Phytochemical and Biological Studies of *Thuja occidentalis* and its clinical evaluation in the treatment of verruca pedis

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By

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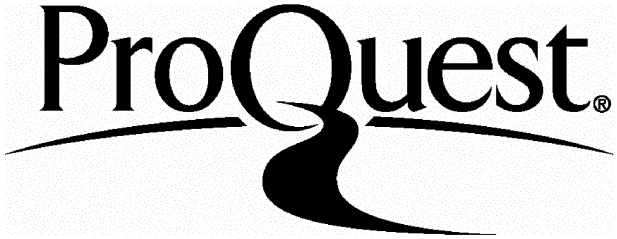

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ABSTRACT

This study undertakes a botanical investigation, phytochemical analysis, biological testing and chemical evaluation of *Thuja occidentalis* in the treatment of verruca pedis.

Clinical observation over 10 years in dermatology and podiatric clinics revealed a high incidence of verruca pedis. Most medical and surgical intervention can cause a great deal of discomfort, as well as post-operative complications. Investigation of medicinal plants for the treatment of podiatric disorders led to clinical pilot studies and a double-blind controlled trial of tagetes species in the treatment of verruca pedis (Khan, 1994).

In the present study, the clinical features of verruca pedis, aetiology and pathology are described and advantages and limitations of traditional treatments are discussed. In the phytochemical study of *Thuja occidentalis*, a flavonoid, and a lignan deoxypodophyllotoxin, were isolated and identified by chromatographic techniques and spectral analysis.

In the clinical study, the effect of *Thuja occidentalis* crude extracts and sub fractions in different organic solvents were tested in an open study and double-blind study over a period of 12 weeks. Ethical approval was obtained from the Clinical Lead of Dermatology Department at the Royal London Hospital and the Director of Research at the Royal London Homoeopathic Hospital. Ninety volunteers were chosen, using inclusion and exclusion criteria and were admitted to the study from a population awaiting podiatric treatment at the Royal London Hospital and the Royal London Homoeopathic Hospital. These were then systematically allocated to 3 groups of 30 in each group. Test samples were prepared on the basis of the effective dose established in pilot clinical studies of *Thuja occidentalis* at 300mg/ml-dose/day for crude extract and 60mg/ml-dose/day for fractions.

The results of the clinical study show that ethanolic crude extract of *Thuja occidentalis* was effective in reduction of verruca size and clearance, with a level of significance at $p<0.001$. The chloroform fraction of *Thuja occidentalis* was shown to be the most effective in reduction of size and clearance with a level of significance at $p<0.01$.

In biological tests, *Thuja occidentalis* crude extract at 1μg/ml and the chloroform fraction at 0.021μg/ml showed no toxicity to human keratinocytes, but at concentrations of 1μg/ml to 1mg/ml and 0.21μg/ml to 0.21mg/ml was toxic to cell growth respectively. These findings concurred with earlier results from a clinical study that activity of *Thuja occidentalis* was located in the chloroform fraction as other fractions did not affect growth rate of cells.

The results of the clinical study and biological tests show a close correlation in terms of safety and efficacy in the treatment of verruca pedis. These encouraging results need further investigation using a larger number of patients over a longer period of time.

CONTENTS

Abbreviations

A.L.A	Amino-Laevulinic Acid
A.P.C	Anaphase – Promoting Complex
B.P.V.1	Bovine Papilloma Virus 1
BrdU	Bromodeoxyuridine
C.C	Column Chromatography
C.I.M.S	Chemical Ionisation Mass Spectrometry
C.M.I	Cell-Mediated Immunity
C.M.V	Cytomegalovirus
C.O.P.V	Canine Oral Papilloma Virus
C.R.P.V	Cottontail Rabbit Papilloma Virus
D.C.P	Diphencyprone
D.M.E.M	Dulbecco's Modified Eagle's Medium
D.M.S.O	Dimethylsulphate
D.N.A	Deoxyribonucleic Acid
D.P.D	Deoxypodophyllotoxin
E.B.V - E.A	Epstein- Bar Virus Antigen
E.D.T.A	Ethylene-diamine-tetra-acetic acid
E.G.F	Epidermal Growth Factor
E.I.M.S	Electron Impact Mass Spectrometry
Et.O.H	Ethanolic hydroxide
E.V	Epidermodysplasia Verruciformis
ELISA	Enzyme-Linked Immunosorbent Assay
F.A.B.M.S	Fast Atom Bombardment Mass Spectrometry
F.A.C.S	Fast Activated Cell Sorting
F.C.S	Foetal Calf Serum
F.D.M.S	Field Desorption
H.F.V	Human Foreskin Keratinocyte
H.I.R	Humoral Immune Response
H.I.V	Human Immunodeficiency Virus
^{1}H.M.N.R	Nuclear magnetic resonance spectroscopy
H.P.L.C	High Pressure Liquid Chromatography
H.P.V	Human Papilloma Virus
H.S.V	Herpes Simplex Virus
H.z	Hertzs
I.A	Independent Assessor
I.C 50	Concentration of an inhibitor at which 50% inhibition of the respose is seen.
L.C.R	Long Control Region
L.D 50	Dose of a compound which causes death in 50% of the organisms to which it has been administered
M.I.C	Minimium inhibitory concentration
m.R.N.A	message Ribose Nucleic Acid

M.S	Mass Spectrometry
M.T.s	Micro-Tubule's
M.T.T	[3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide]
N.C.B	Noncoding Region
N.H.S	National Health Service
N.M.R	Nuclear Magnetic Resonance
N.P/P.E.G	Natural Products/ Polyethylenglycol Reagent
O.C.D	Ornithine Decarboxylase
O.D.R	Optical Density Reader
O.R.F.s	Open Reading Frames
P.B.L	Peripheral Blood Lymphocytes
P.B.S	Phosphate-Buffered Saline
P.C.R	Polymerase Chain Reaction
P.D	Podophyllotoxin
P.I	Propidium Iodide
P.T.L.C	Perpartive Thin Layer Chromatography
P.V	Papilloma Virus
P.V.s	Papilloma Viruses
R.L.H	Royal London Hospital
R.L.H.H	Royal London Homoeopatic Hospital
R.N.A	Ribose Nucleic Acid
S Phase	Synthesis Phase
S.C.I.D	Severe – Combined Immunodeficient
S.D.S	Sodium Dodecyl Sulphate
S.I	Selectivity Index
S.P.F	S – Phase Promoting Factor
T.L.C	Thin Layer Chromatography
T.M.S	Tri-methly Siyl
T.O	*Thuja occidentalis*
T.P.A	Tetradecanoylphorbol Acetate
T.P.S	Thuja Polysaccharide
T.P.Sg	Thuja Polysaccharide g
U.V	Ultra violet spectroscopy
V.L.P	Virus Like Particles
V.S.V	Varicella – Zoster Virus

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CHAPTER 1 –
GENERAL INTRODUCTION

1. INTRODUCTION

Warts are caused by infection of the epidermis with human papilloma virus (HPV). HPVs are divided into separate genotypes on the basis of their deoxyribonucleic acid (DNA) sequence. Different HPV types may preferentially infect either cornified stratified squamous epithelium of skin or uncornified mucous membranes. The appearance of the lesion is influenced not only by viral type but also by environmental and host factors. These viruses infect the epithelia of susceptible individuals to produce tumours that are mostly benign, but very often cosmetically unacceptable. They can also be painful, particularly when they arise on the plantar surface of the foot (Sterling et al., 2001).

The Human Papilloma virus (HPV), the cause of warts/verrucae, is one of a group of highly specialised DNA viruses displaying a remarkable host and target-cell specificity. These induce papillomatous proliferations in skin and mucosa via specific differentiation of the genome-harbouring cell (zur Hausen 1996). Warts can be persistent, annoying and unsightly. They may persist for many years, despite treatment, or may disappear spontaneously. There is no uniformly successful remedy. Therapeutic modalities commonly used include the local application of preparations containing salicylic acid, glutaraldehyde, podophyllin and formaldehyde, or comprise the use of non-conservative treatments such as cryotherapy and electrosurgery (Bunney et al, 1992). However, there are many cases where eradication of the warts are unsuccessful. Failure to treat warts can often result in long-standing distressing problems for patients including socially, physically and mentally. The occurrence of warts is a common feature seen in dermatological and outpatient clinics (Gibson, 1988). Such patients are also found in chiropody clinics. Outcomes of radical therapies in the treatment of warts and verrucae are difficult to evaluate objectively. The patient must ultimately develop an immune response capable of dealing with this agent before the condition can be resolved (Bartolemi and McCarthy, 1977). No reproducible method has yet been developed for HPV propagation *in vitro*, which consequently has

necessitated numerous studies concerning the infectivity in man. Naturally, this has impeded research into the biological properties of the virus and immunological responses to wart infections (zur Hausen, 1996). It is a known fact that most people do not develop antibodies to HPV until the infection has been present for a few months and re-infection can occur despite the presence of these antibodies. Although a second exposure is more likely to result in stimulation of the Humoral Immune Response (HIR), this does not always seem to influence the duration of the wart (Bunney et al, 1992). The appearance of these HPV specific antibodies is probably merely a secondary phenomenon in wart rejection (Von Krogh, 1979). Morison (1975a) states that although there is a close relationship between the method of treatment and development of Cell-Meditated Immunity (CMI), there is no indication that the method of treatment influences the type or presence of antibody. In fact, in many studies, CMI is seen to be the dominant factor involved in spontaneous wart regression (Morison, 1975a) (Tagami *et al.*, 1974).

Since most HPV infections will regress spontaneously by the above pathways, both patient and therapist must maintain a positive favourable outlook. Suggesting that a wart will soon disappear, when in fact it is showing little sign of doing anything at all, is perhaps not such a blatant untruth. Massing and Epstein (1963) stated that 53% of all warts resolve in one year and 67% in 2 years. Genner (1971) remarked that 90% undergo spontaneous recovery within a 4 year period.

Most wart therapies are destructive, invasive or painful and can be very time consuming to both practitioner and patient. There is little evidence to suggest that any particular therapy is noticeably more successful than any other. Much interest nowadays is focused upon more natural remedies that pose less threat to health, harmony and the environment. Coupled with the need for cost-effectiveness within the National Health Service (NHS), which is dogged by inefficiencies (Kemp and Winkler, 1983), and a consequent reluctance on the part of the dermatologist to treat such an unpredictable conclusion (Kemp and Winkler, 1983; Morison, 1975b), it is not surprising that herbal and

homoeopathic therapies have advanced to the forefront of "Complementary Medicine" treatments for complaints such as these.

1.1 AETIOLOGY

Human papillomaviruses (HPV) are small DNA viruses that multiply in the nuclei of differentiated keratinocytes and induce squamous epithelial tumours of the skin or mucosa. The virions are non-enveloped, cubic particles of 55-60nm, which encapsulate a single copy of the double-stranded genome in the form of a protein-complexed minichromosome (Pfister and Fuchs, 1987; Pfister and Fuchs, 1994). The capsid proteins carry type-specific and genus-specific antigenic determinants (Jenson et al., 1980). To establish the viral DNA in the human epidermis, the virions probably have to infect cells of the basal layer. It may take weeks before tumours develop and up to 90% of the infections will have no clinical consequences (Pfister, 1987). Warts, papillomas, or dysplasias arise when virus-encoded proteins stimulate cellular proliferation and interfere with the proper differentiation of epithelial cells. Differentiating keratinocytes become increasingly permissive for HPV, which is reflected by the onset of vegetative viral DNA replication in prickle cells and by the synthesis of structural proteins, starting in the granular layer. Mature virus particles are spread throughout the cell nuclei in the stratum grandulosum and appear embedded in keratin in the stratum corneum (Pfister and Fuchs 1987).

HPV-induced papillomas or acanthomas may develop into carcinomas mainly in three conditions: the skin disease epidermodysplasia verruciformis (EV), dysplasias of the cervix uteri, and laryngeal papilloma of adults (zur Hausen, 1977; zur Hausen, 1989). Carcinomas are not an early consequence of HPV infection but occur on the basis of long-persisting lesions with "latency periods" of many years (zur Hausen, 1986). No mature HPV particles are produced in carcinomas cells but persistence of viral DNA and ongoing expression of viral early genes could be demonstrated in carcinomas of EV patients and in anogenital cancer (see below). Due to the preferential

association with malignant tumours, specific HPV types are regarded as having high carcinogenic potential as opposed to low risk viruses that prevail in predominantly benign proliferations (Pfister, 1987; zur Hausen, 1989).

1.2 PATHOLOGY

All papillomaviruses induce proliferation of cells resulting in tumour formation. Only differentiated stratified squamous epithelial cells undergo a lytic process as the viruses complete their life cycles. Each virus produces very specific lesions but all follow fundamentally similar patterns of cellular proliferation and lysis (Sundberg et al 1997).

Papillomaviruses are remarkably stable in the environment. Natural inoculation by rubbing on communally used posts or other animals probably forces desiccated cornified cells filled with millions of virions into wounds of susceptible hosts. The virus-packed cornified cells undergo dissolution and viruses invade surrounding cells. Target cells probably have surface receptors for normal cytokines by which the virus attaches and enters the cell such as an epidermal growth factor receptor for vaccinia (Eppstein *et al.*, 1985). The receptors for PVs are still undetermined. The primary target cells of PVs are basal cells of stratified squamous epithelia (Cheville 1964). Transformation results in tumour formation. In some species, notably the ruminants (Artiodactyla), fibroblasts appear to be the primary target cells with overlying squamous cells being secondary (Sundberg *et al.*, 1985). This latter observation has been difficult to prove, however, sensitive *in situ* hybridization techniques may resolve this question.

These cell proliferation processes result in formation of three benign tumour types: papillomas (epidermal proliferation on thin fibrovascular stalks), fibropapillomas (epidermal proliferation on prominent fibrovascular structures forming a verrucous pattern), or fibromas (primarily fibroblasts and dense irregular collagenous connective tissue) (Sundberg, 1987a&b).

Epidermal degenerative changes, the second effect of PV infection, are usually limited to the cells in the stratum granulosum of the benign tumours but may include those in the upper stratum spinosum. The cytoplasm of these cells swells and stains poorly; large and/or abnormal keratohyalin granules become prominent; and nuclei may contain amphiphilic inclusions of various sizes. The inclusions contain large crystalline arrays of virions when viewed by transmission electron microscopy. These infected cornified cells are sloughed into the environment as part of the normal exfoliation process thus completing the life cycle of the virus (Sundberg et al 1997).

Regression of lesions is the result of the combined effects of the cellular and humoral immune system. Antibody titres can be detected following experimental inoculation and before regression of tumours. This is associated with a rise in viral genome copy number in tumours (Sundberg *et al.*, 1985; O'Banion and Sundberg, 1987). Infiltration of perivascular lymphocytes into the dermis immediately precludes tumour regression, indicating the role of the cellular immune system (Sundberg *et al.*, 1985). Simultaneous regression of coexisting, sometimes widely separated, benign warts or papillomas occur if these lesions are caused by the same HPV type, suggesting that cell mediated immunity is responsible for spontaneous regression of HPV induced lesions and that such immunity is HPV type-specific (Sundberg et al 1997).

1.3 CLASSIFICATION

The traditional classification of cutaneous warts is based on clinical appearance and location. It includes the following clinical types: verruca vulgaris or common wart, deep hyperkeratotic palmoplantar wart or myrmecia, superficial mosaic type palmoplantar wart, verruca plana or flat wart, and intermediate wart.

1.3.1 VERRUCAE VULGARES (COMMON WARTS)

Commons warts begin as little, flat or dome-shaped, firm, skin-coloured papules. With increasing elevation, their surface becomes rough, irregular and hyperkeratotic. Their size varies from a few millimetres to more than 1cm, and their colour changes to grey or brownish. They may occur singly or in clusters and are sometimes confluent and hypertrophic, especially in the periungual location. They may appear anywhere on the skin, including the thin epithelia of the anal and genital regions and the vermilion of the lips, but they are most commonly localised on the dorsal aspects of the fingers and hands. On the soles, common warts usually appear as *mosaic warts* (Gross and Jablonska, 1997). They are painless, only slightly raised above the skin level, multiple and often so closely set that they may impinge against each other and form a coherent plate of warts. There may be only one large plaque, but nearby, it is common to find several small warts of the same structure nearby. *Endophytic* common warts also appear characteristically in palmar and plantar localisations. They are usually multiple, painless, and have a slightly raised hyperkeratotic surface. Sometimes they are surrounded by a horny wall, which defines the size of the lesion. If they are small, they may resemble keratotis punctata. (Jablonska et al., 1985).

On the face, head, neck and in flexures, verrucae vulgares can become elongated and papillomatous. Depending on their clinical characteristics and size, they are often referred to as *filiform, digitate, or multidigitate warts.* Hyperproliferative, papillomatous common warts with a cauliflower-like, vegetating appearance have been found on the hands of butchers (Orth et al., 1981).

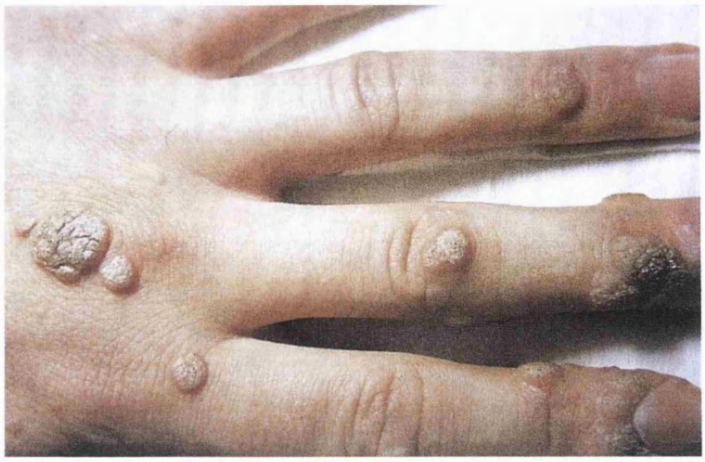


Figure 1. Common warts on the hands in varying stages of development from small, smooth, fresh-coloured papules to large, greyish, keratotic nodules.

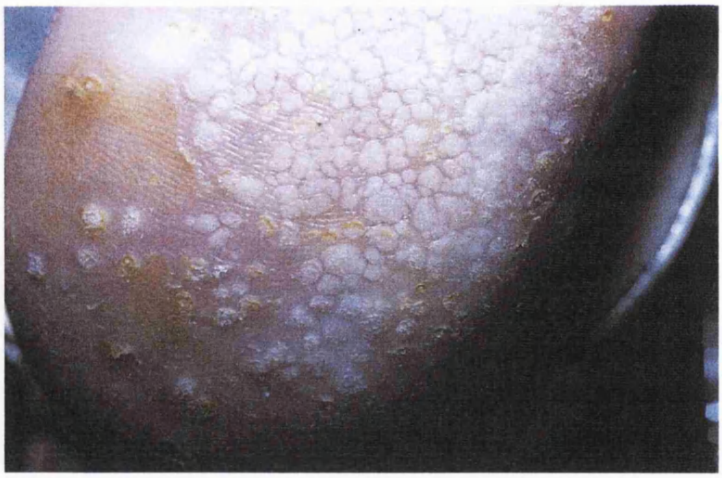


Figure 2. Mosaic plantar warts that have been pared down revealing paving-stone appearance of discrete areas of wart tissue divided only by horny partitions.

