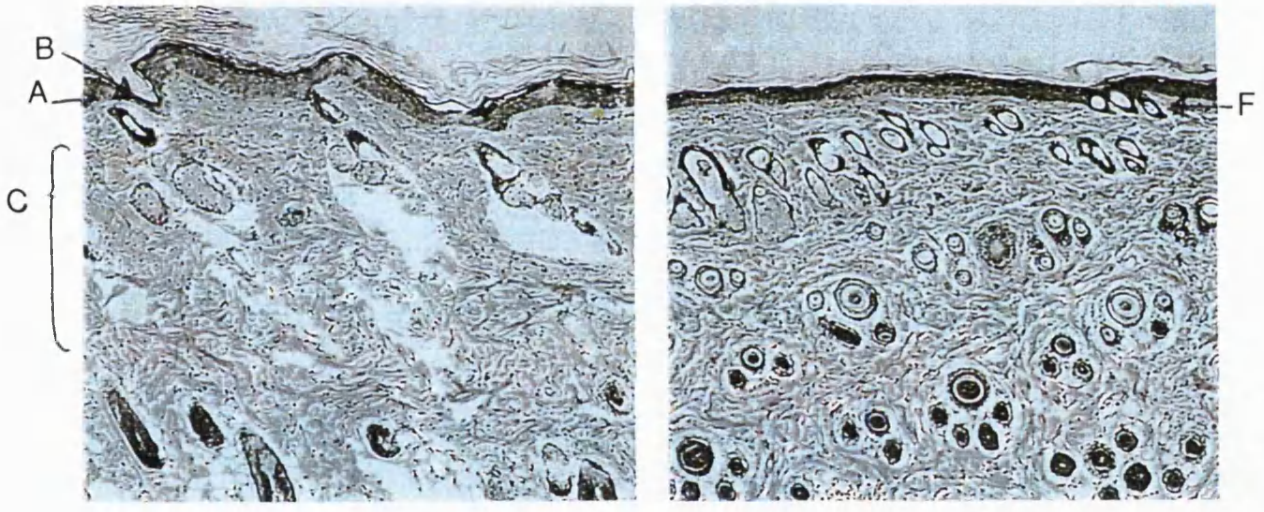
Key

A=Epidermis
B=Stratum Corneum
C=Dermis
D=Tortuous Capillaries
E=Hair Follicles
F= Microabscess

Figure 3.3.7.3. Histological Appearance of Male Wistar Rat Skin Five Days After Treatment With UVB Irradiation and Lutein



a) Control **b) β-Carotene**



c) UVB **d) UVB & β-Carotene**

Stain: Haematoxylin and Eosin Magnification x4

Key

A=Epidermis
B=Stratum Corneum
C=Dermis
D=Tortuous Capillaries
E=Hair Follicles
F= Microabscess

Figure 3.3.7.4. Histological Appearance of Male Wistar Rat Skin Five Days After Treatment With UVB Irradiation and β-Carotene

CHAPTER FOUR

DISCUSSION

4.1. Discussion on methods used to select plants for anti-psoriatic activity

Psoriasis exhibits a wide range of clinical symptoms varying from hyper and parakeratosis, increased epidermal thickening, inflammation through to the production of pustules. Although the pathological and biochemical symptoms of psoriasis have been investigated by others, these symptoms were not used as part of the screening process. In choosing a screening assay it was important to concentrate on the clinical signs of psoriasis as it is these symptoms which are recorded in the past and present published literature with reference to potential anti-psoriatic plants.

The selection procedure of the plants was described as targeted. Plants used in the screening assay were chosen on the basis of their reported ethnomedical use in history for treating psoriasis or inflammatory skin diseases. *G.glabra*, for example, has not been reported for treating psoriasis its anti-inflammatory effects have been reported for over 1,200 years (Shibata, 1994:A). *G.aparine, T.pratense* and *B.perennis* were reported in numerous herbals to help alleviate the symptoms of psoriasis. However past herbals quote that the plants *G.glabra, G.aparine* and *B.perennis* should be used directly onto the skin, whereas *T.pratense* was reported as being taken internally. The alternative modes of application of the plant extracts did have indirect affects on the results of the screening assay, leading to a biased selection of those plants with anti-inflammatory effects when directly applied to the skin. However the objective of the study was to isolate active anti-psoriatic compounds which could be applied directly to the skin in the form of an ointment or cream.

There were several advantages and disadvantages in using either random or targeted selection of plants. Using a targeted selection procedure by researching past herbals

for plants which have anti-psoriatic/inflammatory activity, there was an increased chance that some anti-psoriatic activity would be observed in the assays. However in using such a technique that targeted plants with reported ethnopharmacology the likelihood of discovering a new compound was quite low. If the random selection technique had been chosen the screening programme would have required a greater number of plants and consequently an increased amount of time and resources. The chance of discovering an active anti-psoriatic/inflammatory compound would have been lower, however there would have been an increased chance of perhaps finding a novel active compound.

Choosing the screening assay, which was subsequently used for bioassay guided fractionation of the extracts, was a crucial part in the identification of active anti-psoriatic plant extracts. The screening assay chosen had to target a symptom which was a clinical sign of psoriasis. The assay technique inhibition of chemically induced erythema was chosen for several reasons. The chemical irritant TPA was able to induce skin inflammation and epidermal hyperproliferation followed by hyperplasia of the skin. Erythema and inflammation are both characteristic symptoms of psoriasis ('red scaly plaques'). Inflammation is a localised and protective response activated as the result of an injury to tissue. The injury maybe the result of mechanical or chemical trauma, radiation or infection. At present TPA is believed to induce inflammation as a result of tissue damage resulting in a more prolonged response. Inhibition of chemically induced erythema on the mouse ear had both advantages and disadvantages. The advantages were that it was quick and reliable assay, enabling a relatively high throughput of extracts. Also in using an in-vivo rather that an in-vitro model the complete inflammatory process encompassing all of the circulating components were tested. However chemically induced erythema does assay all of the symptoms of psoriasis and topical application of the extracts excluded the potential anti-inflammatory effects of the extracts if they were taken internally. Models used to screen for hyper and parakeratosis, characteristic symptoms of psoriasis, were complicated and required the sectioning of skin samples, therefore these assays were considered to be inefficient in screening for activity because of the time and resources required.

In using inhibition of chemically induced erythema we were therefore selecting for those plants which displayed anti-inflammatory activity to TPA when applied topically to the skin. All extracts were tested against blood platelet aggregation and normal cell proliferation, however these results did not form the basis for further fractionation of an extract. Although some plants showed strong activity in these latter two bioassays, subsequent fractionation of extracts was based purely on the results of inhibition of chemically induced erythema only.

4.2. Discussion of the results obtained from the screening of the selected plant species

The initial screening assay inhibition of chemically induced erythema clearly identifies those plant extracts possessing anti-inflammatory compounds. When compared to the other two assays, inhibition of ADP and TPA induced platelet aggregation and the inhibition of normal Swiss 3T3 fibroblast proliferation, a correlation was apparent between those extracts expressing significant activity in the three tests.

A number of inter-related pathways can be used to control inflammatory responses, resulting in a number of potential mechanisms to inhibit inflammation. The inflammatory response is initiated and maintained by pro-inflammatory mediators which serve to increase vascular permeability, by causing leukocytes to cross the vascular endothelium, promoting accumulation of leukocytes in injured tissue through chemotaxis and the stimulation of phagocytosis of antigens. The initial response comes from the lysosomal enzymes and polymorphonuclear leukocytes (PMNL's) which release biologically active materials. PMNL's are identified in psoriasis with developing localised and generalised pustulation. The response is increased by the production of chemotactic factors such as 12-HETE, a product of arachidonic acid following the 5-lipoxygenase pathway. It has been reported that within psoriatic patients the PMNL's are more sensitive to chemoattractants which are passively trapped or produced in the epidermis. Psoriatic serum has also been found to contain PMNL's. Pustular psoriasis is an exaggerated model of PMNL accumulation. Following tissue damage a transient vasoconstriction with localised

vasodilatation occurs with an increased blood flow. The mediators of this response include histamines, quinones and prostaglandins. Histamine release leads to increased vascular permeability resulting in extravasation of fluid followed by oedema. Prostaglandins, also a product of the arachidonic acid pathway, potentiate this response. The migration of macrophages into inflamed tissue is a response of acute inflammation. Once the macrophages are situated in the damaged cells they become highly phagocytic and secrete cyclo-oxygenase products and proteolytic enzymes. Blood platelets will also release such products as well as histamines. Therefore as mentioned before there are numerous sites whereby compounds may act upon inflammation and thus control or inhibit the process.

The initial screening programme clearly identified those plant extracts possessing anti-inflammatory compounds. *G.glabra* exhibited activity in both the chloroform, ethyl acetate and ethanol extracts, however the ethyl acetate extract had the strongest anti-inflammatory activity resulting in its selection for further fractionation. The anti-inflammatory activity of the ethyl acetate extract was also reflected in the further two assays used in testing the crude extracts. TPA and ADP induced blood platelet aggregation was inhibited similarly by hexane, chloroform and the ethyl acetate extracts. The chloroform extract at 100μg inhibited ADP induced aggregation by 30% and TPA induced aggregation by 20%. The ethyl acetate fraction of *G.glabra* also inhibited the normal proliferation of Swiss 3T3 cells by 70% at a 1μg dose, 20% more than all other extracts tested. Interestingly there was a considerable difference in the percentage inhibition of cell proliferation when exposed to the ethanol and ethyl acetate extracts. Those exposed to the ethanol extract showed an increase in proliferation by 22%, whilst the ethyl acetate extract inhibited proliferation by 70%. From this evidence one may suggest that there were compounds present both in the ethyl acetate extract and the chloroform extract which significantly inhibited the proliferation of cells. However the ethanol extract may either lack these particular compounds or had additional compounds which were not previously extracted with the less polar solvents therefore resulting in such significantly different results. From the initial screening assays it was concluded that the ethyl acetate extract from *G.glabra* did exert anti-inflammatory activity.

The chloroform extract of *G.aparine* showed the strongest anti-inflammatory activity, against chemically induced erythema, of all the crude extracts tested including those from *G.glabra*, *T.pratense* and *B.perennis*. Again this anti-inflammatory activity of the chloroform extract was reflected in the subsequent two inhibitory assays against platelet aggregation and cell proliferation. Inhibitory activity of platelet aggregation was expressed by both the hexane and chloroform extracts against both ADP and TPA induced aggregation at similar percentage levels. In cell culture the chloroform extract inhibited proliferation of Swiss 3T3 cells at 20μg by 85% and at 1μg by 22%. The ethanol extract inhibited proliferation at 20μg by 58% and at 1μg by 52%. The initial screening assays clearly identified the anti-inflammatory activity of the chloroform extract which led to subsequent fractionation. However is was also established that the ethanol extract although not active against chemical erythema or platelet aggregation did significantly inhibit cell proliferation. Past literature has reported on the presence of anthraquinones in the polar fractions of *Galium sinaicum* which were found to be cytotoxic on the mouse P388 leukaemic cell line (El-Gamal *et al.*, 1995). From this study it was found that free anthraquinones 6, 7-dimethoxy xanthopurpurine, 6-hydroxy-7-methoxy rubiadin, 5-hydroxy-6-hydroxymethyl anthragallal 1,3-dimethyl ether, 7-carboxyl anthragallal 1,3-dimethyl ether showed stronger activity (IC50 1-10.4μg/ml) than the anthraquinone glycosides, anthragallal 1-methyl ether 3-O-rutinoside anthragallol 3-0-rutinoside and alizarin 1-methyl ether 2-0-primeveroside (IC50 48- >100μg/ml). Numerous anthraquinones have also been isolated from the less polar fractions of *G.sinaicum* (El-Gamal *et al.*, 1995). The n-hexane extract had an IC50 of 3.8μg/ml^{-1} against the P388 cell line. From my studies the IC50 values against Swiss 3T3 fibroblasts of the hexane, chloroform and ethanol extracts were 20, 1-10, and 1μg/ml respectively.

The inhibitory activity expressed by both *T.pratense* and *B.perennis* against chemical erythema in all extracts did not prove to be significant. In both cases limited inhibitory activity was observed when dosed at 100μg/ear. However when considering these results it should be remembered that past herbals recommended *T.pratense* to be taken internally as decoction to treat psoriasis. *B.perennis* had been

recommended as an anti-inflammatory agent to be used as a skin wash for treating eczema, but the water extract of *B.perennis* only exhibited anti-inflammatory activity at 100μg/ear.

The extracts of *T.pratense* and *B.perennis* did exhibit considerable activity for inhibition of platelet aggregation and tissue culture. For example the chloroform extract of *T.pratense* exhibited the strongest activity against platelet aggregation, inhibiting TPA and ADP induced aggregation by 52 and 66% respectively. Normal 3T3 fibroblast cell proliferation was inhibited by 98% at 10μg by the chloroform extracts and by 67% at 1μg by the ethyl acetate extract. The isoflavanones, biochanin A, daidzein, formonetin and genistein have been reported in past literature as being present in ethanolic extracts of *T.pratense.* Biochanin A (Chae *et al.*, 1991) has been identified as a potent inhibitor of the carcinogen benzo[a]pyrene as used on hamster embryo cell cultures. Anti-tumourgenic activity by biochanin A has also been expressed against the carcinogens aflatoxin B, 2-amino-anthracene and *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. Therefore it was quite possible that the anti-proliferative effects of the ethyl acetate extract of *T.pratense* could be attributed to biochanin A. Anti-platelet aggregation of *T.pratense* could be attributed to the presence of coumarins (coumestrol, medicagol and coumarin); the 4-hydroxy coumarins are known to antagonise the effects of vitamin K. Vitamin K is a necessary factor of blood clotting by indirectly activating those substance necessary for the conversion of prothrombin to thrombin (Evans, 1996).

The more polar fractions of *B.perennis* significantly inhibited cell proliferation (IC50, ethanol 1-10μg/ml, water 10-20μg/ml), while the non-polar fractions actually enhanced proliferation of cells. The Bellis saponins BS4, BS5, BS6, BS7, BA_1 and BA_2 have all been isolated from the polar fractions of *B.perennis* and they maybe responsible for the inhibition of cell proliferation.

The screening programme successfully identified those plant extracts which possess anti-inflammatory activity. The inhibitory activity was further substantiated from the subsequent tests of inhibition of blood platelet aggregation and inhibition of cell proliferation. From these results the ethyl acetate extract of *G.glabra* and the

chloroform extract of *G.aparine* were further fractionated to isolate active compounds. Although *T.pratense* and *B.perennis* were not significantly active against chemically induced erythema this did not necessarily suggest that they were not active against psoriasis. For example it is likely that the effects of *T.pratense*, if any, would only be expressed when taken internally. It must also be noted that the extracts of both *T.pratense* and *B.perennis* were also significantly active against cell proliferation, an important pathological part of the disease psoriasis.

4.3. *Glycyrrhiza glabra* - Discussion of the isolation and purification of isoliquiritigenin, licochalcone B and echinatin

The purification of isoliquiritigenin and echinatin relied upon the use of column chromatography and more importantly the adsorbent used in the stationary phase. The basis of column chromatography is the separation of solutes in a mixture according to their affinity for a stationary phase through which a mobile phase moves under gravity (Williamson *et al*, 1996). In using a range of adsorbents and solvents the technique of column chromatography could be manipulated to enable purification of a single compound. The solvents may vary according to their polarity and pH, whilst the adsorbents can be classified as either strong (high polarity) for example silicic acid through to weak adsorbents (low polarity) such as cellulose and keiselguhr.

Initial fractionation of the ethyl acetate extract was successfully carried out using silica gel. However subsequent use of this adsorbent was unsuccessful in separating the compounds further. It was found that excessive tailing occurred when using silica for either prep TLC or column chromatography. A wider range of adsorbents were tested of which only polyamide proved to be of any success. Polyamides have a high capacity for phenolic hydroxyl groups via their amide carbonyl functions, hence the elution of phenolics depends on the ability of the solvent to replace the phenol on the hydrogen bond site and fractionation of compounds depend on the number and type of hydrogen bonds formed.

The use of polyamide both in column chromatography and prep.TLC proved essential in the successful separation and consequently isolation of the compounds. Using the technique of bioassay guided fractionation the three chalcones, isoliquiritigenin, licochalcone B and echinatin, were isolated. The percentage yields of echinatin and licochalcone B were 0.0031% and 0.009% respectively, compared to 0.074% and 0.096% as isolated from *G.inflata* by Kajujama *et al.*, (1992). The percentage of isoliquiritigenin obtained from the species *G.uralensis* was 0.082% (Zeng *et al.*, 1990), compared to the yield we obtained 0.0125%. Discrepancies in percentage can easily be attributed to the different species of *Glycyrrhiza* used and the chromatographic methods used to isolate the compounds. However this information does give an indication of the relative quantities of material obtained.