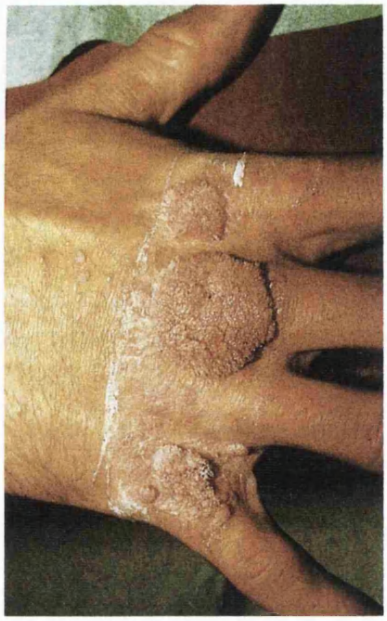


Figure 3. Typical 'butchers' warts, but occurring in a young fisherman. HPV 7 detected.

1.3.2 MYRMECIA

A prototype of Myrmecia is a single, painful palmoplantar wart deeply set in the skin. It is usually associated with considerable tenderness and may show swelling and redness. As it proliferates, the individual lesions do not coalesce. Myrmecia do not only occur on the palm and soles, but also on the lateral aspects of the fingers and toes, under the nails, on the pulp of the digits and, rarely, on the face, scalp, and body. Usually, the deep palmoplantar wart is covered with a thick callus. When this horny plate is removed, the wart tissues appear soft and crumbly and show a white opaque colour. This sort of wart occurs frequently in children between 5 and 15 years of age (Grussendorf-Conen, 1987).

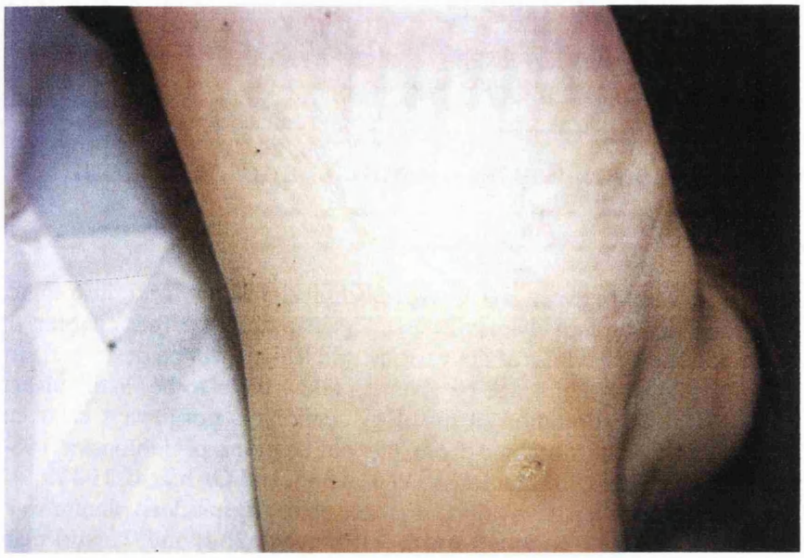


Figure 4. Deep plantar wart (myrmecia) showing keratotic cap and horny collar.

1.3.3 VERRUCAE PLANAE (PLANE WARTS, FLAT WARTS)

Verrucae planae are slightly elevated, small, skin-coloured papules. They always appear as multiple, irregularly disseminated or grouped lesions, sometimes in a linear distribution. The forehead and the dorsal aspects of the hands are most commonly affected; rarely do they occur on the forearms and lower extremities (Grussendorf-Conen, 1987).

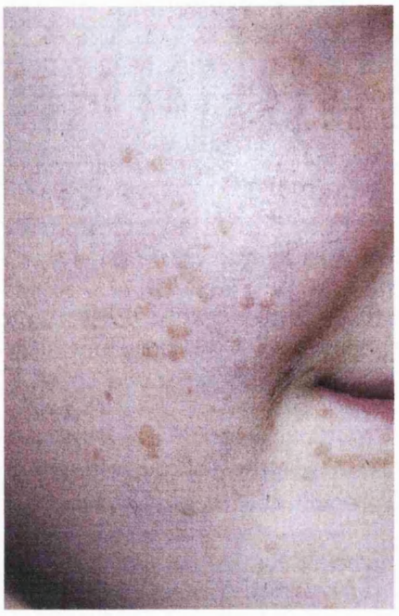


Figure 5. Classically flat-topped plane warts on the cheek of a child. Brownish colour is due to surface keratin. Note the linear arrangement of warts below the lip along a scratch (Koebner's phenomenon).

1.3.4 INTERMEDIATE WARTS

Jablonska et al. (1985) coined the term intermediate warts to describe lesions that could not be classified as common or plane warts, as they combined clinical features of both. If they are hyperkeratotic, raised, and coalescent, these papillomas differ from common warts by a flatter surface. Typical flat warts are often found between the raised ones. The clinical recognition of intermediate warts and their differentiation from early common warts may be impossible (Gross and Jablonska 1997).

1.4 CUTANEOUS WARTS AND INFECTING HPV TYPE

Disclosure of the plurality of HPV types associated with skin warts raises the problem of the relation of clinical morphology to distinct types of HPV. This was first suggested by the characterising of distinct HPV from different types of lesions: HPV 1 from deep plantar warts, HPV 2 from common hand warts, and HPV 3 from plain warts. At present, more than 70 types of HPV are recognised with several sub-types for many of them. Some HPV types are especially associated with cutaneous warts and a great number of them are associated with skin warts in epidermodysplasia verruciformis (Table 1). A great number of different HPV types pertain to only a limited variety of clinically and histologically different sorts of warts as mentioned above (Gross and Jabloska 1997). Table 1 shows how the HPV types are related to clinically defined warts:

Table 1. The Relationship Between Clinically Defined Warts and HPV Types.

Type of skin lesion	**HPV type**
Myrmecia	HPV 1
Common warts	HPV 2, 4, 7, 26-29, 49, 57
Plane warts	HPV 3, 10, 28, 41

HPV 1 is characterised by its enormous virus production, which leads to highly characteristic histological features (Grussendorf, 1980). It is associated with deep plantar warts, such as myrmecia, and only rarely with other common warts. The HPV 1 induced wart has a dome-shaped clinical appearance with a central depression. Nevertheless, the clinical morphology of HPV 1 warts varies, depending on their localisation. Lesions on the face, for example, can have the clinical features of digitate or filiform warts (Egawa et al., 1993).

According to their DNA sequence homology, some of the HPV types show a close relationship to each other, e.g. HPV 2,27,29 and 57. HPV 2 and its subtypes are the prototypes of common warts (Rúbben et al., 1993). The HPV 2 related type 57 can also be found in genital lesions (de Villiers, 1989). Plane warts and intermediate warts are commonly associated with the related HPV types 10 and 28 (Favre et al., 1989). HPV 4 induces small multiple warts and endophytic warts. HPV 4, may be associated with HPV60 and 65. Recently, these types have been associated with pigmented warts (Egawa et al., 1991). Another group of related HPV types are HPV 7 and HPV 40. HPV 7 was first detected in butcher's warts (Orth et al., 1981; Oltersdorf et al., 1986) and later was found in other skin warts and in oral warts in HIV-infected people (Greenspan et al., 1988; de Villiers, 1989). The HPV 7 related HPV 40 could be demonstrated in genital warts and Bowenoid lesions (de Villiers, 1989). Originally, HPV 41 was isolated from facial skin warts of a child. Later on, it was detected in some cases of actinic keratosis and in skin cancer (Grimmel et al., 1988).

1.5 VIROLOGY

When a virus enters the host a normal immune system will produce antibodies against the viral determinants. The viruses themselves are relatively simple in structure comprising a genome of circular, double-stranded DNA enclosed in an icosahedral capsid of 55 nm (Broker and Botchan, 1986). However, although the genome is structurally uncomplicated, it is fairly complex with regards to its expression (Acs et al., 1989). The particle is thought to affect the basal cell of the epithelium, probably after mechanical wounding and subsequent expression of various genes. Viral DNA synthesis and virion production is probably related to the sequence of keratinocyte differentiation. These events are poorly understood, but in essence it is thought the genome is only minimally expressed in the basal cells, while viral DNA and capsid synthesis and assembly occur in the upper layers of the stratum spinosum and granulosum, with the virions probably being shed in the stratum corneum (Bernard, 1990).

HPV gene expression is closely linked to the differentiation status of the infected epithelial cell. Different promoters and alternate RNA splice sites have been shown to be used depending upon the differentiation status of the infected cell (Blair et al., 1998).

Early viral genes involved in viral gene regulation and DNA replication are expressed in the basal and spinous epithelial cell layer. Both early and late gene capsid proteins are expressed and infectious virions assembled only in the most superficial fully differentiated squamous cell layers (Blair et al 1998) (Figure 6.).

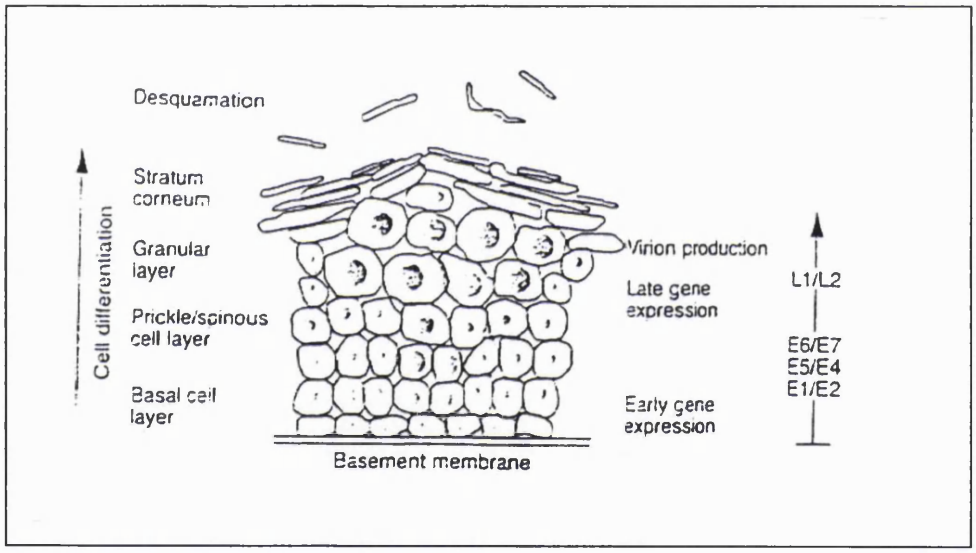


Figure 6. HPV gene expression in stratified epithelium.
(Blair et al 1998).

1.6 GENOME ORGANISATION

The HPV genome is a circular DNA molecule of 7500-8000 base pairs. A large number of HPV types have been sequenced in their entirety or at least partially (Delius and Hofmann, 1994). Their organisation turned out to be remarkably similar. All putative protein-encoding sequences (open reading

frames, ORFs) are located on one DNA strand and occupy comparable positions relative to each other. Three representative genomes are shown in Figure 6. The individual ORFs are classified as "early" (E) or "late" (L) in analogy with other DNA viruses where this designation denotes the expression schedule in the viral life cycle. The expression in non-productive, bovine papilloma virus 1 (BPV1) transformed cells defined the "early" segment of papillomavirus DNA, and a detailed genetic analysis indeed identified typical "early genes," whose products are involved in viral DNA replication, transcription control, and cellular transformation (DiMaio and Neary, 1990). Only E4 seems to be predominantly expressed late in the viral life cycle, possibly playing a role in the collapse of the cytokeratin matrix and in virus release as shown for HPV16 E4 (Doorbar et al., 1991). The "late" ORFs encode the major viral capsid protein (LI) and a minor capsid component (L2) of uncertain localisation (Doorbar and Gallimore, 1987). ORFs L1 and E6 are consistently separated by a 400-1000 base pair monocoding DNA segment that contains the origin of viral DNA replication and numerous transcription control signals. This segment is variably referred to as noncoding region (NCB), long control region, or upper regulatory region.

There are two noteworthy differences in the genome organisation of HPV types belonging to various phylogenetic subgroups (Pfister and Fuchs, 1987; Delius and Hoffmann, 1994). The noncoding DNA segment of EV-associated HPVs has roughly only half the length of the noncoding region of all other HPVs (Figure 7) and there is some evidence for transcriptional control elements integrated into flanking coding sequences (Fuchs and Pfister, 1990). The ORF E5 is located between ORFs E2 and L2 in genital HPVs like HPV 16 (Fuchs et al., 1986) (Figure 7) and in more distantly related cutaneous HPVs like HPV 2 (Hirsch-Behnam et al., 1990). In contrast, there is little space between E2 and L2 in HPV 1 (Danos et al., 1983) and the coding part of E5 completely overlaps L2 (Figure 7). The ORF E5 of HPV 1 is located downstream of a polyadenylation site at the beginning of L2 and therefore is not part of the early transcription unit. The amino acid sequence and the hydrophobicity profile of the putative HPV1 E5 protein show no similarity of E5 of HPV16-related viruses so it is questionable if the E5 ORFs of these viruses

really represent homologous genes. There is no comparable ORF E5 in EV-associated HPVs like HPV8 (Fuchs and Pfister, 1990; Fuchs et al., 1986) (Figure 7).

1.6.1 CELL DIFFERENTIATION-LINKED TRANSCRIPTION

As the replication cycle of papillomaviruses is well known to be closely linked to keratinocyte differentiation, it was not surprising to observe characteristic transcription patterns in individual epithelial layers by *in situ* hybridisation with subgenomic RNA probes. Only very weak transcription of early genes was detectable in the basal layers of low-grade squamous intraepithelial lesions induced by HPV16 or HPV33 (Beyer-Finkler et al., 1990b; Stoler et al., 1992) and in HPV5-positive, benign EV-lesions (Sherman and Alloul, 1992). The same holds true for some HPV6 or HPV11 induced condylomata (Stoler et al. 1989) but the majority of HPV6 induced condylomas showed clear signals in the basal layer with an E6 probe and even more pronounced labelling of all cells within the two to three lowest epidermal layers after hybridisation with an E7 probe (Iftner et al., 1992). This indicates that expression of the viral oncognenes of low risk HPV types is much less restricted in proliferation competent cells. There may have been a special need in evolution to develop mechanisms avoiding undue expression of the more potent oncoproteins of HPV16 (see below) in order to prevent progression to malignancy. HPV16 E6-E7 RNA was detected in the basal layer of cysts induced in nude mice by HPV16 immortalised human keratinocytes (Durst et al., 1991). This change in viral gene regulation may be due to integration of the viral DNA or to the lack of a human dermis.

Probes that were able to distinguish between mRNAs with a coding potential for E6 and E7 failed to detect these transcripts in differentiated layers of many condylomas (Iftner et al., 1992) and revealed no E6 specific RNA in HPV16 infected lesions (Bohm et al., 1991). HPV16 and HPV5 E7-signals were confined to a few nuclei of differentiated cells (Haller et al., 1995; Bohm et al., 1993). Transcriptions of several early genes strongly increases in the more differentiated cells of low-grade lesions, accompanied by the onset of vegetative DNA replication (Haller et al., 1995; Beyer-Finkler et al., 1990a;

Iftner et al., 1992; Bohm et al., 1993). The E1 and E2 signals were mostly confined to the nuclei, which indicates that the levels of translatable mRNAs with a coding potential for these genes are very low in benign lesions. The bulk of the cytoplasmic signals in the middle and upper third of the epithelium appears to represent the E1-E4 mRNA. It is usually more abundant than transcripts from the late genes L1 and L2, which are only present in terminally differentiated keratinocytes of the superficial strata of the epithelium (Haller et al., 1995; Durst et al., 1992; Stoler et al., 1989; Bohm et al., 1993; Higgins et al., 1992). A close topological correlation was observed between the abundant HPV16 transcripts and immunostaining for E4 proteins as well as immunostaining for L1 proteins (Crum et al., 1990). This fits the function of the E4 protein, which leads to the collapse of the cytokeratin network and may thus facilitate virus particle release (Doorbar et al., 1991).

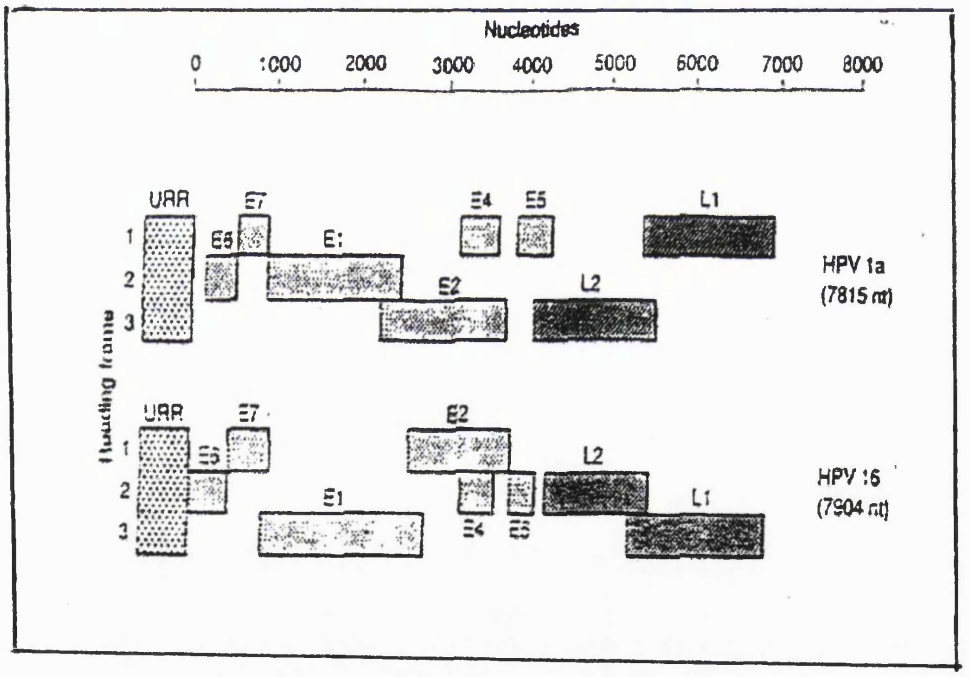


Figure 7. Genome organisation of human papillomaviruses (HPV 1, 8 and 16 – Pfister and Fuchs, 1987; Fuchs and Pfister, 1990; Seedorf et al, 1985; Danos et al., 1983). The circular genomes are linearised for convenience of alignment. Open boxes indicate open reading frames. Dotted lines within the frame represent the first methionine codon, which could serve as a start point of translation. Stippled areas of the genome bars represent coding sequences and black regions stand for noncoding regions. The scale is in base pairs (bp).

1.6.2 FUNCTIONS OF VIRAL PROTEINS

E1 shares a number of properties with SV40 large T antigen (Seo et al., 1993; Sun et al., 1990). It codes for a policistronic RNA, the protein has site-specific DNA binding functions (Ustav et al., 1991), binds and hydrolyses ATP (Seo et al., 1993), possesses ATP-dependent helicase activity (Yang et al., 1993) and is essential for papillomavirus replication (Ustav et al., 1991). It also interacts with cellular DNA polymerase α (Bonne-Andrea et al., 1995). The E1 protein binding site in the origin of replication, localised in the proximal region of the LCR, represents an 18 nucleotides imperfect palindrome (Holt et al., 1994). Bi-directional unwinding of this region is a prerequisite for viral DNA replication (Li et al., 1993). Besides L1, the E1 open reading frame represents the most conserved structure among different papillomavirus types.

The E2 open reading frame encodes at least two and probably three different proteins, all acting as transcription factors (Bouvard et al., 1994). They differently affect viral gene expression and represent major intragenomic regulators by forming dimers at specific binding sites. HPV16 and HPV 18 E2 protein function as transcriptional activators in human cervical keratinocytes (Bouvard et al., 1994; Cripe et al., 1987; Phelps et al., 1990). The C-terminal domain of the HPV 16 E2 gene acts as transcriptional repressor and interferes with the activity of the full length E2 protein (Doorbar et al., 1990).

Deletion of the E2 open reading frame is frequently observed in samples isolated in cervical cancer biopsies and in cell lines derived from this cancer (Schwarz et al, 1985), leading to the speculation that this deletion facilitates transformation of human cells and the transition into a malignant state. Indeed, mutations in the E2 ORF, but also in the E2 DNA binding sites within the viral LCR led to enhanced immortalising activity of HPV 16 DNA (Romanczuk and Howley 1992). In cancer development, disruption of the E2 appears, however, to usually represent a late event since most premalignant lesions do not reveal this modification (Matsukura et al., 1989; Durst et al., 1992). A recent study noted integration of HPV 16 DNA also in advanced

cervical intraepithelial neoplasias (Daniel et al., 1995). Besides its role in transcriptional regulation, E2 proteins interacting with E1 stimulate viral DNA replication (Chiang et al., 1992; Sverdrup and Khan, 1994; Chow and Broker, 1994). They apparently facilitate binding of E1 to the origin of replication (Seo et al., 1993).

The E5 protein is the major transforming protein in bovine papillomaviruses (Schiller et al., 1986; DiMaio et al., 1986; Rabson et al., 1986). In contrast, in HPV infections E5 has only weak transforming activity (Leptak et al., 1991; Leechanachai et al., 1992; Pim et al., 1992). It may cause tumorigenic transformation of mouse keratinocytes, leads to anchorage-independent growth of mouse fibroblasts, and stimulates growth of primary rat kidney epithelial cells in co-operation with the HPV16 E7 gene (Straight et al., 1993; Bouvard et al., 1994). The open reading frame coding for E5 is frequently deleted in cervical cancers (Schwarz et al., 1985), although anogenital low grade intraepithelial neoplasias contain relatively large amounts of E5 mRNA and protein (Stoler et al., 1992; Kell et al., 1994). This may support the assumption that E5 plays a role in early steps of HPV infection but is obviously dispensable for the maintenance of malignant transformation.

The E4 protein seems to be incorrectly assigned as an early gene product. It originates from a viral RNA transcript formed by a single splice between the beginning of the E1 open reading frame and the E4 open reading frame. This mRNA is the major transcript in HPV-induced lesions (Chow et al., 1987a&b). The role of this protein in the life cycle of the virus has yet to be determined. It is not required for transformation or episomal persistence of viral DNA (Neary et al., 1987). The E4 protein is exclusively localised within the differentiating layer of the infected epithelium (Doorbar et al., 1986; Breitburd et al., 1987; Palefsky et al., 1991a&b). It has been speculated that this protein plays a role in productive infection, possibly establishing favourable conditions for viral maturation.

E4 proteins associate with the keratin cytoskeleton of cultured epithelial cells (Doorbar et al., 1991; Roberts et al., 1993). Electron microscopically they can

be localised to tonofilament-like structures in HPV1 warts (Rogel-Gaillard et al., 1992). HPV16 E4 induces a collapse of the cytokeratin network in cultured cells (Doorbar et al, 1991; Roberts et al, 1993). Multiple E4 proteins have been demonstrated in HPV1-infected cells (Doorbar et al., 1986). This may result from differential expression but also from posttranslational modifications and should influence the functional activity of E4 proteins (Boiron et al., 1964).

Even papillomavirus types sharing tissue specifity reveal only limited homology in DNA sequences coding for E4 proteins (Doorbar et al., 1989). The HPV1 E4 protein has been identified as a zinc finger protein (Roberts et al., 1994). The functional consequences of this property are presently unknown.

E6 and E7 proteins are expressed in HPV-positive cancer cells. These proteins may cause immortalisation of human keratinocytes and of a number of other cell types. Those HPV types coding for E6 and 7 genes involved in immortalisation of tissue culture cells and found frequently in malignant tumours and are considered as high risk HPVs, contrasting an apparently low tumorigenic potential of other types, generally designated as low risk HPVs (zur Hausen 1986). E6, and E7 genes code for growth stimulating proteins, in particular, E6 and E7 of specific types are relevant for the progression to malignant growth.

Both proteins of high risk types co-operate in immortalisation and transformation (Hawley-Nelson et al., 1989; Munger and Phelps 1993). E7 proteins of these viruses, however, are able to transform established rodent cell lines by themselves. Similarly E6 represents an independent oncogene since it immortalises human mammary epithelial cells (Band et al., 1990).

In the late 1970s the first papillomavirus genome was successfully cloned in bacteria. This allowed investigation of the molecular biology of this group of viruses to begin. The genome of a number of papillomaviruses has now been sequenced. A most significant observation related to the function of the E6 was made initially by Werness et al. (1990), revealing the binding of the

cellular p53 protein to E6. This was followed by experiments showing this binding promotes the degradation of p53 mediated by the cellular ubiquitin proteolysis system (Scheffner et al., 1990; Huibregtse et al., 1995). P53 acts as a transcriptional activator by binding to specific DNA sequences (Kern et al., 1991) and is required for the growth arrest following cellular DNA damage (Kuerbitz et al., 1992; Lin et al., 1992). Cells without functioning p53 are not arrested appropriately in G1 and display genomic instability (Livingstone et al., 1992; Yin et al., 1992). The transcriptional activation by p53 induced after DNA damage is inhibited by HPV18 E6 (Gu et al., 1994).

E7 protein has an affinity to bind to the retinoblastoma protein. The binding releases the transcription factor E2F from pRB complexes, activating transcription of genes regulating cell proliferation (Bagchi et al., 1990; Bandara et al., 1991). Besides binding pRB, E7 proteins of high risk viruses associate with related proteins, such as p107 and p130, and with the protein kinase p33cdk2 and with cyclin A (Dyson et al., 1992; Tommasino et al., 1993). Recent studies demonstrate that E7 expression in NIH3T3 cells results in a constitutive expression of cyclin E and Cyclin A genes in the absence of external growth factors (Zerfass et al., 1995). Cyclin E activation requires the cd-2 domain, but not cd-1, whereas cyclin A activation requires both domains. The cyclin E activation precedes that of cyclin A. Obviously E7 overrides two Inhibitory functions restricting expression of cyclin E and cyclin A genes. Cyclin D1 expression is not affected by E7. The analysis of E7 mutants indicates that the activation of cyclins E and A co segregates with the ability of E7 to transform (Zerfass et al., 1995). Similarly, transcriptional activation by HPV16 E7 has also been reported from the adenovirus E2 (Phelps et al., 1988; Phelps et al., 1991) and B-myb promoters (Lam et al., 1994).

The L1 product has a molecular weight of 57 000 and is highly conserved among papillomaviruses. It is a protein that is rich in cysteine and which consist of covalently linked subunits that are resistant to disruption with detergents such as sodium dodecyl sulphate (SDS). The L2 product is larger (molecular weight 78 000), but it lacks cysteine and disrupts readily in SDS.

This important difference allows distinctions to be made between papillomaviruses of different species: antiserum to both L1 and L2 produce similar staining patterns in sections of warts, only anti-L1 serum will cross-react with other papillomaviruses. Jenson et al. in 1980 used disrupted BPV particles to produce a group-specific antiserum that has been available commercially for some time (Dako Ltd.) and which is used in many studies to detect HPV antigen (Bunney et al 1992).

1.7 REGRESSION AND IMMUNOLOGY

Skin warts usually regress spontaneously. It is generally stated that 35% of patients lose their warts within 2-6 months (Massing and Epstein, 1963; Bunney et al, 1992), 63% within one year, and 67% within two years. However, there are a number of people who have had warts for decades. The mechanism of wart regression has been the subject of much speculation and research. The spontaneous disappearance, frequently observed simultaneously for multiple warts, may indicate that wart rejection is due to humoral and/or cell-mediated immune reactions towards the wart cells. Numerous studies have been done by different groups of investigators on the humoral or cellular immunity to HPVs using various techniques (Tagami et al., 1974). HPV-specific antibodies seem to have rather less significance in the regression of warts, while there is considerable evidence that cell-mediated immunity plays the major role in the control of HPV infection (Chardonnet et al., 1985).

The incidence of warts increases during immunosuppressive treatment and in persons with cell-mediated immune deficiency. On the other hand, regression of warts has been reported after inflammation or treatment with Dinitrochlorobenzene and with a variety of immune adjuvants. Tagami and colleagues (Tagami et al., 1974) reported as early as 1974 the occurrence of mononuclear cell infiltration in the dermis and epidermis of regressing flat warts. This was confirmed by Berman and Winkelman (1977), who have shown similar changes in resolving common warts. *In situ* investigation of the

cellular immune response is another approach provided by the use of monoclonal antibodies directed against surface antigens of T Cells, B cells and Langerhans cells. It has been demonstrated that Langerhans cells in the epidermis play an important role as antigen carriers, elicitors, and targets of immunological reactions. In skin warts, Langerhans cells are markedly reduced in number, suggesting a decrease in immunological surveillance, which might induce tolerance to HPV (Chardonnet et al., 1985; Grussendorf-Conen, 1987). Involution of common warts, myrmecia and plane warts is associated with a mononuclear cell infiltration, exocytotis, and degenerative epidermal changes, indicating that regression represents a cell-mediated immune rejection of the warts.

1.8 CLINICAL SIGNS OF REGRESSION

A deep plantar wart in regression is dark or almost black and becomes painful. The edges sometimes peel slightly and the superficial part of the wart is dry. In advanced regression, the whole wart is dry and friable (Rasmussen, 1958). Common warts, however, usually shrink silently. Itching sometimes precedes resolution. They involute individually, in part probably due to an immune response of the host, but nearly always due either to simple occlusion of blood vessels in the tumour tissue or to changes in the keratinocytes, which are not favourable to the propagation of HPV (Grussendorf-Conen 1997).

Flat warts in normal people represent a dramatic, systemic, regression phenomenon based on the development of a cellular immune response of the host against HPV-transformed cells (Tagami et al., 1985). Before involution, the warts suddenly begin to enlarge. They become reddened and swell up, causing intense pruritus. In severe cases, extensive vesiculation may appear on the top of the warts. After 2-7 weeks, when the inflammation subsides with associated scaling and crusting, generally all warts have disappeared.

1.9 CLINICAL MANAGEMENT

Sterling et al. (2001) commented on the significance of no individual treatment regime showed complete effectiveness in wart clearance, and that overall regression may need to consider spontaneous regression due to normal viral regression and not due to treatment intervention. Sterling et al. (2001) claimed that long duration of infection in older patients and immunosuppressed patients lead them to be recalcitrant to treatment and unlikely to clear spontaneously.

Sterling et al (2001) defined 3 key aspects of an effective wart treatment: removal of wart without recurrence to induce life-long immunity and leave no scarring. The author defined categories that should be considered when treating warts. All warts do not need to be treated. Treatment should be carried out if the warts, give pain, are unsightly, have chance of malignancy or cause problems with day-to-day life. Consider treatment success rate prior to administrating treatment as average 60-70% clearance in 3 months. Immunocompromised individuals never show wart clearance as normally, as immune response is essential for clearance. Finally, best results of clearance occur in the young who have a short duration of infection (Sterling et al 2001).

Recalcitrant lesions may be defined as those, which fail to respond to treatment after at least one year. Wherever they arise they may represent a frustrating therapeutic experience for both practitioner and patient. A number of novel treatment modalities have been developed in the past two or three decades in attempts to produce more effective therapies and so reduce the incidence of persistence. These treatment regimes, which will be discussed later in the text, are rather different from the more commonly used methods and include immunotherapy, chemotherapy and wart ablation by means of electrosurgery or lasers (Bunney et al, 1992).

1.9.1 CHEMICAL THERAPIES

Strong acid, such as concentrated salicylic or nitric acids and mono- or trichloroacetic acids, were previously popular for treating skin warts but are now only seldom used and then only by experts familiar with their corrosive effects. This also applies to the use of phenol, silver nitrate, or copper sulphate crystals and to blistering agents such as cantharidin. Their effectiveness is no greater than that of gentler treatments and the likelihood and degree of discomfort and complications are greater. Extreme care must be taken when treating diabetic patients because of an increased danger of ulceration (Bunney et al, 1992).

Gibbs et al (2002) carried out a systemic review of local treatments for cutaneous warts, the findings towards salicylic acid showed thirteen trials assessed. Concentration of preparation used varied from 15% to 60%. Standard preparation was between 15% and 26% with or without lactic acid (Gibbs et al 2002). Cure rates of 75% (144 of 191) in placebo controlled trials compared with 48% (89 of 185) in controls (odds ratio 3.91, 95% confidence interval 2.4 to 6.36) (Gibbs et al 2002). The authors documented that cellulitis was seen in one person in a trial with monochloroacetic acid and 60% salicylic acid and minor skin irritation were occasionally seen but overall salicylic acid was not harmful. (Gibbs et al 2002).

Other preparations used in wart treatment include silver nitrate. Silver nitrate causes an "eschar", a change in the keratotic tissue leading to a black pigment. Yazar and Basaran (1994) carried out a double-blind study with 70 patients. The results showed a 43% (15) clearance rate with silver nitrate, whilst the placebo (black ink) showed 11% (4) were completely healed.

Virucidal methods such as formaldehyde and glutaraldehyde are also available to treat warts. Vickers (1961) showed 200 children with plantar warts were treated using 3% formaldehyde solution for a period of nought to eight weeks. The results showed 80% clearance of warts (Sterling et al 2001).

Similarly Hirose et al 1994 showed in an uncontrolled trial that 20% solution of glutaraldehyde applied daily cleared 72% (18 of 25) patients in three months(Sterling et al 2001). It was reported that the skin stained and signs of necrosis were present at doses of 20% applied solutions (Sterling et al 2001).

There are a number of additional treatments for viral warts but they should only be considered after standard therapy has failed. Some of these medications can only be prescribed in hospital clinics because close monitoring of the patient is necessary. In each case the possible benefits of treatment have to be weighed against the potential side effects. It is therefore important to know what each treatment entails so that the best advice can be given.

1.9.2 ANTIMITOTIC THERAPY

5-Fluorouracil is an anti-metabolite that causes local skin sensitisation leading to hypersensitivity reactions and activation of a immunological response to clear the wart (Bunney et al. 1992).

5 –Fluorouracil is often used with salicylic acid, but shows no difference in its activity (Bunney et al. 1992). Gibbs 2002 reviewed all trials carried out from the 1970s to the 1980s and found that treatments were not effective.

The antibiotic bleomycin seems to have gained favour in some quarters when administered intralesionally since it has an antitumour and antiviral action due to its ability to inhibit DNA synthesis in cell and viruses. Since it has an antitumour and antiviral action due to its ability to inhibit DNA synthesis in cell and viruses (Shumer and O'Keefe 1983).

Intralesional bleomycin cure rates ranged from 16% to 94%. Two trials showed bleomycin had a higher cure rate than placebo. Whilst another showed higher cure rate in the placebo. Adverse reactions with bleomycin were reported in 19 out of 62 (31%) of participants but information was not clear as to what the reactions were. Most patients had reported experiencing pain with application of bleomycin (Gibbs et al 2002).

Retinoids have showed applications in the treatment of warts as they have two key actions: they affect the regulation of keratinocytes and have a immunostimulating effect (Bunney et al. 1992). Kubeyinje and C'Mathur (1996) carried out randomised trials with 0.05% tretinoin cream on 25 individuals, their results were 85% clearance (Sterling et al 2001). Topical corticosteroids have a very similar effect, inhibiting cell division and DNA synthesis in the epidermis (Sullivan, 1984).

Podophyllin is a mixture of resins obtained from the rhizomes or roots of podophyllum also known as the Mayapple (Gross et al 1997). A powerful antimitotic agent podophyllotoxin is present within podophyllin, the extract found in the Unites States are different from those found elsewhere in the world, but the active compound remains the same. The onset of these effects is delayed for several hours, and for this only reason experts should apply podophyllin. Excess podophyllin absorption can cause severe toxic symptoms and can lead to fatality. It should be avoided during pregnancy. Adverse signs seen with podophyllin can cause swelling, erythemia, tenderness and burning. Preparation of podophyllin are used at concentration of 25% solution in spirit. They are often mixed in liquid paraffin (Bunney et al. 1992). Kraus and Stone 1990 reported that cure rates seen with podophyllin range were between 22% to 77% with a mean of 50%, but the reoccurrence rate was seen to be 74% (Bunney et al. 1992).

Podophyllotoxin is the most active of the lignans and studies have shown that podophyllotoxin alone gives the same therapeutic results as the crude podophyllin resin. Its action is to bind to tubulins, which are essential for mitotic cell division, preventing tubulin polymerisation into microtubules (Loike and Horwitz 1976) (Mansono-Martinez 1982). Biological effects include mitotic arrest of eukaryotic cellular division in metaphase but also nucleoside transport inhibition (von Krogh and Maibach 1982).
In a number of single blind prospective trials comparing patient-applied podophyllotoxin with podophyllin resin, podophyllotoxin shows superior efficiency (Gross et al 1997).

Mazurkiewicz and Jablonska (1990) reported wart clearance in 68% (17 of 25) of men treated with 0.5% podophyllotoxin solution, 38% (7 of 24) of resin-treated patients after one to two courses. In most of these cases there were cases of local reaction and sensitivity to applied areas but considerably lower than those treated with podophyllin (Gross et al 1997).

Von Krogh (1982) formulated the concept of patient-applied low dose podophyllotoxin. The rationale behind this proposition was to decrease local tissue reactions, to minimise the dose of drug thus eliminating the potential for systemic reactions, and to increase efficacy.

Specific antiviral drugs have also been attributed with some success: Imiquimod, a patient-applied topical 5% cream has been found to be clinically efficacious and safe in the management of condylomata acuminata and other warty manifestations of human papillomavirus infections. It is an imidazoquinoline, a novel synthetic compound which is an immune response stimulator, enhancing both the innate and acquired immune pathways (particularly T helper cell type 1-mediated immune responses) resulting in antiviral, antitumour and immunoregulatory activities. The mechanism of action of imiquimod involves cytokine induction in the skin, which then triggers the host's immune system to recognize the presence of a viral infection or tumour, ultimately to eradicate the associated lesion (Garland 2003).

Another area of development in HPV infection treatment are vaccines. Virus-like particles (VLP) subunit vaccines composed of the major capsid protein L1 are in experimental stages. They are so far shown to be safe and immunogenic and early results show efficacy. These vaccines induce strong cell-mediated and humoral immune responses and may have therapeutic potential (Stanley 2003).

1.9.3 IMMUNE STIMULATION

Immunotherapy with universal allergic contact sensitisation diphencyprone (DCP) has been successful in the treatment of recalcitrant viral warts (Buckley et al 1999). In a study carried out, it was reported that patients sensitised with DCP 88% (42) were cleared of all warts over a period of 5 months. Adverse effects occurred in 27 of 48 patients (56%), most commonly blistering and eczematous eruptions (Buckley et al 1999).