Initial identification of the chalcones was shown by the presence of yellow spots on TLC which developed a red colour after spraying with H_2SO_4 followed by heating, a characteristic of chalcones. Chalcones are C_6-C_3-C_6 compounds which lack a central heterocyclic ring. The numbering system of chalcones are unique in that the A-ring carbons are identified with primed numbers and B-carbons with unprimed numbers. The reason for this was that chalcones were recognised as structurally related to acetophenones where ring carbons were identified by primed number (Harborne & Mabry, 1982). Isoliquiritigenin is a well known chalcone, having hydroxyl groups at positions 2' and 4' of the A-ring and a B-ring hydroxyl at position 4. These three hydroxyl groups were clearly identified in the ^{13}CNMR spectrum at δ161.1 (H-4), 165.97 (H-2') and 168.01(H-4') respectively. The bathochromic shift of band I (367-430nm) on addition of NaOMe was a clear indication of the free 4 hydroxyl group. The addition of $AlCl_3$ also caused a bathochromic shift of band I, an indication of the hydroxyl group at C-2'. Isoliquiritigenin occurs in *Dahlia* species but is most often encountered in members of the Leguminosae.

Licochalcone B and echinatin are structurally distinct from most chalcones in that the A and B rings appear to be reversed and they lack the oxygen functionality at either C-2' or C-6'. The term retrochalcone was coined to recognise the existence of such compounds. Furuya *et al.* (1971) isolated the first known retrochalcone echinatin from the tissue culture of *G.echinata*. Further investigations proved the A-ring of

echinatin was derived from a phenylpropanoid precursor while its B-ring was of acetate-malonate origin. Licochalcone B was first isolated by Saitoh and Shibata (1975) from the roots of an unknown species of *Glycyrrhiza*. The molecular structures of licochalcone B and echinatin are identical, with the exception of the additional hydroxyl at C-3 of licochalcone B, and therefore gave similar shifts on ^{1}HNMR spectrum. The shifts for the protons at α and β were again similar in both licochalcone B and echinatin. An additional shift was found on the spectrum of echinatin at δ6.556 which could be assigned to H-3; within licochalcone B this signal was not present because of the hydroxyl. This can be observed on the ^{13}CNMR of licochalcone B with the additional signal at δ149.85 a clear indication of the additional OH group at C-3. The proton at H-5 in echinatin appears as a double doublet coupling to H-6 and H-4. However in licochalcone B the shift appears as a doublet because coupling only occurs with H-6. Both echinatin and licochalcone B exhibited bathochromic shifts of band I an indication of the free 4 hydroxyl group. Licochalcone B also exhibited a bathochromic shift of band I with the addition of $AlCl_3$/HCl an indication of the presence of phenolic hydroxyls near to the carbonyl group; this shift did not occur with echinatin.

4.3.1. Isoliquiritigenin, licochalcone B and echinatin and their bioactivity in relation to psoriasis

Inflammatory experiments in mouse skin using phorbol esters have provided a useful model to study the anti-inflammatory and anti-psoriatic effects of test compounds. TPA is a tumour promoter and on application to mouse skin leads to skin inflammation and various biochemical alterations in the epidermis, such as prostaglandin synthesis and ornthine decarboxylase (ODC) (Yamamoto *et al.*, 1991). The cellular receptor for TPA is protein kinase C which on activation leads to a number of cellular responses. Previous work has demonstrated that TPA induced inflammation can be inhibited by lipoxygenase and cyclo-oxygenase inhibitors (Inoue, 1989), therefore cyclo-oxygenase products of arachidonic acid may have a role in the TPA activated skin inflammation, tumour promotion and ODC induction.

Using the technique of bioassay guided fractionation, the experimental model being the inhibition of chemically (TPA) induced erythema on the mouse ear, we successfully isolated three chalcones two of which were classified as retrochalcones. The two retrochalcones exhibited much lower IC50 values as compared to the chalcone isoliquiritigenin. The IC50 of licochalcone B and echinatin were 5-10μg/ear and 1-10μg/ear respectively when applied before TPA as compared to 20-50μg/ear for isoliquiritigenin. Application of TPA before the test compounds increased the IC50 value to 50-100μg/ear and 10-20μg/ear for licochalcone B and echinatin respectively. Echinatin and licochalcone B were named retrochalcones based on their characteristic properties of their biosynthetic pathways and chemical structures. Licochalcone B and echinatin posses no hydroxyl group at the C-2' position as found in all other non-retrochalcones, including isoliquiritigenin, therefore one could say the absence of this hydroxyl group in ring A gives rise to stronger anti-inflammatory properties identified in the retrochalcones. The only structural difference between echinatin and licochalcone B was the additional hydroxyl group at C-3 of licochalcone B. Therefore the additional functional group in the B-ring at C-3 may be responsible for the decrease in inhibitory activity against TPA induced erythema. The compounds β-glycyrrhetinic acid, indomethacin and dithranol were also tested so a comparison of the results could be compared against known inhibitors of inflammation. Dithranol is used in the treatment of psoriasis, sometimes in conjunction with UVB. In treating TPA induced erythema dithranol appeared quite ineffective in reducing the erythema response. Indomethacin is a known cyclo-oxygenase inhibitor, thus inhibiting the production of prostaglandins. The inhibitory effect of echinatin (IC50, 1-5μg/ear) was comparable to indomethacin (IC50, 5-10μg/ear). β-glycyrrhetinic acid is also derived from *G.glabra* and exerts an inhibitory effect similar to isoliquiritigenin. Lee *et al.* (1996) suggested that the anti-inflammatory effects of β-glycyrrhetinic acid may be the consequence of either its interaction with steroid receptors or a direct inhibition of PKC.

Shibata *et al* (1991) found the retrochalcone licochalcone A (0.5mg) exhibited anti-inflammatory action towards mouse ear edema (TPA 2μg or arachidonic acid 2mg), anti-tumour promoting action in-vivo against mouse papilloma formation (initiated

by DMBA and promoted by TPA) and the inhibition of ^{32}Pi incorporation to phospholipids of HeLa cells promoted by TPA. They suggested that a competitive interaction of licochalcone A with the TPA receptor was responsible for the inhibitory activity. Arachidonic acid edema is caused by increased production of endogenous leukotrienes and prostaglandins and can be inhibited by 5-lipoxygenase inhibitors but not non-steroidal and cyclo-oxygenase inhibitors, whereas TPA edema can be inhibited by non-steroidal, lipoxygenase and dual lipoxygenase and cyclo-oxygenase inhibitors. Therefore it was suggested that arachidonic acid edema was inhibited by suppression of leukotrienes at the inflammatory site and TPA induced edema was inhibited by interaction with TPA receptors at the cell membrane. Previous studies by Yamamoto *et al.* (1991) found that isoliquiritigenin, the non-retrochalcone, could inhibit epidermal ornithine decarboxylase (ODC) induction, TPA induced edema, DMBA and TPA initiated skin papilloma formation and TPA stimulated prostaglandin E_2 (PGE_2) production in intact epidermal cells. However isoliquiritigenin was reported not to inhibit 12-lipoxygenase and cyclo-oxygenase in the sub-cellular fractions. Interestingly they reported that indomethacin inhibition of ODC was counteracted by PGE_2 application, although this was not the case with isoliquiritigenin. A suggested mechanism of action other than inhibition of PGE_2 production was made, perhaps through lipoxygenase inhibition acting on those cells other than target epidermal cells. Isoliquiritigenin was also reported not to inhibit PKC and did not inhibit superoxide production from PMN's. From our initial study and reports from the literature we could say that the inhibitory action of the compounds isolated from *G.glabra* appear to be different to indomethacin, in that the inhibitory action of indomethacin was not affected if TPA was applied before or after. The anti-inflammatory action of the chalcones depended on whether they were classed as retrochalcones or not, also the presence of the hydroxyl at C-3 of the retrochalcone had a significant affect on the anti-inflammatory activity.