Rosado-Cancino et al (1989) reported wart trials with dinitrochlorobenzene vs placebo showed efficacy with active ingredient. The data showed cure rates of 80% (32 of 40) and 38% (15 of 40) respectively. No precise data was found on adverse effects (Gibbs et al 2002).

1.9.4 CRYOTHERAPY

Cryotherapy is widely used by dermatologists to treat a variety of benign and malignant skin lesions including viral warts. Such treatments have been employed since the nineteenth century when salt and ice mixtures were used to alleviate pain as well as to treat superficial skin infections and tumours. Currently three main freezing agents are employed: liquid nitrogen, which boils at -195.8°C, carbon dioxide snow (-79°C), and nitrous oxide gas (-70°C), which is used as a refrigerant in closed probe systems. The exact techniques used and the freezing times vary on depending on the agent and the nature of the lesions. Like other viruses and bacteria, human papilloma virus is resistant to the effects of cryotherapy of warts, therefore depends on primarily damage to, or destruction of, host cells (Benton et al., 1992).

The effect of cryotherapy on wart clearance is demonstrated by necrotic destruction of keratinocyte infected HPV and cell-mediated response induced by local inflammation (Sterling et al 2001).

Gibbs et al (2002) reported 16 trials using cryotherapy. The pooled data from two trials showed no significance in cure rates between active treatment

versus placebo. Two large trials showed no significant difference between cryotherapy and salicylic acid.

Pooled data demonstrated that "aggressive" cryotherapy methods (various definitions) were more effective than "gentle" cryotherapy. Cure rates of 52% (159 of 304) and 31% (89 of 288) were seen respectively. Reports of adverse effects were pain and blistering noted in 64 out of 100 (64%) (Gibbs et al 2002). Three trials examined optimum treatment intervals. No significance was seen between 2-4 weekly intervals, higher rates of adverse effects were seen with shorter intervals (Gibbs et al 2002).

1.9.5 SURGICAL PROCEDURES

The surgical removal of a wart is frequently demanded by patients under the mistaken impression that if a wart is "cut out", it will be the end of the matter. Removal of all visible wart tissue does not however necessarily eradicate all the infected cells and recurrence following surgical removal is high.

Morison (1975a) concluded that the removal of the bulk of the viral antigen could be responsible for an absence of an immune response following surgical removal. In addition, a real drawback to surgical excision is the resultant scarring, which itself is often cosmetically unacceptable and sometimes incapacitating if it occurs in sites such as the fingertips or on the weight bearing areas of the foot. Subsidiary arguments against the surgical removal of warts for all but exceptional cases are the need for anaesthesia to control postoperative pain, possible secondary infection, and inconvenience. Whilst actual excision is not advised, warts can be removed by curettage with or without cauterization, cauterization alone, by electrodesication or by laser (Bunney et al 1992).

Gibbs et al (2002) identified that no randomised trials to study carbon dioxide, laser, surgical excision, curettage or cautery, formaldehyde, podophyllotoxin or podophyllin have been carried out, leading to anecdotal evidence of their effectiveness.

Street and Roenigk (1990) and Sloan et al (1998) demonstrated 64% to 71% cure rates of individual warts in 12 months but post-operative pain and scaring may be possible (Sterling et al 2001).

Other methods include photodynamic therapy, which involves uptake of a chemical e.g. amino-laevulinic acid (ALA), by abnormal cells. This is followed by photo-oxidation from irradiation by a laser or non-laser light of affected tissue (Sterling et al 2001).

Gibbs et al (2002) identified a trial by Stender et al (2000). 40 adults reported 56% cure of warts treated with ALA photodynamic therapy compared with 42% treated with placebo photodynamic therapy. Adverse effects of ALA treatment reported during the trial included burning and itching (Stender et al 2000).

1.9.6 COMPLEMENTARY MEDICINES

The recognition of warts can be traced back as early as the times of the ancient Greeks and Romans (Bafverstedt 1967). Warts or *condyloma* as known by the Greeks were well known. They also coined the term *myrmecia*, a term still in use to describe painful plantar warts, which was derived from the Greek word for an anthill. Genital warts were known as *thymia* due to their resemblance to wild thyme leaves or more commonly as *ficus*, meaning figs. (Oriel 1997).

The Roman author Celcus (AD25) wrote about three types of warts in his *De medicina*: myrmeciae, *thymia* and *acrochordon*, which most often affected children and were usually multiple, these may have been referring to common or maybe filiform warts (Oriel 1997).

As well as the widely used term verruca, from the Latin for a hill, which was coined by a German physician, the word condyloma is still in use today, the other terms were more or less out of use by an early date (Oriel 1997).

There were many different types of methods to treat warts throughout the ages. Celsus for example, suggested burning warts off. Whereas a fellow Greek, Galen, tried to rid the problem by sucking out and biting of the warts from the patient's feet. In the eighteenth century Daniel Turner wrote extensively about curing warts by using everything from the extracts of plant juices, medicinal plasters, cutting them off to burning them and could not understand why there were still so many sufferers (Bunney et al 1992).

The treating of warts has been historically linked to the use of folklore myth and magic throughout the world. It was reported for example that Sir Francis Bacon used the fat from pork to rub on his warts and watched them melt away in the sun (Bunney et al 1992). In spite of all these interesting tales, the clinical management of warts has only been made possible recently due to being unable to classify the entire human papillomavirus (HPV) (Bunney et al 1992).

The only common denominator in all those methods is an intensive therapeutic approach to the patient's warts. Every therapeutic procedure implies a suggestive component (Vollmer, 1946) and it is therefore reasonable to assume that with the exception of radiotherapy and surgery, all methods used for the treatment of warts act mainly as suggestion. Hellier (1951) deduced from his survey into the therapeutic effects of fractional doses of X-rays that any benefit obtained could only be the result of suggestion.

According to Rulison (1942) the average duration of untreated warts is between 2 and 3 years. Following treatment by suggestion, the majority of warts disappear within 2-12 weeks. Since spontaneous regression is seen to be very similar morphologically an acceptable theory is that suggestive therapy accelerates the process of spontaneous healing.

The link between local hyperaemia and regression has already been mentioned and the effect of psychic influences on capillaries is a known fact. It is assumed that treatment by suggestion brings about an emotional reaction

and a psychogenic impulse, which is in some manner linked to and associated with that part of the body, which is being treated (Rulison 1942).

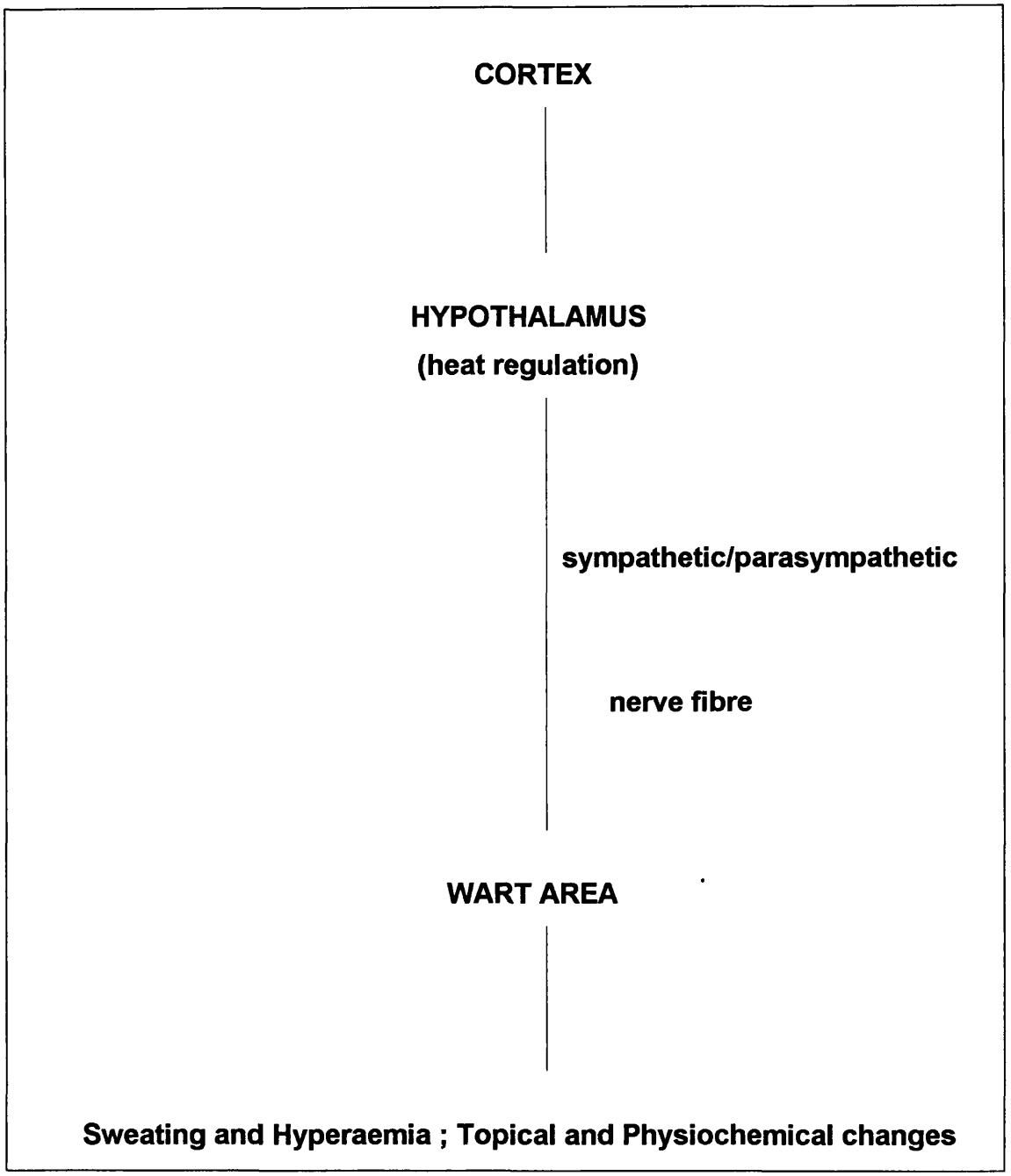


Figure 8. The influence of the nervous system in wart regression.

The connection between allergy (i.e. reactions involving histamine release) and psychology is well known to dermatologists, as in asthma-hayfever-eczema and thus could be the reason for dermatology being one of the first branches of medicine to recognise officially the curative powers of suggestion. In 1955 the BMA officially recognised hypnosis and hypnotic suggestion as a legitimate therapeutic technique in the treatment of warts and has been shown to have a good effect in Ullman and Dudek's report in 1960.

Table 2. **Cure rates of warts using suggestion therapy (Massing & Epstein, 1963).**

Therapists	Suggestion Therapy	% Rate (months) 1	2	3	4
Bloch	Untreated	1%	2-3%	4%	6%
	Treated	24%	39%	-	44%
Memmesheimer &	Untreated	3%	7%	-	29%
Eisenlohr	Treated	16%	20%	-	24%
Massing & Epstein	Untreated	-	11%	13%	24%

However, results seem to depend upon the convincing approach of the therapists, the creation of a certain treatment atmosphere and the "suggestibility" of the subject.

Boericke's Materia Medica with Repertory (1927), lists several preparations, which can be used in the homoeopathic treatment of warts, including *Thuja*, sulphur, causticum and ant.crudum. Prescription depends upon the symptomatology and includes Nitricum Acidum (Nitric Acid), which is given for exuberant "warty" excrescences and is said to select the areas where skin and mucous membranes meet as its preferred site action. Incidentally this is also the area where viral particles are nowadays known to proliferate and nitric acid is an established conventional wart therapy.

Many such examples of preparations, which have recognition in both medical and Homoeopathic practices can be found. Other more innovative modes of alternative therapy are becoming increasingly favoured nowadays; one example in Vaga therapy (or electro-acupuncture). This begs comparison with the use of mild electric currents (or faradization) in suggestion therapies.

The rationale for use of *Thuja occidentalis* (*abor vitae*) in wart treatments is well established in homeopathy. Its main action is on the skin and genito-urinary organs producing "fig-like warts and condylomata" and it is said to

have specific antibacterial action and vaccination effect in viral skin infections of this nature (Boericke, 1927).

Personal observation of wart treatment has lead to anecdotal evidence of the medicinal properties of plants. These include the use of garlic, lemon juice, banana skin and tea-tree oil, all of which have some success but have not been researched further.

1.10 THE GENUS *THUJA*

There are four species of *Thuja* found in China, Japan and North America: *Occidentalis*, *Orientalis*, *Plicata* and *Standishii* (Bloom, 1972).

Thuja occidentalis known as the White Cedar or American *Arbor Vitae* originates from the Eastern United States from Nova Scotia in the North, to Tennessee in the South. It is usually columnar in appearance, reaching its maximum height of 20m. The branches are sprawled, with flattened branchlets and spray-like leaves. They have conspicuous resin glands, dark green on the upper surface, pale green underneath and turning bronze in winter. *Thuja occidentalis* is a native of North America. It has a coniferous pyramidal appearance, with flattened branches, the twigs in one plane bearing small scale like leaves (Bloom 1972).

Thuja orientalis, the Chinese Arbor Vitae, is considered a distinct genus and bears the generic name Biota. A large rounded conical tree which reaches a height of 10m. The branches and branchlets are erect, with flattened sprays bearing small green leaves. These leaves are the same colour on both sides. The species is further distinguished by being less aromatic than the other *Thuja*'s and by the recurved cone scales (Bloom 1972).

Thuja plicata, the "Western Red Cedar" is one of the most important timber trees in North America, but it is also of great ornamental value, being widely used for screens and hedges. It is a fast growing pyramidal tree with

cinnamon red or brown shredding bark. The leaves are a mild shining green above, with whitish markings beneath, borne of large flattened sprays. The foliage has a very pleasant odour when crushed (Bloom 1972).

Thuja standishii, the Japanese Arbor Vitae, also known as *Thuja japonica*, is a small to medium size tree in cultivation, although in its natural state in Japan it reaches 35m. Much slower growing than *Thuja plicata*, the species it most closely resembles, it differs by having larger and broader leaves, the branchlets being mostly flattened, the base is a broad pyramid, the leaves are dark green above and conspicuously silver-white beneath (Bloom 1972).

Figure 9. Photos of *Thuja* Species – Bloom, 1972

Thuja occidentalis 'Holmstrup' – A 10 year old specimen.

Thuja orientalis 'Aurea Nana' – A 10 year old specimen.

Thuja plicata – A 10 year old specimen.

Thuja Standishii – A 10 year old specimen

1.11 PHYTOCHEMISTRY OF *THUJA OCCIDENTALIS*

Thuja contains a volatile oil, flavonoids, mucilage and an astringent. *Thuja* extracts stimulate nerves, uterus and heart muscles in addition to having expectorant, diuretic, an astringent and counter irritant properties. *Thuja* is used to treat respiratory tract infections together with antibiotics in the treatment of bacterial skin infections and *Herpes Simplex*. Other possible uses include the treatment of bronchitis, rheumatism, trigeminal neuralgia and streptecoccal tonsillitis. The drug is used externally as an ointment for arthralgia, arthritis and muscle rheumatism. In general the drug is toxic. The toxicologically harmless polysaccharide and glycoprotein fractions of the drug are usually the parts used for therapeutic purposes (PDR, 1998).

Chang et al. (2000) evaluated the bioactive constituents of *Thuja occidentalis*. He found that an ethyl acetate-soluble extract of the combined leaves and twigs of *Thuja occidentalis* was found to inhibit 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced ornithine decarboxylase (ODC) in cultured mouse

epidermal ME 308 cells. Bioassay-guided fractionation of this extract led to the isolation of six active constituents (*1-6*), namely (+)-7-oxo-13-epi-pimara-14,15-dien-18-oic acid (*1*), (+)-7-oxo-13-epi-pimara-8, 15-dien-18-oic acid (*2*), (+)-isopimaric acid (*3*), (1S,2S,3R)-(+)-isopicrodeoxypodophyllotoxin (*4*), (-)-deoxypodophyllotoxin *(5*), and (-)-deoxypodorhizone (*6*). Compounds *1* and *4* are new natural products, and their structures and stereochemistry were determined using spectroscopic methods. Compound *1* is a diterpene acid and the structure determined was (+)-7-oxo-13-epi-pimara-14,15-dien-18-oic acid (Chang et al., 2000). Compound *4* showed a molecular ion peak at m/z 398.1366, indicating a molecular formula of $C_{22}H_{22}O_7$. The ^1H and ^{13}C NMR spectra of this isolate were similar to those of the known lignan, (-)-deoxypodophyllotoxin. Compounds *1-6* were evaluated for inhibition of the transformation of murine epidermal JB6 cells, inhibition of ornithine decarboxylase induction with murine epidermal ME 308 cells, and cytotoxic activity against KB cells.

Tanaka et al. (2000) isolated seven labdane-type diterpenoids from the stem bark of *Thuja standishii* (Gord.) Carr. (*Cupressaceae*) and their analogues showed strong inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). Among these compounds, 15,16-bisnor-13-oxolabda-8(17), 11E-dien-19-oic acid was revealed to have the strongest inhibitory effect on the EBV-EA activation, being stronger than that of beta-carotene which has been intensively studied in cancer prevention using animal models. 15,16-bisnor-13-Oxolabda-8(17), 11E-dien-19-oic acids were also found to exhibit the excellent anti-tumor promoting activity in two-stage mouse skin carcinogenesis test using 7,12-dimethylbenz[a]anthracene and TPA.

Hassan et al. (1996) showed that a polysaccharide of *Thuja occidentalis*, *Thuja* polysaccharide g (TPSg), an anti-human immunodeficieny virus (HIV-1) agent, it enhances in vivo haemopoetic progenitor cells recovery, in sub lethally eradicated mice (in vitro). Gohla et al. (1988) had earlier reported the effect of TPSg and were shown to be an inducer of the CD4+ fraction of the human peripheral blood T-cell subset. Furthermore, it could be demonstrated

that TPSg is a potent inhibitor of the expression of HIV-specific antigens and of the HIV-1 specific reverse transcriptase.

Gohla et al. (1988) reported that the arborvitae or *Thuja occidentalis* L., one of the *Cupressaceae*, has rarely been investigated until now. Several authors have demonstrated that allopathic extracts of this plant could be used as strong antiviral agents directed against plant and animal viruses. Polysaccharide fractions with molecular weights ranging between 20,000 and 1,000,000 and higher have been isolated from the aqueous alkaline extract of the herbal parts of T. *occidentalis* L. by ethanol precipitation and fractionation by ultra filtration. High molecular subfractions of Thujapolysaccharides (TPS) proved to be highly mitogenic on peripheral blood leukocytes. The alcalic immune phosphatase-antiphosphatase and Pappenheim staining methods that the mitogenic and cluster-forming activity of TPS cause T cell induction, in particular, of CD4-positive T-helper/inducer cells as opposed to B cells demonstrated it. The CD4+ T-helper/inducer cell induction is connected to an increased production of IL-2.

The cluster-forming ability and mitogenity of TPS correlates well with the 3H-thymidine uptake and seems to be IL-1 and IFN-gamma dependent as could be shown by blocking the mitogenic effect using anti-IL-1- and anti-IFN antibodies.

Whether it is possible to use these polysaccharide fractions as an adjuvant in the therapy of immune deficiency syndromes and cancer must now be further investigated.