Blood platelets (thrombocytes) are small colourless disc-shaped fragments; their interactions with vascular wall components and coagulation proteins play a key role in the physiological processes: homeostasis, thrombosis, arteriosclerosis, immune disorders and cancer metastasis (Beretz and Cazenave, 1991). On rupturing the blood vessel platelets release serotonin-containing granules which lead to the

constriction of vascular smooth muscle, slowing or stopping the blood flow. Various compounds are present in the blood such as hormones (adrenaline, vasopressin), autacoids (ADP, serotonin, platelet activating factor) and factors of coagulation. Ligands located on the platelet plasma membrane have primary receptors for the activating ligands such as ADP, thrombin, collagen and PAF. On binding of the activating ligand to the receptor an interaction occurs with the intracellular mechanisms through GTP-binding proteins. The activation of the intracellular pathway involves cAMP, cGMP, Ca^{2+}, hydrolysis of phosphoinositides by phospholipase C yielding IP3 and diacylglycerol, hydrolysis of phospholipase A2 yielding arachidonic acid which will be metabolised into TXA2. Next membrane receptors are expressed for adhesive proteins (fibrinogen, thrombospondin) on the surface of the activated platelets. These events lead to platelet aggregation (Beretz and Cazenave, 1991). On adhering to the collagen exposed at the injured site the damaged endoethelial platelets release thromboplastin converting plasma prothrombin into thrombin. Thrombin then converts fibrinogen into fibrin which polymerises into a fibrillor matrix entrapping platelets and blood cells giving rise to a homeostatic plug, a blood clot. ADP and TPA were both used as platelet aggregation antagonists. ADP is referred to as an activating ligand and the primary receptors as mentioned before are found on non-activated platelets. These receptors are linked to intracellular events by the regulatory proteins binding GTP (G-protein) in a transduction system. TPA is reported to cause an irreversible platelet aggregation with human platelet rich plasma. The initial aggregation was hypothesised as the induction of ADP release, followed by a second aggregatory response due to release of endogenous aggregatory material from granules. TPA induced aggregation is reported as being associated with Ca^{2+} dependent protein kinase C (Naka *et al*, 1983).

In testing the chalcones for inhibition of platelet aggregation two positive controls were also tested for comparison. Aspirin was used as a cyclo-oxygenase inhibitor; ADP induced aggregation was therefore inhibited through the prevention of the arachidonic acid pathway (inhibition:- ADP 19%, TPA 0.8% both at 100μg). Colchicine was used as an inhibitor of TPA induced aggregation, responding by inhibiting the microtubule assembly of platelets. TPA and ADP induced aggregation was inhibited at 4% and 24% respectively by colchicine. Isoliquiritigenin the non-

retrochalcone inhibited ADP and TPA induced aggregation by 8% and 6% respectively at 100μg. However the two retrochalcones licochalcone B and echinatin inhibited ADP induced aggregation by 14% and 1.7% respectively and TPA induced aggregation by 32% and 17% respectively, at 100μg. Interestingly echinatin was unable to inhibit ADP induced aggregation but inhibited TPA induced aggregation very strongly and quite significantly more than licochalcone B, suggesting that the absence of the hydroxyl group in echinatin increases the inhibitory response to TPA induced aggregation only. Kimura *et al.*, (1993:B) have studied the effects of isoliquiritigenin and licochalcone B on platelet aggregation and found some differences in activity between these two chalcones. Licochalcone B was found to inhibit the formation of 12-hydroxy-5, 8, 10-heptadecratrienoic acid (HHT), thromboxane B_2 (TXB_2) and inhibited thrombin induced platelet aggregation. Isoliquiritigenin could only inhibit HHT and TXB_2 at lower levels, increased the formation of 12-HETE and had no effect on arachidonate metabolism and thrombin induced aggregation. They also reported that licochalcone B strongly inhibited the Ca^{2+} influx from extracellular medium and Ca^{2+} mobilization from calcium pools stimulated by thrombin. Naka *et al* (1983) reported that TPA induced aggregation was associated with the Ca^{2+} dependent enzyme protein kinase C. Within the blood platelet studies carried out we found that licochalcone B and echinatin inhibited TPA induced aggregation by 17% and 32% respectively, compared to 6.6% by isoliquiritigenin (all at 100μg). From these data it could be suggested that the inhibitory action of retrochalcones is through the inhibition of the Ca^{2+} influx which results in the inhibition of the protein kinase C enzyme.

Swiss 3T3 mouse fibroblast cells have been used extensively to analyse mechanisms of mitogenic stimulation by growth factors. The stimulation of mitogenesis in Swiss 3T3 cells by TPA has been attributed to their ability to bind and activate the PKC enzyme (Roberts *et al.*, 1997). Prostaglandin F-2 alpha (PGF2 alpha) is reported as triggering protein tyrosine kinases in Swiss 3T3 cells independent of PKC activation although PKC and PTK act concertedly to cause mitogenesis (de Asua and Gain, 1997). These cells provided a useful model system to investigate the inhibitory effects of the isolated compounds from *G.glabra* on cell proliferation.

The three compounds isolated from *G.glabra*, the crude ethyl acetate extract and β-glycyrrhetinic acid were tested on the inhibition of normal growth of Swiss 3T3 fibroblasts. β-glycyrrhetinic acid was used as positive control compound as its inhibitory activity had been reported in past literature in Swiss 3T3 fibroblasts. From the data it was clearly seen that echinatin exerts significant inhibitory activity, comparable to β-glycyrrhetinic acid and the ethyl acetate extract. The anti-proliferative activity of the ethyl acetate extract could therefore be primarily attributed to the presence of echinatin and perhaps to a lesser extent the other chalcones. At 1μg the inhibitory activity of echinatin was 82% as compared to 23% for β-glycyrrhetinic acid and 70% for the ethyl acetate extract. Isoliquiritigenin and licochalcone B exerted significant inhibitory activity against Swiss 3T3 fibroblast proliferation between 1 and 20μg. However the inhibitory activity of licochalcone B (31%) and echinatin(82%) was significantly different at 1μg/1ml, therefore one could suggest the activity decreases in the retrochalcones with the presence of the hydroxyl group at C-3. Ramanthan *et al.* (1993) reported on the inhibitory activity of 2-OH chalcones and other flavanoids and concluded that chalcones were more effective than flavanoids in suppressing cell proliferation and that the potency was attributed to the $C_{2,3}$ double bond and the open C-ring. They also observed that the mechanism of action of the chalcones was different to the cytotoxic drug colchicine. They reported colchicine as an effective inhibitor of mitosis and microtubule assembly in-vitro, while flavanoids and chalcones have been reported to inhibit cell growth by increasing cyclic AMP levels or by affecting DNA and RNA polymerase activity. De Vincenzo *et al.* (1995:B) reported on the effects of chalcones on ovarian cancer cell growth and found at concentrations of 0.1 to 10μm chalcones inhibited ovarian cancer cell proliferation and [^{3}H] oestradiol ([^{3}H]E2) binding to type II EBS. On considering the structure related variation in their results they concluded that the presence of an α-β double bond, the hydroxylation in 3 or 2 of ring B and the absence of a prenyl group were important to both anti-proliferative and binding activity. They also said that the free 4' hydroxyl was considered as critical to activity and that variation in activity caused by the introduction of an additional hydroxyl group on the B ring depended on its position. An increase in activity was also found

if the hydroxyl was located on carbon 3 or 2, but decreased if the hydroxyl was located at C-4. The chalcones have also been identified as inhibiting established cancer lines and colony formation of primary ovarian tumours. The results we obtained suggested that compounds absent of the C-2'hydroxyl, termed as retrochalcones, were significantly more active than non-retrochalcones and that the addition of the hydroxyl at C-3 in the B ring decreases the inhibitory activity of the retrochalcone. Whether the presence of the hydroxyl at C-4 decreased any inhibitory response is unknown, but this could be encompassed in future work.