Roth (1993) isolated a lignan called deoxypodophyllotoxin from *Thuja Occidentalis* and demonstrated that it has an antiviral effect on the Influenza virus *in vitro*. Beuscher and Kopanski (1986) demonstrated, using the plaque-reducing assay with HSV-1, that an extract of *Thuja occidentalis* had an antiviral effect on Herpes Simplex (I) Virus (*in vitro*).

Offergeld et al. (1992) reported that *Thuja* polysaccharide g fraction (TPSg) was shown to be an inducer of the CD4+ fraction of the human peripheral blood T-cell subset. Furthermore, it could be demonstrated that TPSg is a potent inhibitor of the expression of HIV-1-specific antigens and of the HIV-1-specific reverse transcriptase. This report deals with the cytokine pattern induced by TPSg in human peripheral blood lymphocytes (PBL) and purified monocyte/macrophage cultures. In addition, a further characterization of the CD4+ T-cell fraction stimulated by TPSg was performed by FACS analysis. TPSg induces IL-1 beta, IL-2, IL-3, IL-6, gamma-IFN, G-CSF, GM-CSF, and TNF-beta production in PBL cultures; and IL-1 beta and IL-6 in monocyte/macrophage cultures. Enzyme-linked immunosorbent assays (ELISAs) demonstrated that no IL-4 was produced by PBL cultures under TPSg influence.

Sharma et al. (1990) demonstrated in vitro antimicrobial effects of *Thuja* leaf extract on gram positive and negative bacteria and fungi. Guerin et al. (1996) reported the effect of allergic rhinitis caused by *Thuja occidentalis*. Grimm (1991) reported allergic contact dermatitis from *Thuja occidentalis* extract.

Gibson et al. (1986) carried out double-blind investigations in rheumatoid arthritis using homoeopathically prepared *Thuja* with successful results in reduction of inflammation around joints. Pay (1980) observed that homoeopathic *Thuja* with phosphorus and calcarea carbonate helped to reduce high serum cholesterol levels, with the *Thuja* having been tried on 53 patients suffering with hypercholesterolaemia.

Sehgal (1967) demonstrated that when *Thuja occidentalis* was taken internally homoeopathically and topically applied in an herbal form, he found an antibacterial activity on warts and verrucae. Khan (1994) carried out a trial to evaluate a preparation of Tagetes *signata*. He found this to be an effective treatment of verrucae pedis in children and adults with a clearance rate of 80% in a period of three months.

These initial studies have paved the way to further evaluate phytotherapy preparations in the treatment of viral warts. It is hoped that *Thuja occidentalis* will have a beneficial therapeutic effect, which intern lead to the discovery of normal therapeutic targets for future Chemotherapy.

1.12 AIMS OF THE PRESENT STUDY

1. To extract, separate, purify and characterise the chemical components of *Thuja occidentalis* using thin layer, column and paper chromatography and to identify the isolated compounds using proton nuclear magnetic resonance spectroscopy and mass spectroscopy.

2. *In vivo*: to investigate the effect of *Thuja occidentalis* crude extract and fractions in the treatment of verruca pedis.

3. *In vitro*: to investigate the effect of *Thuja occidentalis* crude extract and fractions on human epidermal keratinocytes infected with human papilloma virus and to compare with the activity on normal cells. To evaluate the mode of action of *Thuja* extract and fractions at cellular level.

4. To correlate the results obtained *in vivo* and *in vitro* and compare statistically for significant differences.

Chapter 2 -

Botanical Investigation and Isolation, Purification and Chemical Characterisation of Components of *Thuja occidentalis*

2.1 INTRODUCTION

Although the *Thuja* genus is relatively small, it has produced many fine garden plants. In many ways they are quite similar to Chamaecyparis (a conifer), which is originally from North America and Japan and is widely grown and planted in Western Europe, forming shrubs or trees of mainly conical habit and reproducing freely from seed. The tendency for many clones of similar appearance to get into general cultivation has produced similar problems of horticultural identification to the Chamaecyparis. Three species of *Thuja*; *occidentalis*, *orientalis* and *plicata* have given us useful garden forms. They all have certain characteristics in common, flattened branchlets and scale-like overlapping leaves. One or two species have distinctly aromatic or pungent foliage, the choice of words to describe this is usually dependant upon individual tastes (Bloom, 1972).

The *Thuja*s are adaptable to most soils, it grows more successfully where there is adequate moisture and good drainage. *Thuja orientalis* is the only species which could not be considered completely hardy in the coldest climates. Because this conifer also dislikes exposed positions and drying winds, it can sometimes be difficult to transplant. Although hardy in Great Britain it will not normally survive Scandinavian winters nor above zone 6 in the United States. Two species, *occidentalis* and *plicata* make excellent hedges. The considerable variety of sizes and colours represented make the *Thujas* an invaluable genus for inclusion in any garden (Bloom, 1972). The name *Thuja* is applied indiscriminately to several species: *orientalis*, *plicata*, *standishii* and *occidentalis* (Bloom, 1972). For the purpose of this study, *Thuja occidentalis* has been investigated.

Figure 10. *Thuja occidentalis*.

Table 3.The botanical nomenclature of *Thuja occidentalis* is described as:

Kingdom	Planta
Phyla	Phaenoegamous
Class	Monocotyledonae
Order	Apetalous
Tribe	Coniferae
Family	Cupresacease
Genus	*Thuja*
Species	*occidentalis*

2.2 MACROSCOPIC AND MICROSCOPICAL FEATURES OF *THUJA OCCIDENTALIS*

2.2.1 AUTHENTICATION OF PLANT MATERIAL

A sample of *Thuja* was obtained from the School of Pharmacy Garden, Enfield, North London, and identified by the Royal Botanic Gardens, Kew, London as the "*occidentalis*" species in comparison with other *Thuja* species, e.g. *standishii*, *plicata* and *orientalis*.

The fresh leaves of *Thuja occidentalis* when rubbed between the fingers produce a powerful balsamic odour. One-year shoots show little lignification and branch widely. The small scale leaves are in four rows and lie close to the stem. On young trees they are narrow and linear, on mature trees broadly triangular, lying close to the stem like overlapping roof tiles with little or no convexity, lighter in colour without the whitish lines that mark the stomata. The dorsal and ventral leaves each have a dorsal resin gland and the lateral leaves do not. The leafy branches are dark green on top and significantly lighter in colour on the underside (British Herbal Pharmacopia, 1974).

2.2.2 MACROSCOPICAL FEATURES

Stem, Leaves, and Root (Figure 11-13): The plant is a narrowly clavate, 12 to 21m high tree with short horizontally spread branches and red-brown, striped, peeling trunk. The trunk is usually branched from the base up. The leaves are scale-like, crossed opposite, imbricate, flattened on the branch side and folded at the margins. They are dark green above and matt-green beneath. The scales on the upper part of the branches have a globular glandular swelling (PDR, 1998).

Thuja occidentalis has small lengthened and flattened, green twigs, bearing paired, decussate leaves about 3mm long, the keeled alternately lateral pairs partly concealing the flat rhomboidal, facial pairs which are pressed close

together. Not more than 2% of the stems are over 4mm in diameter. The odour and taste are strongly camphoraccous (PDR, 1998).

Flower and fruit: The male flowers are dark brown, the female flowers are yellow-green. They are monoecious, the male in small, terminal catkins and the female almost star-shaped. The ripe cones are brown-yellow, 6 to 8 nm long, ovate and covered in coriaceous, obtuse scales. The lower ones are patent at the tips. The seeds are brown-yellow, 3 to 5 mm long and approximately 1 mm wide. They are narrow winged the whole way around (PDR, 1998).

2.2.3 MICROSCOPICAL FEATURES

Microscopical features include elongated epidermal cells. These are lignified in parts and are covered with a crusty cuticle.

For the purpose of identification of image structures present in *Thuja occidentalis*, scanning electron microscope (Phillip XL20) and the technique described by Rudall (1994) was carried out by Dr D McCarthy, Department of Electron microscopy, School of Pharmacy. The stomata, as shown in Figure 14 and Figure 15, are typically anomocytic. Like the epidermis, the stomata are lignified. Their shape may be described as broadly elliptical and large, being sunken beneath the subsiding cells.

This represents the microscopy for *Thuja* species with each species resembling each other. The purpose of this was to confirm the structures present in *Thuja* and clearly identify *Thuja occidentalis*.

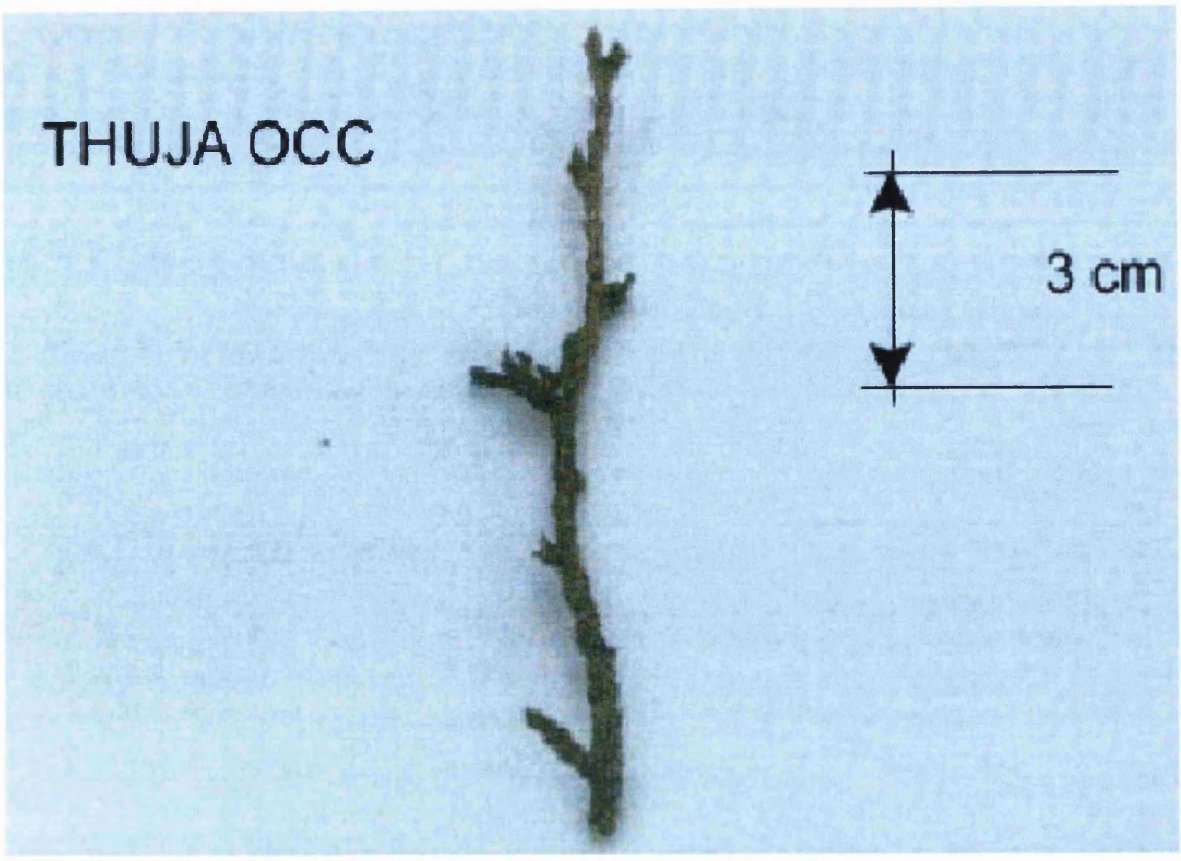


Figure 11. Stem of *Thuja occidentalis.*

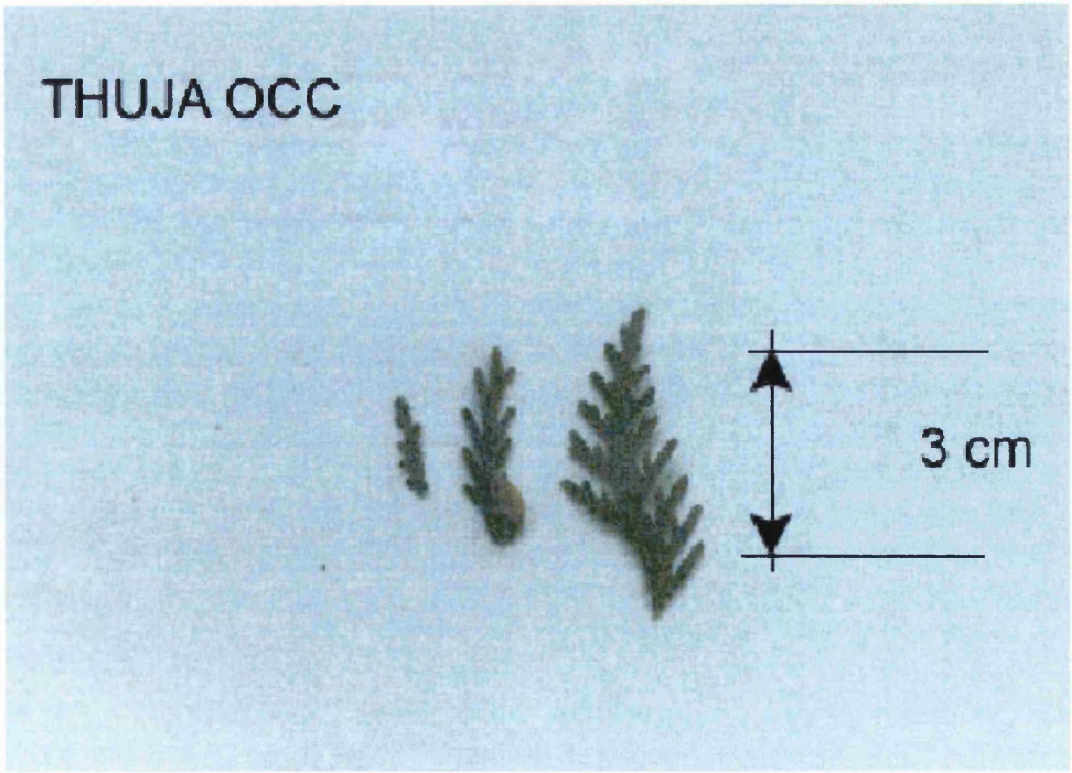


Figure 12. Leaves of *Thuja occidentalis*.

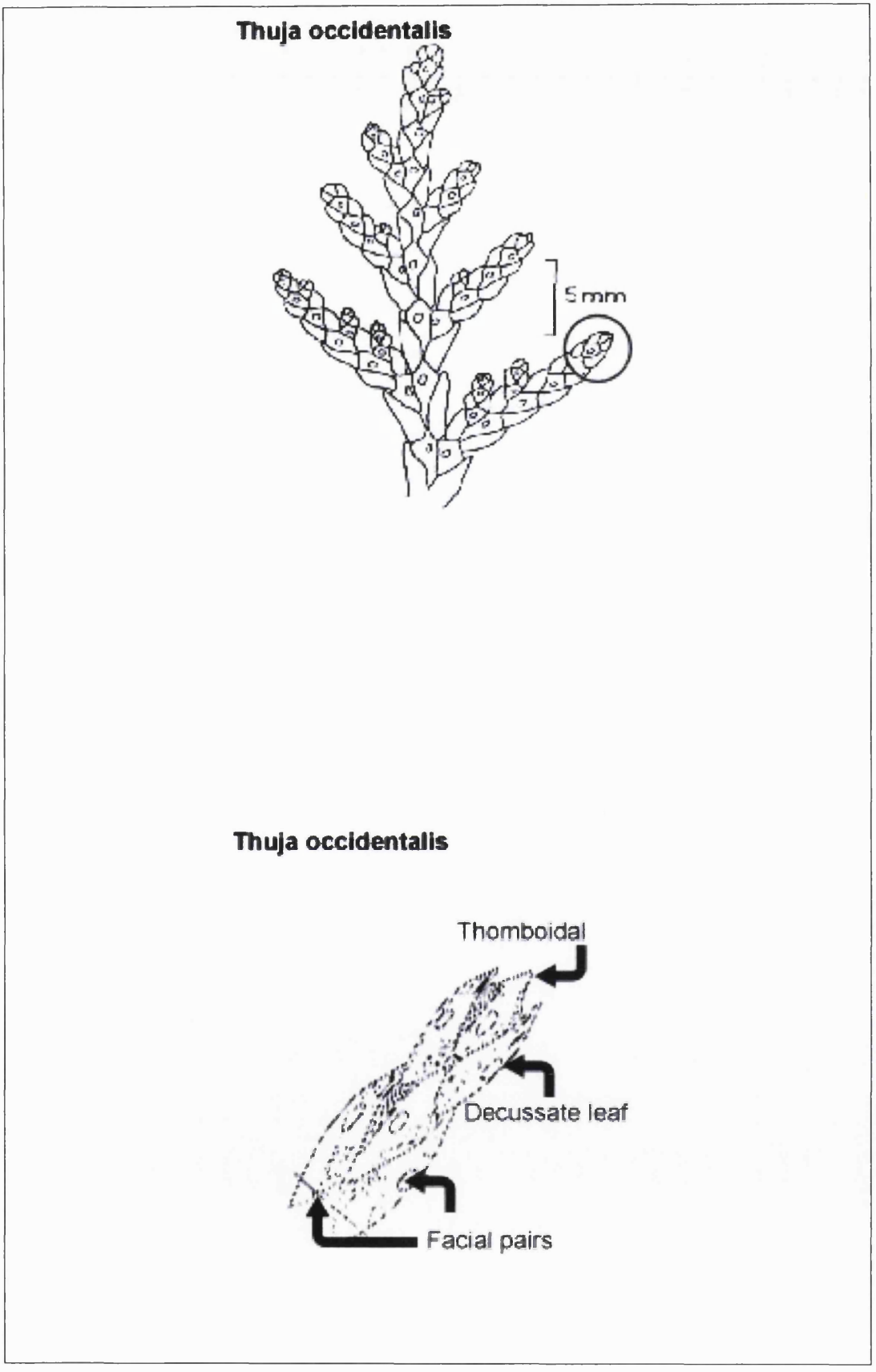


Figure 13. Macroscopical diagram / view of *Thuja occidentalis*.

2.2.3.1 ELECTRON MICROSCOPY

Nikon photo microscopy was used to identify the stomata in fresh leaves of *Thuja occidentalis*.

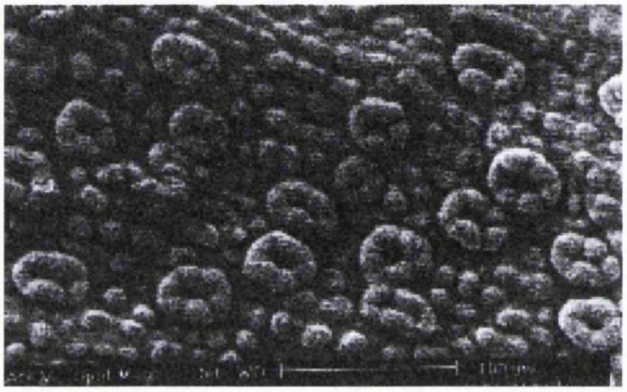


Figure 14. Stomata x 231 magnification *Thuja occidentalis* showing a standard measurement bar of 100μm.