Further studies involved the inhibition of TPA induced Swiss 3T3 cell proliferation, either when TPA was added at the same time point or 24 hours after the test compound. The two retrochalcones exhibited significant inhibitory activity (echinatin 99% and licochalcone B 70%, 20μg/1ml), compared to the non-retrochalcone (isoliquiritigenin 28%, 20μg/1ml). Again β-glycyrrhetinic acid was used as a positive control but proliferation was only significantly inhibited at 10μg and above. From these data as before we can suggest that the retrochalcones exhibit stronger activity than non-retrochalcones against TPA induced Swiss 3T3 cell proliferation and the presence of the hydroxyl at C-3 of licochalcone B decreases this activity. If the stimulation of Swiss 3T3 cells by TPA is the result of TPA binding and activation of the PKC enzyme, one can hypothesise and say that the retrochalcones could be inhibiting an influx of Ca^{2+} which is required for the functioning of the PKC enzyme normally activated by TPA, as was apparent in the inhibition of blood platelets by licochalcone B. The anti-tumourigenic activities of 40 chalcone derivatives was studied by Iwata *et al.*, (1995); they used the in-vitro test phosphorylation of phospholipids promoted by TPA in HeLa cells to screen for anti-tumourigenic activities as it showed a good parallelism with in-vitro experiments. In using this assay a structure-activity relationship was shown whereby the presence of free hydroxyls at the C-3 position seemed to be essential, since the O-methylation of these compounds decreased the inhibitory potency. These data contradict my findings in that the absence of the hydroxyl at C-3 actually enhanced quite significantly the anti-proliferative effects. They also concluded that the presence of a hydroxyl or methoxyl on the A ring of chalcones possessing C-3 hydroxyl on the B-ring decreases the inhibitory activity. The addition of chalcones 24 hours before the TPA

increased the inhibitory activity of the chalcones compared to when they were added at the same time point as TPA . This result could be expected as the chalcones would be expressing a natural cytotoxic affect within the first 24 hours. Numerous studies have been done on the effects of β-glycyrrhetinic acid on cell proliferation and for this reason it was used as a positive control. Mi Lee *et al* (1996) reported on the inhibition of histamine synthesis by β-glycyrrhetinic acid in mast cells co-cultured with Swiss 3T3 fibroblasts and found that at 50μM β-glycyrrhetinic acid inhibited about 80% of histidine decarboyxlase activity. Kitagawa *et al* (1984) reported on the inhibition of TPA stimulated 3-O-methyl-glucose transport in Swiss 3T3 mouse fibroblasts;however, the mechanism of action was undecided. It was suggested that TPA induced stimulation of hexose transport was due to the enhancement of the number of hexose carriers. β-Glycyrrhetinic acid was found to inhibit both forms of TPA induced proliferation at 10 and 20μg only, concentrations tested below this were found to induce proliferation, quite substantially at 1μg/1ml when applied before the TPA. The chalcones exhibited a much stronger anti-proliferative effect.

Ultra-violet ray B induced dermatitis in the rat was chosen as a model for human psoriasis. Rat UVB dermatitis reacts quite differently to human, guinea-pig or mouse skin in that it is characterised by dermarcated brownish-red lesions, scale formation, micro-vascular dilation, intra-epidermal accumulation of polymorphonuclear leukocytes with micro-abscesses, mononuclear cell infiltration and hyperproliferation of epidermal cells (Nakaguma *et al.*, 1995). Macroscopically UVB induced a faint erythema for a period of 30 minutes immediately after exposure, a second phase of erythema occurred between 24 and 48 hours with scale formation occurring at 48-72 hours; no haemorrhaging was visible. Histologically at two days intra-epidermal infiltration of PMN, with mononuclear cell infiltration and micro-vascular dilation is said to occur. Three days after irradiation intra-epidermal micro-pustules develop as do micro-abscesses in the stratum corneum. On the fifth day after irradiation when skin samples were taken the epidermis had thickened, between 4-6 cells were counted, and the micro-abscess remained. Under normal conditions the rat epidermis is comprised of two keratinocytes layers including the basal layer. The human epidermis however has ten layers, therefore if one observes an increase to 4-6 layers in the rat

when exposed to UVB this could correspond to an increase of 30 layers or more in the psoriatic patient.

The crude ethyl acetate extract which proved active against TPA induced erythema and the isolated compounds from *G.glabra* were tested for their inhibitory activity against UVB induced erythema and skin inflammation. β-glycyrrhetinic acid was also tested as a control compound. The experimental design of the experiment incorporated a statistical analysis which eradicated any variation which may occur between animals. Unfortunately due to insufficient quantities the retrochalcone echinatin could not be tested. Licochalcone B and isoliquiritigenin significantly inhibited the proliferation of epidermal cells induced by UVB. Isoliquiritigenin inhibited the proliferation of cells by 81% which was significantly different from the control UVB exposed skin; licochalcone B similarly inhibited the proliferation of cells by 80%. In all test groups the UVB control was always found to be significant from the control at the 1% level. β-Glycyrrhetinic acid did inhibit the proliferative response of the epidermal cells but not to the same extent as the retrochalcones. This would suggest that the chalcones are either stronger inhibitors or are inhibiting a different pathway to β-glycyrrhetinic acid. These inhibitory responses are visible on the photographs taken of the skin sections. The control sections unexposed to UVB show a normal epidermis about 6μm in thickness. On exposure to UVB there is a 60-100% increase in the epidermal thickness with the formation of micro-abscesses which are visible on the photographs of the skin sections. The chalcones do inhibit cell proliferation and to a degree inhibit the formation of micro-abscesses. The inhibitory response by β-glycyrrhetinic acid to these physiological responses to UVB were not quite so apparent.

Matsui and DeLeo (1990) reported that UVB radiation shares a number of biological effects with the phorbol ester tumour promoter TPA. They found that both would induce the stimulation of arachidonic acid release, increase prostaglandin E_2 production (PGE_2) and inhibit the proliferation of epidermal growth factor (EGF) binding and cellular proliferation in cells. However they reported that UVB had no effect on PKC activity and phorbol ester binding. Only UVA was found to induce PKC activity and is therefore specific to 320-400nm. This response is also distinct

from other tumour promoters. UVA acts not only as a complete carcinogen but also promotes UVB carcinogenesis. These data suggest the mechanism of action for the chalcones is perhaps not through the inhibition of PKC activation but in the inhibition of prostaglandin synthesis via the inhibition of the cyclo-oxygenase. Peters *et al* (1977) found indomethacin and pirprofen inhibited UV induced erythema and suggest that prostaglandin synthesis and release was the primary mechanistic process evolved in the production of erythema. It is possible that the chalcones could inhibit PKC, as was suggested with TPA induced erythema and platelet aggregation, as well as inhibiting prostaglandin synthesis.

These biological results lend support to the recommendation that *G.glabra* may be tested in clinical trials for the treatment of psoriasis. Initially pilot studies could be done using either the active compounds extracted from *G.glabra* or the crude ethyl acetate extract, applied in the form of an ointment. In treating psoriatic patients it is likely they will already be on some form of conventional treatment and therefore treatment with *G.glabra* should initially be considered for use as an adjunct therapy. Attention would be paid to symptoms such as dryness, scaling and redness of the skin as well as the patients' quality of life before and after treatment. Further trials of the most active compounds would involve more patients and carried out as a double-blind placebo trial. It is unlikely that the compounds of *G.glabra* will cure psoriasis long term, however it is quite possible that the disease may be controlled and possibly put into remission. The compounds from *G.glabra* may also not have the side effects associated with present psoriasis therapies.

4.4. *Galium aparine* - Discussion of the isolation and purification of lutein, anhydrolutein I, 2',3'-anhydrolutein II and 3-hydroxy-3'-methoxy-β-ε-Carotene (compound 315A)

Lutein, all-E-anhydrolutein I, all-E-2',3'-anhydrolutein II and compound 315A were identified as xanthophylls, a group of compounds which were oxygen derivatives of the carotenoids. The purification of these compounds relied upon the use of column chromatography and prep.TLC, both of which were prepared using silica gel. Once established that the compounds being isolated were carotenoids care was taken to minimise their exposure to air thus preventing oxidation. This was done by scraping the relevant band of the prep.TLC before it had dried and storing the compounds under nitrogen gas at a low temperature. The columns were also covered by aluminium minimising the exposure of compounds to light.

The sorbisil column was subsequently used for large scale separations, however final purification of the compounds could only be achieved through prep.TLC. Separation of all four compounds was carried out using the same techniques. Detection on TLC was not a problem since the carotenoids were coloured.

Initial identification of lutein as a carotenoid was established through its colour and UV spectrum, which was characteristic of a carotenoid. On establishing the *m/z* ion as 568 the ^{1}HNMR spectrum was easily matched to the ^{1}HNMR data of lutein already published in the literature. The same procedure was used for matching the ^{13}C data. The protons attached to the carbons with hydroxyls were easily distinguished on the ^{1}HNMR at δ4.00 (H-3) and δ4.251(H-3'), similarly on the ^{13}CNMR the carbons were identified at δ65.94 (C-3) and δ65.10 (C-3'). The proton assignments were supported by 2D^{1}H, ^{1}HCOSY and 2D^{1}H, ^{1}H NOESY experiments, and the ^{13}C assignments supported by a ^{13}C-^{1}H one bond correlation experiment.

Compound 315B was identified as a mixture of anhydrolutein I and 2',3' anhydrolutein II. These two compounds possess the same *m/z* ion, the same UV spectrum (+/- 1/2nm) and the same RF values which made initial identification that there were two compounds present impossible. These two compounds have been

reported by Khachik *et al.*, (1995) as the two dehydration products of lutein which they isolated from the extracts of ripened black paprika. They subsequently reported that anhydrolutein I and 2',3'-anhydrolutein II were the major dehydration product of lutein found in human plasma. Although only present in small quantities within blood plasma their data were matched to ^{1}HNMR and ^{13}CNMR obtained from the synthetic compounds. An HPLC system was used to try and separate these two compounds in 315B but with no success. The ^{1}HNMR showed a number of shifts which not only suggested there were two compounds, but were also characteristic of the two compounds. For example the methyl groups of 16', 17' and 18' of the two compounds were not identical and therefore the extra shifts indicated there was more than one compound and secondly that the structural differences occur on the primed ring. The ^{1}HNMR data was matched to the ^{1}HNMR from the synthetic compounds produced by Khachik *et al.*, (1995). The shifts corresponding to the protons of C-2' give a clear indication that the two compounds were likely to be anhydrolutein I and 2',3' anhydrolutein II. Anhydrolutein I has two hydrogen atoms at C-2' and the corresponding shifts were at δ1.91 and 2.01, however 2',3' anhydrolutein II has only one hydrogen at C-2' due to the double bond between C-2' and C-3', therefore a corresponding shift was found at δ5.324.

The ^{1}H-NMR of 3-hydroxy-3'-methoxy-β-ε-carotene (compound 315A) was very similar to that of lutein. Initially it was thought perhaps lutein had undergone oxidation to form lutein epoxide ($C_{40}H_{56}O_3$), which is structurally very similar to lutein. However, this possibility was ruled out because the molecular weight of lutein epoxide is 584 and the M^+ of compound 315A was at *m/z* 582. Also, on addition of 0.1M HCl to an ethanolic solution of 315A no spectral shift to a lower wavelength (18-40nm) was observed, a characteristic of the epoxides. The ^{1}HNMR spectrum of compound 315A differed from that of lutein in that the signal for H-3' shifted to a higher field and there was an additional three proton singlet at δ3.64. From this evidence it was deduced that compound 315A is the 3' methyl ether of lutein, 3-hydroxy-3'-methoxy-β-ε-carotene. Unfortunately insufficient material was available for further NMR experiments which may have supported the hypothesis. The ^{1}HNMR data of the methyl groups were matched to data already in the published literature (Hertzberg *et al*, 1985), However, data for the methoxyl group and remaining

CH_2 groups were not reported by them. The literature was extensively searched for NMR data corresponding to 3-hydroxy-3'-methoxy-β-ε-carotene, the databases Scifinder and Beilstein identified the relevant reference. As of yet there appears to be no published data for the complete ^{1}HNMR or a ^{13}CNMR spectrum of 3-hydroxy-3'-methoxy-β-ε-carotene. This is the first report of 3-hydroxy-3'-methoxy-β-ε-carotene being isolated as a natural product.

4.4.1. Lutein, anhydrolutein I, 2',3'-anhydrolutein III and 3-hydroxy-3'-methoxy-β-ε-carotene (compound 315A) and their bioactivity in relation to psoriasis

The bioassay guided technique, inhibition of chemically induced (TPA) erythema on the mouse ear, was used successfully to fractionate the chloroform extract of *G.aparine* and isolate the compounds lutein, anhydrolutein I, 2',3'-anhydrolutein II and 3-hydroxy-3'-methoxy-β-ε-carotene. All of these compounds are classed as xanthophylls or oxygenated carotenoids.

In testing for inhibition of chemically induced erythema β-carotene was also assayed. β-Carotene is a naturally occurring pigment in dark green and yellow/orange vegetables and fruits. Apart from serving as a major dietary source of retinol in humans, β-carotene and its metabolite retinoids have been shown to have anti-cancer and cancer preventative properties. Retinoids are also used in the treatment of psoriasis. The previous results of indomethacin and dithranol were used so the anti-inflammatory effects of the isolated compounds could be compared against two known anti-psoriatic drugs. Lutein and the mixture of anhydrolutein I and 2',3'-anhydrolutein II (compound 315B) significantly inhibited chemically induced erythema; both had IC50 values of 1-5μg/ear. This inhibitory response was comparable to the cyclooxygenase inhibitor indomethacin and the retrochalcone echinatin. 3-Hydroxy-3'-methoxy-β-ε-carotene (compound 315A) did not give significant activity against the chemically induced erythema; the IC50 was established as higher than 20μg, but due to insufficient quantities attained the compound could not be tested at higher concentrations. However the hypothesised

structure 3-hydroxy-3'-methoxy-β-ε-carotene was very similar to lutein except that the hydroxyl at C-3' was replaced by a methoxyl, therefore it could be suggested that the possible addition of a methoxyl significantly decreased the anti-inflammatory activity. β-Carotene although a recognised anti-carcinogen the IC50 of this compound was between 20-50μg/ear, substantially lower than the oxygenated carotenoids.

PKC as mentioned previously is the recognised receptor of TPA and it is suggested that cellular responses mediated through this enzyme give rise to skin inflammation and tumour promotion among other biological events. Tumour promotion such as TPA also has a powerful effect in eliciting the release of reactive oxygen species from PMNs. PMNs have an important defensive role in elaborating reactive oxygen species helping to destroy invading organisms , but become harmful when there is an overproduction for example when induced by TPA. It is suggested that the scavenging effects of the carotenoids, with respect to photosensitiser triplets, singlet oxygens and free radicals give rise to their therapeutic properties. In reacting with these free radicals carotenoids should theoretically prevent their damaging effects, for example lipid peroxidation, enzyme destruction and damage to both RNA and DNA.

β-Carotene among other carotenoids have been found to have biological functions in humans, including photoprotection, singlet oxygen quenching, immunomodulation and anti-cancer properties. Chen *et al.*, (1993) reported on the effects of β-carotene on papilloma formation and the conversion of papillomas to carcinomas in a two-stage protocol with one application of the initiator DMBA and 20 weekly applications of the promoter TPA to Sencar mice. From this study they concluded that papilloma formation was increased in the female sexes, but a 600μg/g dose of β-carotene inhibited the conversion of papillomas to carcinomas. They concluded that the effects of excess β-carotene on carcinoma formation was partially due to the action of retinoic acid formed from β-carotene, rather than due to the intrinsic properties of β-carotene. Kanoshima *et al*, (unpublished results) found that the carotenoid crocin delayed the formation of papillomas again when DMBA was used as the initiator and TPA as the promoter.

The inhibitory responses of lutein, anhydrolutein I, 2'3'-anhydolutein II and β-ε-carotene-3-ol,3'-methoxy against ADP and TPA aggregation produced interesting results. The inhibitory activity of these compounds against ADP induced aggregation was significantly more than the chalcones from *G.glabra* and were comparable to aspirin. Lutein exerted an 18.9% inhibitory response at 0.1μg against ADP induced aggregation and 34% at 100μg. The mixture of anhydrolutein I and 2',3'-anhydrolutein II exhibited strong anti-platelet activity against ADP induced aggregation, 51% at 100μg. The inhibitory response of β-carotene and aspirin were 33% and 19% respectively, at100μg. TPA induced aggregation was similarly inhibited by lutein and 315B, 27% and 38% respectively at 100μg. The inhibitory responses were stronger than the positive control compound colchicine. Compound 315A also strongly inhibited TPA induced aggregation by 31% at 100μg. The oxygenated carotenoids did express significantly more inhibitory activity over the non-oxygenated carotenoid β-carotene. TPA exerts its aggregatory control by the induction of ADP release and a second response via the release of the endogenous aggregatory material from serotonin granules.