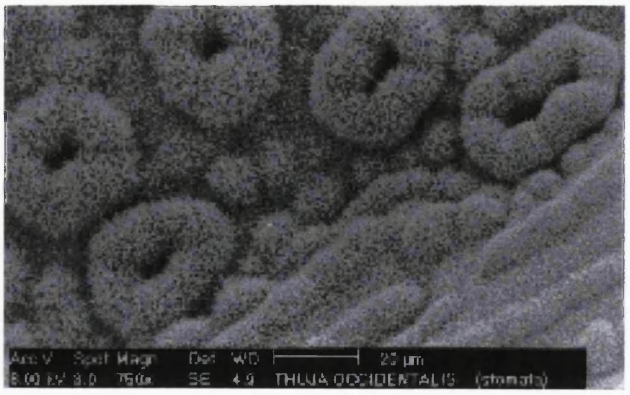


Figure 15. Stomata x 750 magnification *Thuja occidentalis* showing a standard measurement bar of 20μm.

2.3 INITIAL METHODS AND PROCEDURES

2.3.1 PLANT MATERIALS

A *Thuja occidentalis* tree was cultivated in the School of Pharmacy garden at Myddleton House, Enfield. Cuttings were collected between April to September and brought to the laboratory, separated, washed and air dried at normal room temperature. Leaves were ground using a Moulinex coffee grinder.

2.3.2 TECHNIQUES FOR ISOLATION AND IDENTIFICATION OF CHEMICALS IN PLANT MATERIALS

There are a number of well-established chromatographic and spectroscopic techniques for phytochemical analysis. Extraction of plant material is by means of maceration or Soxhlet extraction depending on the nature of the chemical constituents (Markham, 1975; Harborne and Williams, 1975).

All solvents, reagents and chemical were supplied by BDH Ltd or Sigma Ltd, unless otherwise stated in text and are listed in Appendix 4.

2.3.3 EXTRACTION PROCEDURES

Extraction of *Thuja* was carried out initially to provide samples for evaluation in clinical studies and then to separate and identify the active components using phytochemical analysis.

Two principal schemes were used for extraction of *Thuja occidentalis* leaves and bark. Both depended on an initial warm extraction of the plant material with 70% aqueous ethanol in a soxhlet continuous extraction apparatus. In flowchart 1 (Figure 16) the crude extract was then partitioned in different

organic solvents and samples used in the clinical evaluation. The clinical evaluation took the form of a pilot study with 30 individuals and a dose of 300mg/ml of *Thuja* extract, in a solution of 70% ethanol, was prepared for daily administration to the verruca lesion over a three month period. Flowchart 2 (Figure 17) shows the procedure for isolation and identification of the chloroform fraction of *Thuja occidentalis.* The chloroform fraction was separated using two different techniques, column and preparatory silica gel plates. The fractions were separated, then were further purified using Sephadex and identified using NMR and mass spectrometry.

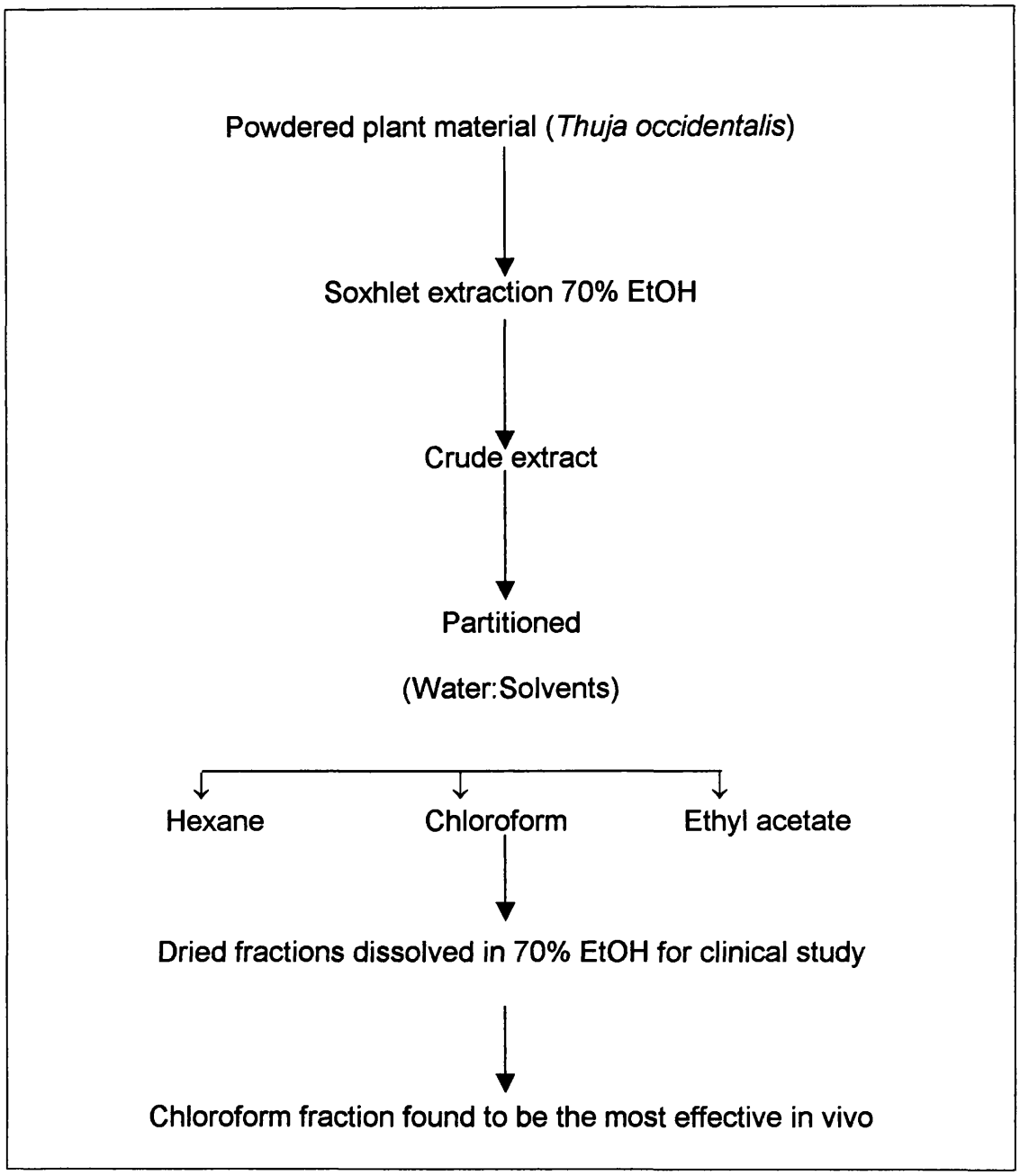


Figure 16. Flowchart for extraction of plant material by partition

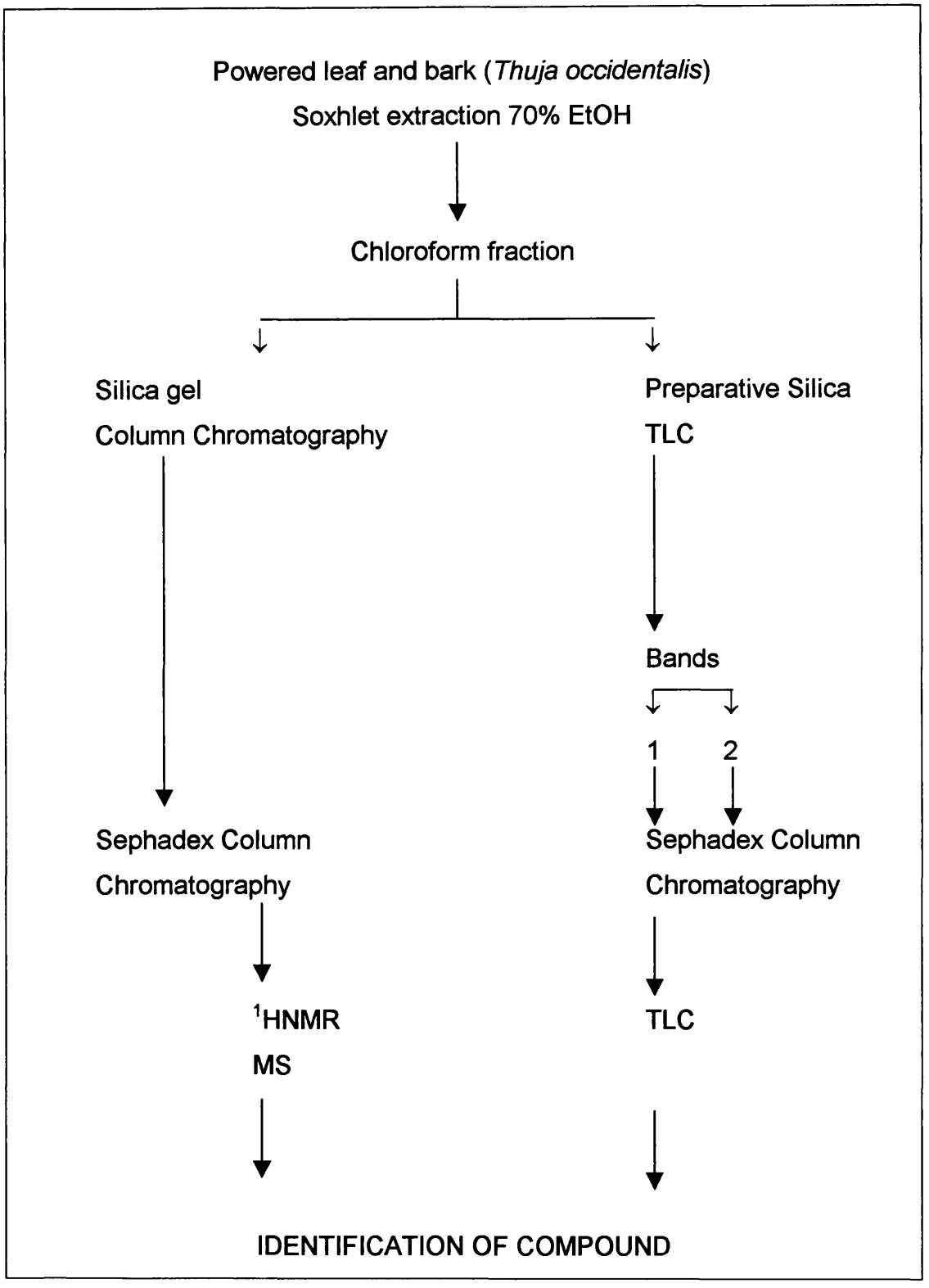


Figure 17. Flowchart for identification of plant material by column chromatography.

2.3.3.1 EXTRACTION OF *THUJA OCCIDENTALIS*

Powdered *Thuja occidentalis* (50g) was extracted in 6 litres of 70% ethanol (EtOH) in Soxhlet extraction until exhaustion. The process was repeated eight times. Each extraction time was extended to 12 to 14 hours at 65°C when the extract was judged to be complete. The extracts were allowed to cool and then evaporated under reduced pressure at 40°C, using a rotary evaporator. The remaining water residues were dried on an evaporating dish over a water bath at 65°C. The crude extract of *Thuja occidentalis* yielded a sticky, green residue. Samples were collected, yields determined and dried under nitrogen and stored at -5°C for further studies. The total of crude *Thuja* extract was 82.19g from 400g of *Thuja* raw material. The crude extract of *Thuja*, yielded a mean of 10.27g per 50g from the *Thuja* raw material. (Data is presented in Table 4).

Table 4. **Soxhlet extraction of crude extract of *Thuja occidentalis* leaf and bark**

Number	**50g x 8 = 400g leaf & bark yield (g)**
Total	82.19g
Mean	10.27g

The ethanolic crude extract of *Thuja occidentalis* (20g) was dissolved in an equal volume of distilled water and hexane (500ml) in a separatory funnel and partitioned. The hexane layer was separated and the extraction process repeated with all three further aliquots of fresh hexane. The combined hexane fractions were dried with a small amount of anhydrous sodium sulphate, filtered and dried under reduced pressure at 40° on a rotary evaporator. The whole extraction process was repeated a further three times, yielding a total of 24.43g of the hexane fraction per 80g of *Thuja* crude extract, with a mean of 5.51g per 20g of *Thuja* crude extract.

As each of these extraction processes was completed, the aqueous layer was partitioned with equal volumes of chloroform. The chloroform layer was separated and the extraction process repeated with all three further aliquots of fresh chloroform. The combined chloroform fractions were dried with a small amount of anhydrous sodium sulphate, filtered and dried under reduced pressure at 40°C on a rotary evaporator. The whole extraction process was repeated a further three times with the consecutive batches to yield a total of 16.88g of chloroform fraction per 80g of *Thuja* crude extract, with a mean of 4.22g per 20g of *Thuja* crude extract.

Finally the aqueous layer from each portion was partitioned with an equal volume of ethyl acetate. The ethyl acetate layer was separated and the extraction process repeated with all three further aliquots of fresh ethyl acetate. The combined ethyl acetate fractions were dried with a small amount of anhydrous sodium sulphate, filtered and dried under reduced pressure at 40°C on a rotary evaporator. The whole extraction process was repeated a further three times to yield a total of 12.03g of ethyl acetate fraction per 80g of *Thuja* crude extract, with a mean of 3.00g per 20g of *Thuja* crude extract.

The remaining water layer was concentrated and dried in an evaporating dish over a water bath at 65°C. This yielded 27.66g water fraction per 80g of *Thuja* crude extract, with a mean of 6.92g per 20g of *Thuja* crude extract.

The yield of each fraction in organic solvent was compared in terms of quantity of chemical components (Table 5). This shows that the difference in weight of material extracted in solvent varies with each fraction. Such sequential extraction is a well-established procedure for the separation of plant materials and components according to their polarity. The hexane, chloroform and ethyl acetate fractions of *Thuja occidentalis* showed that the non-polar compounds were separated in hexane and chloroform and more polar materials in ethyl acetate.

Table 5. Partition of ethanolic crude extracts of *Thuja occidentalis* in different organic solvents. 20g of Leaf and bark yield.

***Thuja* extract partitions**				
	Hexane partitioned	Chloroform partitioned	Ethyl acetate partitioned	Water partitioned
Mean yield	5.61g	4.22g	3.00g	6.92g

The fractions of *Thuja* were prepared for clinical studies and used in pilot studies (Chapter 3), each fraction of *Thuja* was applied at a dose of 60mg/ml per verruca lesion per day. The results were that the Chloroform fraction proved to be the most active in the treatment of verruca pedis (Section 3.1.6). The Chloroform fraction was further evaluated phytochemically to identify compounds present, the study involved the characterisation and identification of compounds in *Thuja* chloroform fraction using column and preparatory chromatography and then identification of compounds with NMR and Mass Spectrometry.

2.3.4 PHYTOCHEMICAL ANALYSIS OF CHLOROFORM FRACTION OF *THUJA OCCIDENTALIS* PLANT

2.3.4.1 CHROMATOGRAPHIC TECHNIQUES

Thin layer chromatography (TLC), preparative thin layer chromatography (PTLC), and column chromatography (CC) are used for separation and purification. CC is considered more useful for large-scale extraction of crude plant material (Harborne, 1973; Markham, 1975; Hostettman et al., 1986).

A variety of packing materials are used as adsorbents, for example, silica gel, cellulose, polyamide and Sephadex LH-20. Polyamide column gives better separation for flavonoids glycosides, whereas silica gel has been found useful for separation of flavonoid aglycones (Hostettman et al, 1986; Wagner et al, 1984). Solvents useful for silica gel TLC are chloroform : methanol (50:50), chloroform : ethyl acetate (60:40) and for polyamide column water : methanol : chloroform (40:20:5) (Ulubelen et al, 1980)

toluene : methanol (95:5). Sephadex LH-20 has been widely used for purification and separation of flavonoids. For flavonoid glycosides water-methanol (80:20) and for aglycones methanol 100% HPLC grade has been used (Hostettman et al, 1986).

THIN LAYER CHROMATOGRAPHY (TLC)

Analytical TLC was carried out on pre-coated, alumina-backed silica gel GF_{254} plates (Merck Ltd). A small amount of ethanolic crude extract of *Thuja occidentalis* was dissolved in a little volume of ethanol and examined by TLC on analytical silica pre-coated plates (Merck) using S8 and S9 solvent systems (Appendix 5) (prepared silica gel and analytical analysis). These two different solvent systems were used for detection as S8 is optimum for the detection of lignans and S9 is optimum for the identification of flavonoids. The samples were applied to the plates with capillary tubes and allowed to air dry. The plates were developed in a tank surrounded by chromatographic paper saturated with the appropriate eluting solvent. The plates were observed under UV light (254nm), before and after spraying with 5% ethanolic PEG 4000 or 80% sulphuric acid.

Silica gel TLC plates were used to monitor the fractions obtained from column chromatography of *Thuja occidentalis* using solvent system chloroform: methanol (50:50). The silica gel plate was observed before and after spraying with 80% sulphuric acid. Fractions were analysed by prepared silica plates and column chromatography.

DETECTION PROCEDURE

Natural products/polyethyleneglycol reagent (NP/PEG). The plate is sprayed with 1% methanolic diphenylboric acid-ß-ethylamino ester, also known as diphenylboryloxyethylamine (NP), followed by 5% ethanolic polyethyleneglycol 4000 (PEG) (10 ml and 8 ml, respectively), was used to detect the phenolic spots. Intense fluorescence is produced immediately or after 15 min in UV-365nm. PEG increases the sensitivity (from 10µg to 2.5µg). The fluorescence behaviour is structure-dependant. Alternatively, the

plate is sprayed with 60% sulphuric acid and heated at 100^0C for 3-5 min, and evaluated in visible light or in UV-365nm. With concentrated sulphuric acid (H_2SO_4) coloured (visible light) zones often appear immediately. Sulphuric acid was used for silica gel TLC plates for development of phenolic spots (Appendix 5).

PREPARATIVE CHROMATOGRAPHIC PROCEDURE OF *THUJA OCCIDENTALIS*

Crude extracts and fractions were loaded on to the plate 2 cm from the base and run in solvent systems. The plate was first run in a solvent mixture of chloroform : methanol for 5cm then dried. It was then further run in a toluene acetone mixture (S8), until it reached the solvent front. The second solvent system used contained a mixture of ethyl acetate, formic acid, glacial acetic acid and water (S9) and run from origin to solvent front. The plates were observed under UV 256nm and UV 365nm. The bands detected were then scraped off and dissolved into HPS Grade Methanol, this was filtered using Whatman filter paper. A small amount was analysed using an analytical silica gel TLC using solvent systems S8 and S9. The filtrate was then dried under pressure and weighed, and sent for mass spectrometric analysis. As the pilot studies carried out using the partitioned *Thuja* extract had found promising results with the chloroform fraction, it was deemed necessary to further investigate and identify activity in this fraction.

Colour reactions following the use of the S8 and S9 solvent systems lead to the detection of two compounds, one belonging to the Lignan family and the other to the Flavonoid family. These were identified by comparison of TLC Rf values with literature (Roth, 1993) as being deoxypodophyllotoxin from the S8 system and isokaempferol. The less polar band was seen as deoxypodophyllotoxin and the more polar band was believed to be a flavonoid isokaempferol, a flavonoid related to kaempferol from the S9 system (Tables 6 and 7).