Lutein and the mixture of anhydrolutein I and 2',3'-anhydrolutein II (compound 315B) were tested for inhibitory activity against normal Swiss 3T3 cell growth, however 3-hydroxy-3'-methoxy-β-ε-carotene was not tested because of insufficient quantities of material. The anti-proliferative response shown by the chloroform extract of *G.aparine* was similar to lutein in that at the higher concentrations approximately an 80% inhibitory response against proliferation of the cells was recorded. Also at the lower concentrations (0.01μg) they enhanced the growth to a level which was significant from the control. Compound 315B did not inhibit the growth so strongly at 20μg (47%), but at lower concentrations the inhibitory response was still recorded. β-Carotene exhibited a normal dose response curve. The oxygenated carotenoids expressed significant inhibitory activity at both 20μg and 0.01μg against TPA induced Swiss 3T3 cell proliferation. β-Carotene did inhibit TPA induced proliferation, but was not recorded as significant from the control at 10, 1 and 0.1μg. When the cells were dosed simultaneously with TPA and the test

compounds the following results were observed. At 0.01μg 315B inhibited cell proliferation by 43%, but at 0.1 and 1μg the inhibitory response was 38 and 33% respectively. This type of response was observed for all three carotenoids, whereby the lower doses exhibited a higher percentage inhibition. However when the compounds were added 24 hours prior to the TPA a normal dose response curve was recorded. A similar response was observed when the test compounds were added to the cells without the addition of TPA. 315B exhibited greatest inhibition at 10μg; the percentage inhibition was recorded as 68%, whereas β-carotene was 17% and lutein 26%. The inhibitory response could be attributed to the initial 24 hour incubation period with the compounds whereby the cells would respond as they did in the assay without the addition of TPA. Therefore at the 24 hour time point a relative percentage of the cells may have already died or the proliferation of cells will have already slowed down. An unexpected result was observed in that a large increase in the proliferation of the cells was recorded for those dosed with low concentrations (0.1 and 0.01μg) of β-carotene. At 0.1μg the proliferation of cells had increased by 52% relative to the control cells with TPA only.

Krinsky (1994) has reported on several findings of the effects of carotenoids on the inhibition of cell growth. A 70μM dose of β-carotene or canthaxanthin was found to inhibit the proliferation of cultured human squamous cells (SK-MES lung carcinoma or SCC-25 oral carcinoma), whereas no effect was observed on the growth of normal human keratinocytes. Canthaxanthin (100μM) was also found to inhibit the growth of three tumour cell lines but stimulated the growth of 3T3 cells (a non-tumour cell line), but their conclusion was that canthaxanthin had a direct effect on inhibiting tumour cell growth. They suggested that the selected inhibitory effect on tumour cell growth was due to a cytokine secreted when carotenoids were added directly to human peripheral blood mononuclear cells. This cytokine is known to inhibit four out of six tumour cell lines, but only has a mild toxic affect on normal diploid fibroblast cells.

β-Carotene is considered as an anti-carcinogen and has been studied by numerous research groups for its anti-tumour activity. Scita and Wolf (1994) have studied the

effects of β-carotene on the glycoprotein fibronectin (FN). Fibronectin is a disulphide linked dimer which occurs in insoluble forms in the extracellular matrix of many cells and in the soluble form in plasma. Fibroblasts in culture will secrete FN into the media and bind it to the cell surface and serves to adhere cells to their substratum. TPA stimulates the release of FN and it is thought that FN release is required for the onset of tumour promotion or abnormal cell proliferation as induced by TPA. β-carotene was found to inhibit the synthesis of FN and release, however it had no effect on the binding of FN to the cell surface. The response was similar to retinoic acid (RA) but to a lesser extent. However RA was found to effect the attachment of FN to the cell surface, whereas β-carotene inhibited FN production. Their conclusion was that the inhibitory action of β-carotene on TPA induced cell proliferation was that it was incidental and that RA and β-carotene have different mechanisms of action. Within my research it could be said that perhaps the inhibitory action of carotenoids on normal cell growth could be attributed to the inhibition of FN synthesis. However the inhibitory response exhibited by the carotenoids on TPA induced proliferation (TPA and test compounds added at the same time point) was greater than normal cell proliferation. This would suggest that the carotenoids are either acting in a competitive manner for the receptor of TPA (PKC) or they are inhibiting a subsequent metabolic pathway initiated by PKC.

Tsushima *et al.*, (1995) studied the inhibitory effects of 51 carotenoids on the Epstein-Barr virus (EBV) activation activity of TPA in Raji cells, a widely used primary screening test for anti-tumour promoting activity. From this study the oxygenated carotenoids β-cryptoxanthanthin, lutein and lactucaxanthin showed the strongest inhibitory activity, superior to β-carotene. These results support my results in that the oxygenated carotenoids are more active than the non oxygenated carotenoid, β-carotene. There results also suggested that carotenoids having a 3-hydroxy-β- and /or 3-hydroxy-ε- end group had stronger activity than those with only a β- and/or ε-end group. In general they found no cytotoxic effects on the Raji cells except at higher concentrations for carotenoids with an epoxy-ketone and/or allene group. Although no mechanism of action was suggested they did conclude in suggesting the essential moiety for the activity of carotenoids was considered to be

the 3-hydroxy-ε-end group. Within my research all carotenoids tested, except β-carotene, had the 3-hydroxy-ε-end group and all proved to be more active than β-carotene.

The chloroform extract of *G.aparine*, lutein and β-carotene were tested for inhibition of physically (UVB) induced erythema. The mixture of anhydrolutein I and 2',3'-anhydrolutein II (315B) and β-ε-carotene-3-ol,3'-methoxy (315A) could not be tested due to insufficient quantities of material. The chloroform extract (71%) was more active in the inhibition of cell proliferation induced by the UVB as compared to both lutein (52%) and β-carotene(51%). This would suggest that perhaps more than one compound is exhibiting an anti-inflammatory effect within *G.aparine*. It was also found that the crude extract applied on non-irradiated skin induced the proliferation of cells. The same effect was also exhibited by lutein and β-carotene. The extent of anti-inflammatory activity induced by UVB and the slight inflammatory activity on non-irradiated skin did not vary depending on whether it was oxygenated or not, however the compounds were only tested at one concentration.

Carotenoids have been found to delay the appearance of tumours and lower the tumour yield in mouse skin either induced by chemicals or UVB irradiation. β-Carotene at 700μg and 30mg/g diets was effective in preventing skin tumours induced by repeated exposure to UVB. However one study with β-carotene supplemented at 100μg/g diet increased the tumour yield in mice exposed to UVB radiation. There are numerous reports of the differences in the effects of β-carotene on papilloma formation, and it is suggested that β-carotene differentially modulates different types of skin tumours induced by UVB radiation and chemical carcinogens (Chen *et al.*, 1993). The effects of excess dietary β-carotene could be partially due to the action of retinoic acid formed from β-carotene.

The suggested mechanism of action for the carotenoids are anti-oxidant, metabolic immunomodulatory or an unknown property of the carotenoid molecule. Much work on the anti-inflammatory/carcinogenic activity of the carotenoids have relied on the anti-oxidant effects. The basis of these statements are on the scavenging effects of

carotenoids with respect to photosensitiser triplets, singlet oxygen and free radicals; the carotenoids react with the free radicals preventing the damaging effects. The possible damage to RNA and DNA could result in the altering of the genotype of cells which is linked to cancer. The anti-oxidant effects of carotenoids with reference to psoriasis is in their interference with the production of superoxide ions released from the increased levels of polymorphonuclear leukocytes. The metabolic properties of carotenoids (in particular β-carotene), is the metabolism of β-carotene into retinoic acid (retinoid). Active metabolites of the retinoids include etetrinate and isotretinoin (derivatives of retinoic acid), which are used in the treatment of severe or resistant psoriasis and eczema. Acitretin, a metabolite of etetrinate, is recognised as an anti-inflammatory and anti-proliferative by decreasing levels of epidermal IL-I and increasing polyamine synthesis. However the carotenoid, canthoxanthin, cannot be metabolised into retinoids, but still exhibits an anti-carcinogenic effect.

Using the carotenoids from a photochemical point of view was a considered form of treatment for a type of porphyric disease known as erythropoetic protoparphyria (EPP). It is believed that the mechanism of action is analogous to the protective effect of carotenoids in photosynthesis. The carotenoid is thought to intercept the protoporphyrin triplet ^3PP to prevent the formation of 'biologically damaging' singlet oxygen:

$$^3PP + Car \rightarrow PP + {}^3Car$$

and/or

$$^3PP + O_2 \rightarrow PP + {}^1O_2$$

$$^1O_2 + Car \rightarrow O_2 + {}^3Car$$

where Car = carotenoid
PP = protoporphyrin
O_2 = oxygen

However the use of β-carotene gave cosmetically unacceptable skin coloration and is no longer used in the treatment of this disease. One could therefore assume that the use of carotenoids as a topical treatment for psoriasis would also be similarly unacceptable. However this does not out rule the use of carotenoids systemically.

Interestingly a case control study from Italy using 316 psoriatic and 366 control patients (Naldi *et al.*, 1996) found psoriasis to be positively associated with body mass index and significantly inversely related with observed intake of carrots, tomatoes, fresh fruit and the index of β-carotene. Kalvi *et al.*, (1985) also

documented a decreased prevalence of psoriasis with increased intake of fresh vegetables and fruit. Carrots, tomatoes and fresh fruit are an important source of carotenoids. It was also found that carotenoid serum levels of psoriatics are lower than control patients. Their suggestion was that the anti-oxidant and immune stimulatory effects of the micro-nutrients are the basis for such effects. Psoriatic lesions are characterised by the intra-epidermal penetration of polymorphonuclear leukocytes. During this process it is well established that oxygen derived materials such as superoxide (0^{2-}) play a central part. The carotenoids β-carotene, α-carotene, lutein and lycopene were also found to be significantly lower in those patients with cystic fibrosis (Homnick *et al.*, 1993). Inflammation and chronic infection appear to play a significant part in the pathophysiology of lung injury in cystic fibrosis.

The disease psoriasis is often believed to be an interaction of genetic predisposition with environmental factors, such as diet. It is therefore possible that dietary factors do influence psoriasis and its clinical expressiveness. The results of my studies do indicate that carotenoids have anti-inflammatory properties both in the in-vitro and in-vivo models also the inversely proportional relationship of fruit and vegetable intake of psoriatics is a second indication that these compounds do have anti-psoriatic properties. Whether the activity is intrinsic to the carotenoids or due to the metabolic action in the formation of retinoids, which are already used in the treatment of psoriasis, could be questioned.

CONCLUDING REMARKS

Psoriasis is a common and complex disease, with 2-3% of the Caucasian and 7% of the Japanese population suffering. The prognosis of a person suffering from psoriasis depends on genetic and numerous environmental factors. At present no cure is available and treatments focus on the management of the disease. However, treatments are varied both in their effectiveness and safety, which is primarily due to the different forms of psoriasis and their ranges in severity. Within this study two different groups of compounds have been isolated, the chalcones and oxygenated carotenoids, which have exhibited potential anti-psoriatic activity.

This study adopted a single approach to assess the four plant species for their potential anti-psoriatic activity. The in-vivo test inhibition of chemically induced mouse ear erythema was used as an effective screening assay successfully identifying those extracts which possessed anti-inflammatory and potential anti-psoriatic compounds. The same assay was subsequently used as a bioassay guided technique to isolate these active compounds.

From the plant species *G.glabra* one chalcone, isoliquiritigenin, and two retrochalcones, echinatin and licochalcone B, were isolated and identified as potential active anti-psoriatic compounds. Isolation of the retrochalcones could only be achieved through using a polyamide column and polyamide preparative T.L.C. plates. The two retrochalcones exhibited greatest activity in all four bioassays which led to the conclusion that absence of the C-2' hydroxyl resulted in a significant increase in activity. The absence of the hydroxyl at C-3 in the B-ring of the retrochalcone also increased the activity of these compounds. The suggested mechanism of action of these compounds was attributed to the inhibition of Ca^{2+} influx from extracellular medium and mobilisation from calcium pools as concluded by Kimura et al., (1993). This effect would directly inhibit the activation of the Ca^{2+} dependent enzyme protein kinase C, which is activated by TPA. TPA was used in three out of the four assays, however the chalcones also exhibited inhibition against UVB induced erythema. Rat UVB was effectively used as a model of psoriasis and the isolated and purified compounds were tested accordingly. Isoliquiritigenin and licochalcone B both

inhibited rat UVB induced dermatitis. UVB induced cell proliferation is said not to be activated by PKC (Matsui and DeLeo, 1990), although showing similar biological effects with the phorbol ester tumour promoter TPA. This led to the conclusion that the mechanism of action of these retrochalcones, with regards to inflammation and psoriasis, is through the inhibition of prostaglandin synthesis via the cyclo-oxygenase pathway as well as PKC inhibition. However, further studies would be required to substantiate these hypotheses.

A group of oxygenated carotenoids from *G.aparine* were isolated as active anti-inflammatory compounds. The method of extraction and isolation of these compounds was easier than the chalcones from *G.glabra* and did not require polyamide materials, although additional care had to be taken to prevent oxidation or degradation from the air or light. The compounds lutein and the mixture of anhydrolutein I and 2',3'-anhydrolutein II proved most active from *G.aparine*. Interestingly retinoids, which are metabolites of β-carotene are already used in the treatment of psoriasis, for example etetrinate and isotretinoin. The retinoids are said to decrease levels of epidermal IL-I and increase levels of polyamine synthesis. Carotenoid intake in the form of fresh fruit and vegetables is also inversely related to psoriasis; one suggestion is that the intrinsic properties of carotenoids inhibit the formation of singlet oxygens and free radicals thereby preventing the formation of superoxide ions released from PMNs. It can, therefore, be concluded that the carotenoids found in *G.aparine* do exhibit anti-psoriatic activities which can be attributed to either their metabolic or intrinsic properties. Although the carotenoids isolated were not novel as were the suggested mechanisms of action, the results of the study do substantiate past reports that the use of *G.aparine* is beneficial in treating psoriasis. It can also be confirmed that the absence of fresh fruit and vegetables in the human diet is directly related to the presence and severity of inflammatory and proliferative skin diseases. The potential use of carotenoids directly onto the skin is however cosmetically unacceptable, as established through the use of β-carotene in the treatment of the disease erythropoetic protoporphyria. It could be concluded that the use of carotenoids in treating psoriasis would probably be most effective through a controlled diet.

The etiology of the disease psoriasis is unknown, but shows a benign epidermal hyperplasia and abnormal differentiation plus dermal inflammation. Most models used to identify potential anti-psoriatic compounds, including the ones used in this study, possess only some of the characteristic features of psoriasis. UVB induced dermatitis was probably the most important model because it exhibited the greatest number of similarities, however it was not a practical assay to be used for screening large numbers of extracts. Identifying a clinical sign of psoriasis which can be visually seen was important in establishing the suitable screening assay to identify the potential anti-psoriatic compounds. Future work would entail the establishment of the exact mechanisms of action of the chalcones and carotenoids isolated. For example, there are numerous hypothesised mechanisms of action for the chalcones including inhibition of protein kinase C. If time had permitted a slightly modified form of the assay protein kinase C activation by Trition X-100 mixed micelles would have confirmed such a statement. Another example is the assay of NADPH-oxidase activation, which would have been useful to test for the anti-oxidant affects of the carotenoids from *G.aparine.* Further examination of the compounds should be done with a view to determine the mechanism of action using enzyme assays relevant to inflammation and hyperproliferation, for example, the inhibitory activity of the compounds on lipoxygenase and cyclo-oxygenase compounds which play important roles in the arachidonic acid metabolism and consequently inflammation.

In summary it can be confirmed that the plants *G.glabra* and *G.aparine* do possess compounds which have therapeutic effects in treating psoriasis. The same statement cannot be made for *T.pratense* and *B.perennis.* It is possible that these two plants do possess anti-psoriatic properties but they were not exhibited in this particular screening assay. The anti-psoriatic activities of the carotenoids isolated from *G.aparine* can be attributed to their anti-oxidant effects or metabolites. It is rather unfortunate that the compounds isolated from *G.aparine* are already known compounds and that the metabolites are used in treating psoriasis, but this is an example of a draw back in using bioassay guided fractionation. However, the results do support the use of *G.aparine* as a herbal treatment for psoriasis and reinforce the importance of a healthy diet. Although the chalcones isolated from *G.glabra* were

not novel it was the first time that they had been investigated for potential anti-psoriatic activity. The results support past literature in that *G.glabra* can be beneficial in treating the symptoms of psoriasis.

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