2.3.5 COLUMN CHROMATOGRAPHY (CC)

Thuja occidentalis crude dried ethanolic extract (3g) was separated on a silica gel column. A glass column was packed with cotton wool plugs and silica gel 60F (70-100mesh) 4.5 x 60cm column. The slurry was prepared with chloroform and poured first into the column, then the crude extract was added and finally a small layer of acid-washed sand, which was covered with filter paper. The column was eluted first with chloroform (100%), chloroform: methanol mixtures of increasing polarity (90:10, 80:20, 60:40, 40:60, 20:80), methanol (100%), methanol: water mixture (70:30, 50:50, 30:70) and pure water.

27 fractions each of 20ml were collected, concentrated to low volume and analysed on silica gel TLC using solvent system chloroform: methanol (50:50). The plates were examined under UV light at 245nm and 365nm. Fractions showing same colour spots and Rf value were combined. *Thuja* yielded five fractions with different colour intensities. They were further separated on a small silica gel column to aid with separation as a smaller column allows more precise amounts to be separated. This procedure resulted in five fractions of *Thuja occidentalis*. The fractions showing similar colours were combined, *Thuja occidentalis* on TLC plates showing presence of purple and yellow and brown colour spots were concentrated and chromatographed on TLC analytical silica plates and developed in S8 and S9 (Appendix 5) solvent systems.

2.3.5.1 SEPHADEX LH-20 SIZE EXCLUSION CHROMATOGRAPHY

Sephadex LH-20 (Pharmacia) is used as a final step for the preparation of a pure compound before analysis. Hydrophilic gels such as those prepared from starch, agar, agarose (a component of agar), polyacrylamide, polyvinylcarbitol and cross-linked dextrans have been used for the fractionation of proteins, peptides, amino acids and polysaccharides. The particles of such gels possess pores formed by the molecular structure of the gel and when packed into a column and percolated with a solution, they permit large molecules of solute, which do not enter the pores, to pass

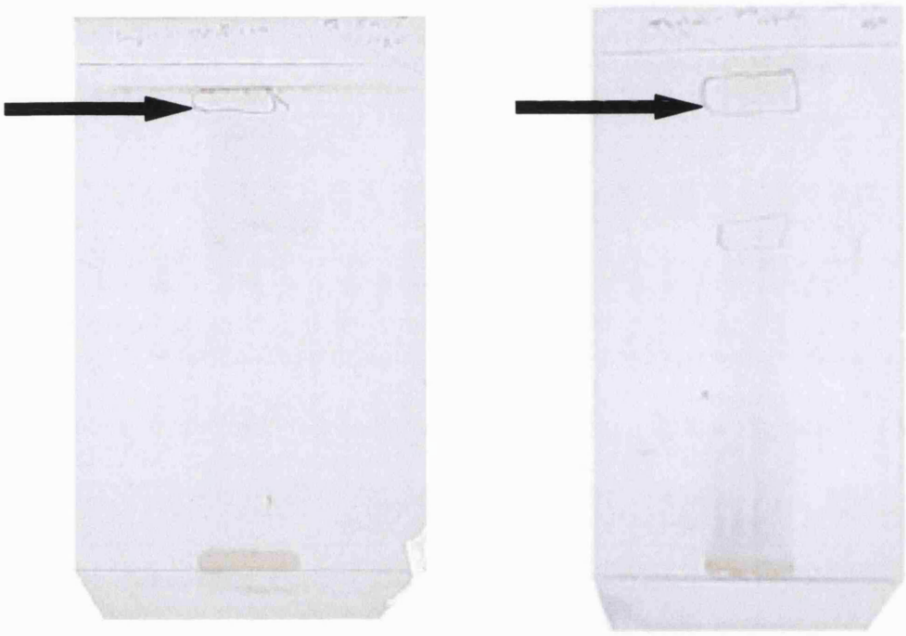
rapidly down the column with the solvent via the intergranular interstices. On the other hand, small molecules, which are able to enter the gel pores become evenly distributed (on equilibrium) across the column and pass more slowly down its length.

The dextran gels ('Sephadex') are formed by cross-linking dextrans (polymers of glucose in which linkages are almost entirely of the 1:6-α-type) with α-epichlorohydrin. The distance apart of the cross-links determines individual pore sizes, and gels covering a molecular weight range of up to about 200,000 are produced. Each gel type possesses a range of pore sizes so that below the size-limit of complete exclusion of the large molecules, different sized solute molecules will enter the gel to a greater or lesser extent and so will vary in their elution rates. Obviously the method is most applicable to mixtures containing large molecules of various sizes and to the separation of large molecules from small ones (as in desalting operations on partially hydrolysed proteins).

A Sephadex column was soaked for three hours in methanol HPLC grade, poured into a small glass column and left overnight before use. *Thuja occidentalis* fractions were purified with methanol HPLC grade and dried under nitrogen for spectral analysis. The colour bands of *Thuja occidentalis* fractions were separated on prepared TLC plates and extracted with methanol HPLC grade. The extracts were filtered through cotton wool, purified on Sephadex LH-20, chromatographed on analytical TLC silica gel plates and observed under UV (365nm). *Thuja occidentalis*, showed a single purple spot, which was identified by ^{1}HNMR and MS as a flavonoid possibly Kaempferol 3-methyl ether and a brown spot which was identified as deoxypodophyllotoxin (Figure 18).

Separated chloroform fraction of *Thuja occidentalis.*

1. Chloroform fraction **2. Chloroform fraction**



Band A
= Kaempferol 3-methyl ether

Band B
= Deoxypodophyllotoxin

The separated chloroform fraction of *Thuja occidentalis* were chromatographed on analytical silica pre-coated plates **1** and **2.** Plate **1** was developed in ethyl acetate: formic acid: glacial acetic acid: water and observed under UV light at 365nm with ammonia vapour. The Rf value is presented in Table 6. And colour reaction in Table 7. Plate 1 showed a major band **A**, yellow in colour, which was presumed to be a flavonoid, Kaempferol 3-methyl ether (Wagner et al, 1984).

Plate **2** was developed in chloroform: methanol to 5cm then toluene: acetone to solvent front and observed under UV light at 365nm with ammonia vapour. The Rf value is presented in Table 6, and the colour reaction in Table 7. Plate 2 showed a major band **B**, brown/red in colour, which was presumed to be deoxypodophyllotoxin compared with reference compound.

Figure 18. TLC Chromatographic profile of chloroform partition fraction.

Table 6. Rf values of *Thuja occidentalis* compounds under UV light at 365nm.

Isolated compounds and authentic sample	**RF values of silica gel plate**	
	$CHCl_3$:MeOH (90:10) Toluene:EA (65:35)	EA:formic acid:glacial acetic acid:water (100:11:11:27)
Thuja occidentalis chloroform fraction	0.75	0.86
Deoxypodophyllotoxin (Authentic sample)	0.75	___
Kaempferol (Literature)	___	0.82

Table 7. Colour reaction of *Thuja occidentalis* compounds in visible and UV light at 365nm with and without ammonia vapour.

Isolated compound and authentic sample	**Visible light**	**λ = 256nm**	**λ = 365nm**
	Daylight	UV	UV/Ammonia
Thuja occ. chloroform partition bands 1 and 2	Invisible	Brown	Red/brown
	Invisible	Purple	Yellow
Deoxypodophyllotoxin	Invisible	Brown	Red/brown
Kaempferol	Invisible	Purple	Yellow

The Rf values and colour reactions shown in Tables 6 and 7 relate to *Thuja occidentalis.*

2.3.5.2 SPECTRAL DATA OF DEOXYPODOPHYLLOTOXIN

Deoxypodophyllotoxin was obtained from *Thuja occidentalis* as a greeny/yellow residue in the multistage separation and purification procedure as specified. The components were principally identified by NMR and mass spectrometry.

2.3.6 SPECTRAL ANALYSIS PROCEDURES

Nuclear magnetic resonance and mass spectrometry were used for identification and structural elucidation of the compounds isolated from *Thuja occidentalis*.

2.3.6.1 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (^{1}HNMR)

^{1}HNMR is an important technique for the structure analysis of flavonoids. It has been used to determine the number of protons present in the flavonoid skeleton and its substituents (Markham, 1975). Most flavonoid glycosides have low solubility in deuteriochloroform ($CDCl_3$), therefore the trimethylsilyl ether (TMS) derivative of the flavonoid is used. However, highly methoxylated flavones and flavonols are sufficiently soluble in the commonly used solvents $CDCl_3$ or CCl_4.

A study of the benzene-induced shifts of OCH_3 protons compared with shifts for these protons in $CDCl_3$ and CCl_4 may prove useful in the determination of the position of methoxyl substitution in flavonoid nucleus (Rodrigues et al., 1972).

Spectra of compounds isolated from *Thuja occidentalis* were determined in CD_3OD with TMS as internal standard on a Bruker WM-250 or 500MHz spectrometer. The chemical shift value (δ) was recorded in ppm and the coupling constant (J) as Hz. The analysis was carried out at the NMR Unit, School of Pharmacy.

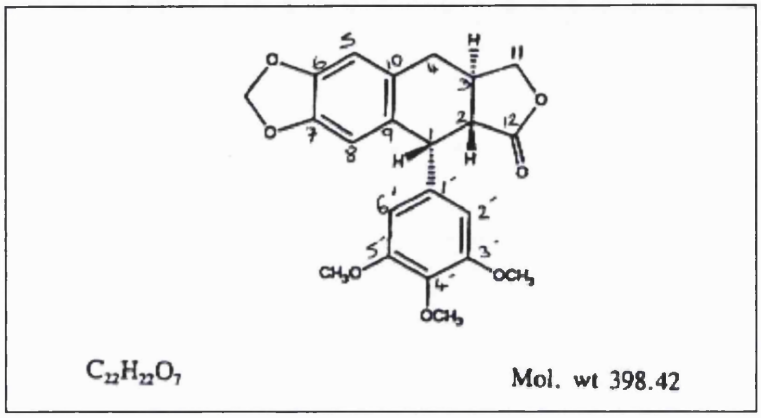


Figure 19. Chromatographic and spectral data of deoxypodophyllotoxin. Deoxypodophyllotoxin is also known as Anthriscin, Hernandion or Silicolin.

While the NMR (Figure 20a) of deoxypodophyllotoxin residue did show some contamination it was clean in the downfield region indicating the required compound. The spectra showed aromatic protons, H-8 at δ6.51. The chemical shifts for DPD of other protons are: both H-2' and H-6' at δ6.37 methylenedioxy protons at δ5.94, H-1 at δ4.60, H-2 and H-3 at δ2.81 and δ2.82 respectively, H-4β at δ2.78. The signals H-11α at δ3.81 and H-11β at δ4.59 overlapped the strong methoxy signals at methoxy 3.75, 3.8 and 3.82.

These results were compared with the purified sample of DPD (Figure 20b), which had been prepared by a reduction sequence, supplied by Dr. Watt.

The method of Brewer et al. (1979) was used to prepare Deoxypodophyllotoxin from Podophyllotoxin. The NMR assignments of these figures are represented above, the sample was used as a comparative with the isolated sample of Deoxypodophyllotoxin from *Thuja occidentalis*.

Table 8. Proton NMR Chemical-shift Assignments for Deoxypodophyllotoxin; a comparison of reference sample with isolated sample.

The purified sample of DPD		
The Spectra Showed	Authentic sample DPD	Isolated sample DPD
H- 5	δ6.66	
H- 8	δ6.51	δ6.51
H- 2'& H- 6'	δ6.32	δ6.37
H- 1	δ4.60	δ4.60
H- 2	δ2.72	δ2.81
H- 3	δ2.73	δ2.82
H- 4α	δ3.07	
H- 4β	δ2.78	δ2.78
H- 11α	δ3.92	δ3.81
H- 11β	δ4.45	δ4.59

These data show that the compound present is identical in structure to the purified sample with some degree of contamination present (Brewer et al., 1979).

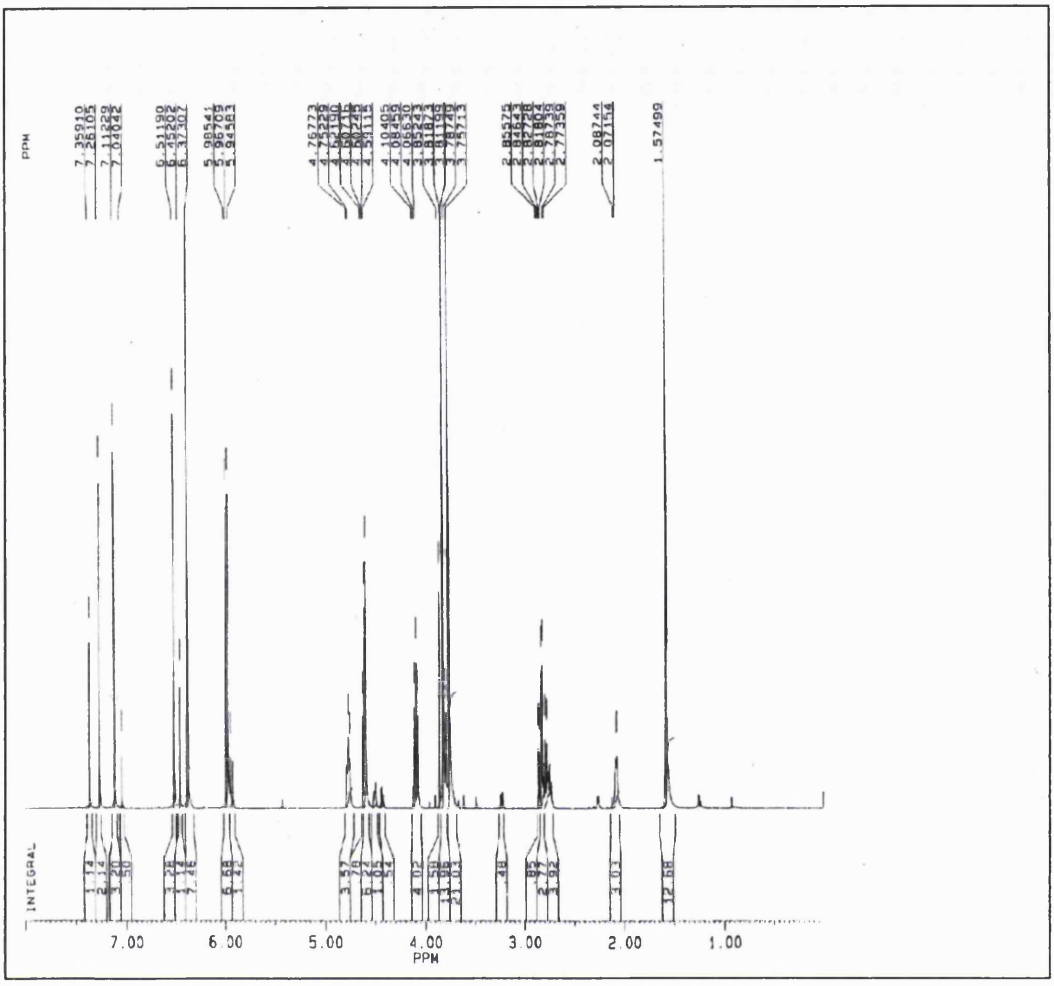


Figure 20a. NMR of isolated Deoxypodophyllotoxin from *Thuja occidentalis*, ^{1}HNMR (500MHz, CD_3OD) ppm.

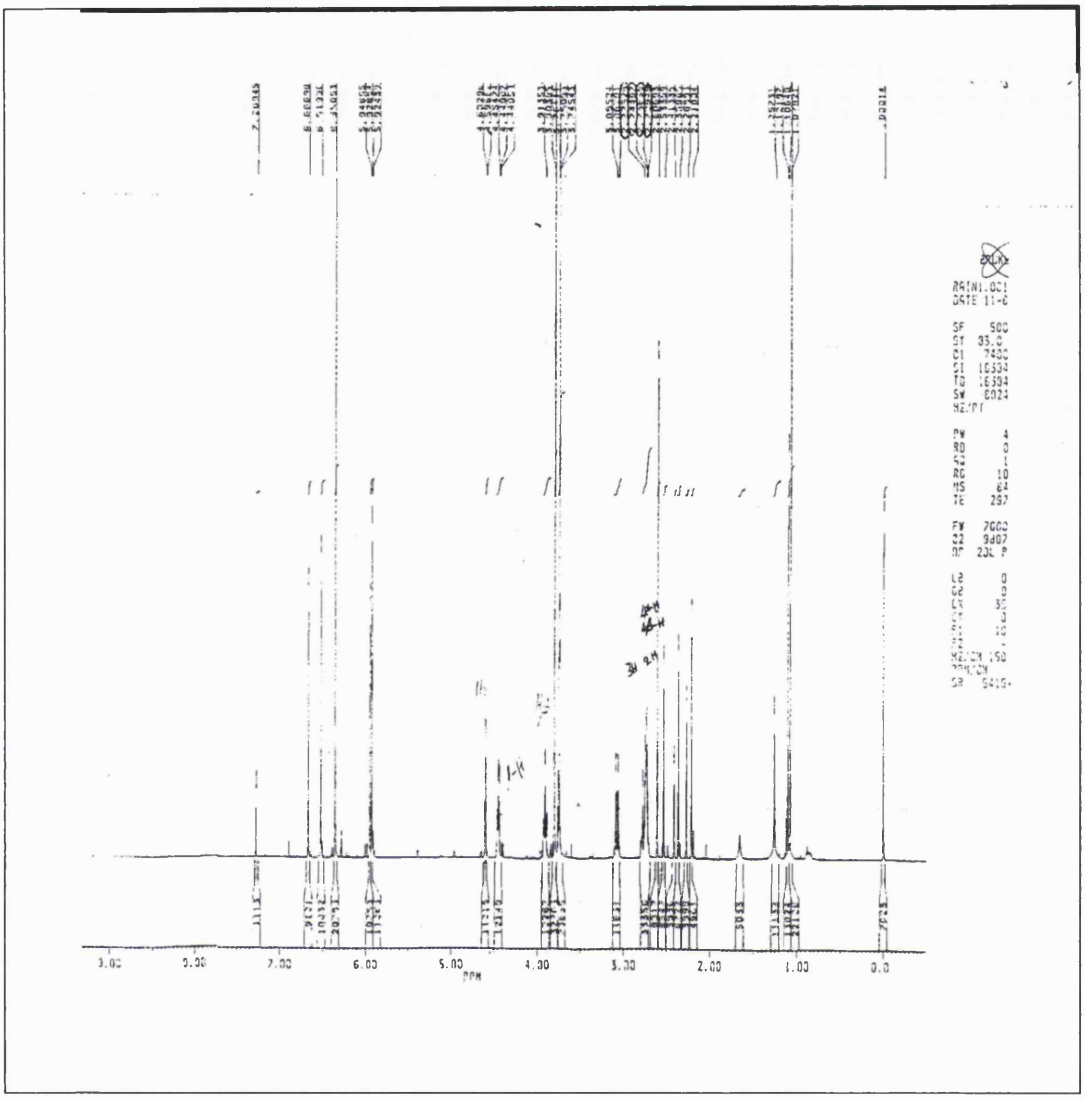


Figure 20b. Reference sample of Deoxypodophyllotoxin
(PD reduction product fraction 3).

2.3.6.2 MASS SPECTROMETRY (MS)

MS is an important aid in the structure elucidation of both flavonoid aglycones and glycosides, especially when less than 2mg of the compound is available for analysis.

A number of MS techniques have been developed. In electron impact mass spectrometry (EIMS), most flavonoid aglycones yield M^+ as the base peak. Other major peaks include $M\text{-}H^+$ and from methoxylated compounds $M\text{-}CH_3^+$, and M^+HCO and $M^+\text{-}COCH_3$. These are the most useful fragmentations in terms of flavonoid identification especially those which involve cleavage of intact A and B ring fragments. Such ions are designated as A_1, A_2, and B_1, B_2 respectively. The retro Diels-Alder (RDA) process known as Pathway I and Pathway II derive some of these ions. Pathway I corresponds to an RDA cleavage.

There are other diagnostic fragmentation patterns for different classes of flavonoid aglycones. Pathway I process produces two different ions $A1^+$ and $B1^+$, the ratio of one to the other being indicative of the charge distribution with the parent ion. In contrast, Pathway II yields predominantly a single charge species $B2^+$ (Kingston, 1971; Mabry and Markham, 1975).

Chemical ionisation mass spectrometry (CIMS) is an effective technique for analysis of flavonoids. It is applied using methane gas a reactant to flavonoids aglycones to give molecular ions and also diagnostic fragments (Ulubelen et al., 1980).

Field desorption mass spectrometry (FDMS) is applied to a number of O^- glycosides to establish fragments resulting from both the Aglycone and saccharide moieties (Ulubelen et al., 1980).

Fast atom bombardment mass spectrometry (FABMS) is a technique of mass spectrometry used for the study of polar molecules (Watson, 1999). The techniques of DDMS and FABMS have been applied to the study of flavonoid glycosides.

The purified sample of deoxypodophyllotoxin was identified with using mass spectrometry. The results verified structurally deoxypodophyllotoxin. Molecular weight of 397.3 was partially low probably due to fragmentation or instrument calibration. The overall results were compared to an authentic sample supplied by Dr. Watt and reviewed with literature (Brewer et al., 1979).

Mass spectrometry m/z (relative intensity %): 397.3 (100%), 229.3 (10%), 313.4 (10%) 415.3 (5%).

The molecular weight of deoxypodophyllotoxin is 398 with the peak at 397.3 corresponding to this.

Rf value of deoxypodophyllotoxin was 0.75 in Chloroform:Methanol and then Toluene:Ethyl acetate. The colour of the compound was brown when viewed under UV light. When the plate was sprayed with 80% H_2SO_4, the colour of the spot changed to red/brown. The Rf value and colour reaction of the compound was compared with published data (Markkanen 1981). Dr R. Watt supplied the authentic sample of deoxypodophyllotoxin, Centre for Pharmaceutical Analysis, School of Pharmacy, University of London.

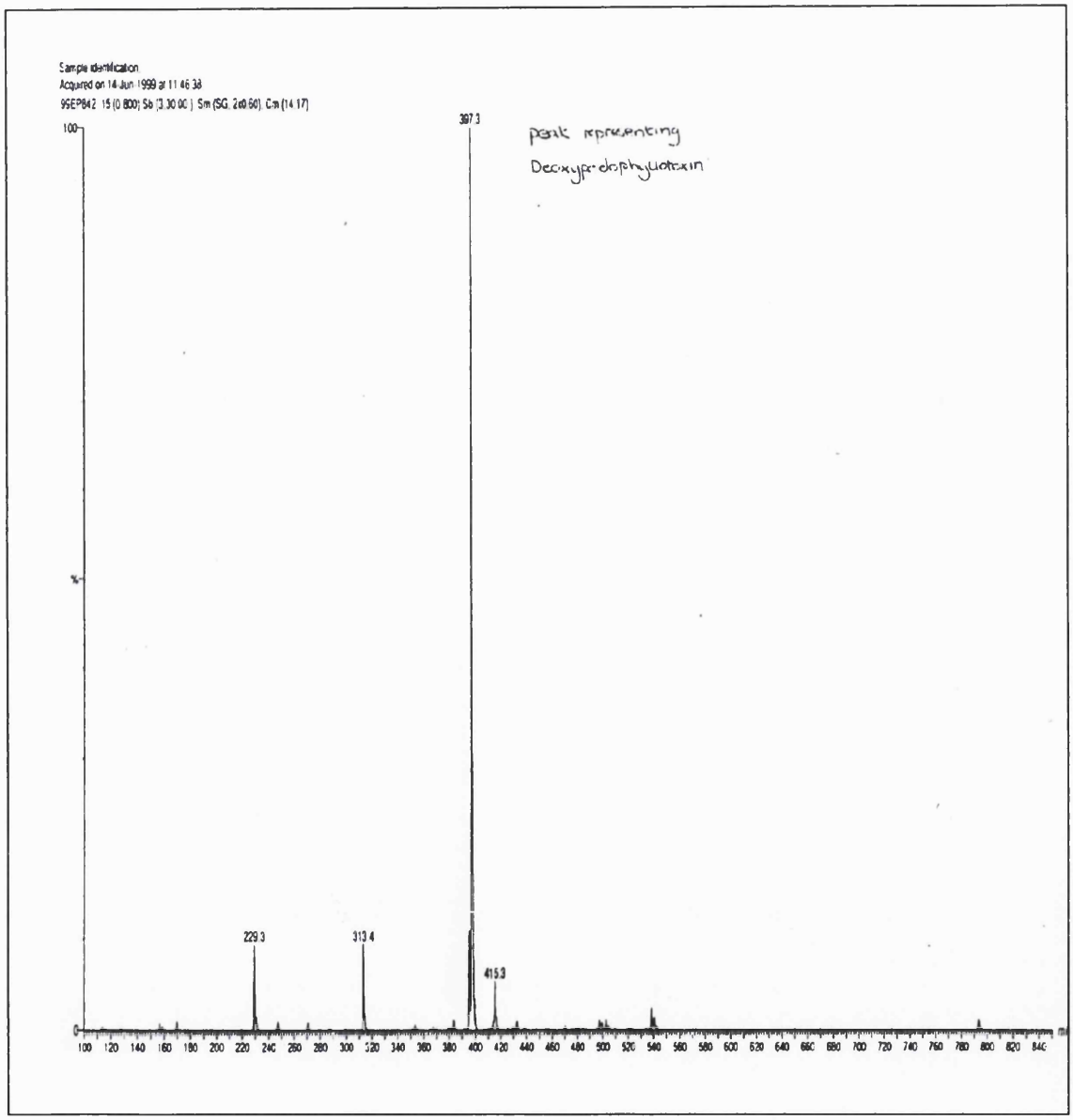


Figure 21. Mass spectrometry of Deoxypodophyllotoxin from *Thuja occidentalis*

2.3.7 CHARACTERISATION OF A FLAVONOID ISOLATED FROM *THUJA OCCIDENTALIS.*

Thuja occidentalis is reported to contain the flavonoids quercetin, kaempferol 3-methyl ether and mycertrin. The compound that was isolated and showed Rf value of 0.86 in solvent system S9. When viewed under UV light a deep purple colour appeared, when exposed to ammonia vapour, it changed colour to yellow (Table 7). The Rf value and colour reaction of the compound was compared with published data (El-Emery and Ali, 1983). An authentic sample was not available, therefore Kaempferol was used as a control to indicate the class of compound present.

The colour reaction and Rf value indicated that a kaempferol related compound was present. Further investigation with published data gave inconclusive results to the structure of the compound with the possibility that it may resemble Kaempferol 3-methyl ether (isokaempferide). The mass spectrum of the compound isolated from *Thuja occidentalis* showed the following peaks: Mass spectra of the compounds isolated from *Thuja occidentalis* were determined by direct insertion at 70eV in methanol as solvent using a ZAB-1F (3VG-Micromass Ltd) at inlet temperature 170°C to 240°C in the Department of Chemistry, School of Pharmacy.

M/z (relative intensity %): 303.5 (100%), 287.5 (55%), 317.5 (50%), 399.3 (20%), 221.5 (60%).

NMR analysis was unfortunately not conclusive due to the number of other compounds found in the sample.

2.3.7.1 MASS SPECTRAL ANALYSIS OF THE FLAVONOID (KAEMPFEROL 3-METHYL ETHER) FROM *THUJA OCCIDENTALIS*

The base peak for all flavonoids is the molecular ion M^+. The fragments A_1^+, $[A+1]^+$, B_1^+ and B_2^+ are present in all spectra. These diagnostic ions only occur in flavones, iso flavones and flavonols (Grayer, 1989). Additionally

Mabry & Markham (1975) have found other diagnostic ions in flavonols, including $[M-H^+]$, $[M-H_2O]^+$, $[M-OCH_3]^+$ and $[M-CH_3CO]^+$.

Two of the most important diagnostic fragments from flavonols are $[A+1]^+$ and B_2^+ and substitution of the A- and B- rings can be deducted by analysing the mass spectral values of these fragments (Mabry and Markham 1975). In flavonols, these fragments basically have mass spectral values of 121 and 105 respectively; in the spectra of compound kaempferol 3-methyl ether $[A+1]^+$ has a mass spectral value of 153 instead of 121, indicating that there are two additional oxygen atoms on the A-ring.

Pathway-I reaction produces two different ions A_1+ and B_1+, the ratio of one to the other being indicative of the charge distribution with the parent ion. In contrast, Pathway-II yields predominantly a single charge species B_2+. The molecular weight of the skeleton for most flavonoids is 222. It is useful to note that the parent flavone itself gives M+ at m/z 222, A_1+120 and B_1+102 and B_2+105 and that the presence of hydroxyl, methoxyl and deuteriomethyl groups increases the m/z value. Two major peaks are seen generally on Mass Spectrometry. One with the highest m/z value represents the molecular weight and the other m/z value the aglycone.

Additional detected mass spectrometry of kaempferol 3-methyl ether was carried out by Chaves et. al. (1998) showed LC-PB-MS experiments to determine optimum conditions for the effect of increasing analyte transfer efficiency using ammonium acetate at concentration 0.01 to 0.1M in the mobile phase. The results reported, are shown in table 9 for kaempferol 3-methyl ether the EI mass spectra of flavonoids from crude extract, also showed fragments which suggested that same ions could result from impurities derived from plant extraction.

Table 9. LC-PB-EI-MS diagnostic ions in the spectra of Kaempferol 3 -methyl ether (Chaves et al., 1998).

Ion **M/I**	**M/E**	**Percentage relative to other intensities**
M+	300	100
[M- I]+	299	84
$[M- H_2O]^{\pm}$	282	18
$[M- CO]^{\pm}$	272	5
$[M- CHO]^{+}$	271	25
$[M- OCH_3]^{+}$	269	14
$[M- CH_3CO]^{+}$	257	62
A_1^{+}	152	11
$[A_1- H]^{+}$	153	24
$B_1^{\pm}$	118	12
B_2^{+}	121	46

Table 9 reports the EI diagnostic fragments and their relative intensities for kaempferol 3-methyl ether.

No precise match was found between the spectra of the isolated sample and the literature however the resemblance of the fragmentation patterns, indicates the sample is of the flavonoid class. Therefore one lignan and one flavonoid had been isolated from the extraction which are deoxypodophyllotoxin and a flavonoid possibly kaempferol 3-methyl ether.

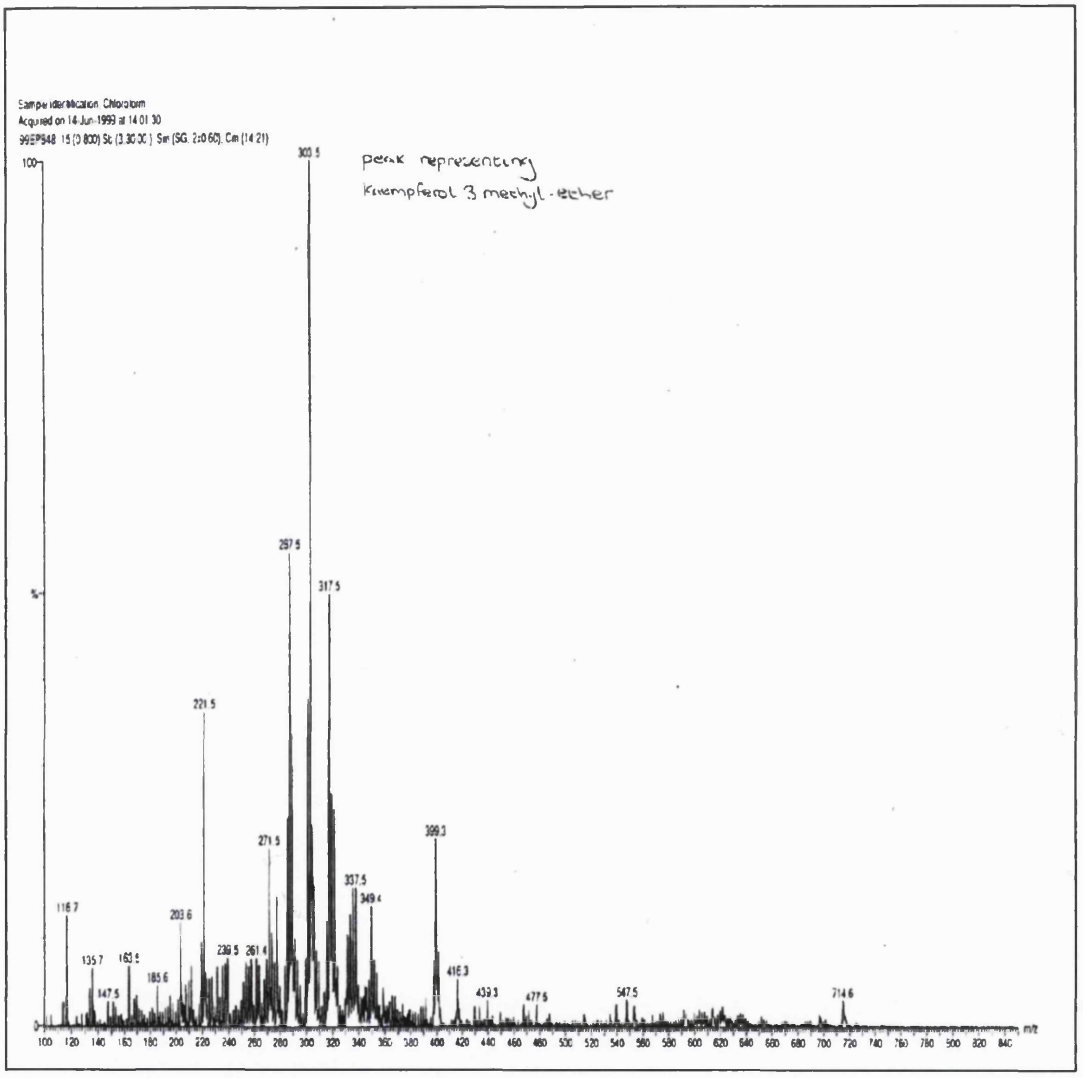


Figure 22. Mass spectrometry of Flavonoid isolated from *Thuja occidentalis*.

2.4 DISCUSSION

2.4.1 SEPARATION AND ISOLATION

The chemical constituents from *Thuja occidentalis* crude extracts and chloroform fractions were separated using different chromatographic procedures with an appropriate solvent system listed in Appendix 5. Detection of chemical compounds of *Thuja occidentalis* was carried out with and without ammonia vapour under UV light at 254nm and 365nm and spraying with NP/PEG or concentrated sulphuric acid and the colour reaction observed.

Silica gel column was used for separation of the flavonoid aglycones and glycosides with the appropriate eluting solvent system. Cellulose and polyamide columns are known to be the most suitable for separation of flavonoids but silica gel column was also found satisfactory. In previous studies, Lo, (1994) and Maier and Groll, (1995) separated flavonoids using the same methods described above.

One problem experienced with the isolation work was the small yields of resultant pure compounds. This can be partly explained by the loss of materials during separation and in consequence there is a need for the separation method to be checked and re-evaluated (Vasange, 1996). According to Harborne, (1973), however, flavonoids as plant phenolics can be a considerable nuisance to the plant biochemist because of their ability to complex with protein by hydrogen bonding. When plant cell constituents come together and the membranes are destroyed during the isolation procedures, the phenols rapidly complex with protein and as a result, there is often inhibition of enzyme activity in crude plant extracts. On the other hand, phenols are themselves very susceptible to enzymatic oxidation and phenolic material may be lost during isolation procedures, due to the action of specific "phenolase" enzymes present in all plants.

2.4.2 PURIFICATION AND IDENTIFICATION

The isolated compounds from CC were purified on Sephadex LH-20 using methanol HPLC grade. In order to obtain pure compounds, the process was repeated several times before spectral analysis. Rf value and colour reaction of the purified extracts and fractions of *Thuja occidentalis* were determined using TLC silica gel plates as presented in Figure 18 and Table 6 and 7.

Polar and non-polar compounds from ethanolic crude extracts of *Thuja occidentalis* were partitioned with hexane to separate fatty materials and some highly methoxylated flavonoid aglycones such as apigenin, leutolin, kaempferol and quercetin. The ethyl acetate separates mono- and diglycosides.

Thuja occidentalis is made up of many different groups of compounds. These include Monoterpenoids, Flavonoids, Lignans and Tannins. When separating the fractions of *Thuja occidentalis* it was possible to identify the different groups of compounds present in each fraction, generally compounds that are less polar separated out in less polar solvent like Hexane, as the polarity increases, lignan would be present in Chloroform fraction and Flavonoids would separate in Ethyl acetate. From the clinical results in Chapter 3, it was interesting to see that the results identified that Chloroform fraction was most therapeutically effective with some positive result found with Ethyl acetate. This led to a more intense investigation of these samples. Initially, when screening the sample chromatography solvent, which would help to identify, Lignan and flavonoid were used (S8 and S9).

The TLC results were conclusive in identifying the presence of Deoxypodophyllotoxin. Also present was a flavonoid, which was not identified conclusively. Referring to literature, *Thuja occidentalis* contains 3 types of flavonoids namely Quercetin, Kaempferol 3-methyl ether and Myrecetin (PDR,1998). The fraction sample was plotted onto a TLC plate with reference marker of Quercetin, Myrecetin and Kaempferol (unable to get Kaempferol 3-methyl ether). These would be able to give indication of

flavonoid present in sample using Wagner et al (1984) as a guide. The TLC plates arrived with 3 spots, 2 clearly corresponded to quercetin and myercetin, but the other was slightly higher to the kaempferol spot. Thus indicating that this spot relates to Kaempferol 3-methyl ether which would be present in this region and the colour reaction was as expected.

This spot was further investigated using NMR and mass spectrometry. The results were not clear and contaminated. It was judged that there is a flavonoid present which may have a role on wart/ verruca regression, but is not the superior component. It was clear that the isolated compound Deoxypodophyllotoxin was the major component on wart regression and the primary active compound.

From the extraction of *Thuja occidentalis,* 30mg of deoxypodophyllotoxin and 10mg of a flavonoid were obtained using multi-stage purification from the chloroform fraction. Deoxypodophyllotoxin was clearly identified, but the flavonoid isolated was not. It was indicated to be the kaempferol derivative kaempferol 3-methyl ether, which has been reported in literature of *Thuja occidentalis* (PDR, 1998).

Chapter 3 -

Clinical Evaluation of *Thuja occidentalis* in the Treatment of Verruca Pedis.

3.1 INTRODUCTION

A number of terms are used in scientific research when looking for a specific answer to a specific question in an organised, objective and reliable way. The process of enquiry may include experimentation but it always involves collection, analysis and interpretation of data for the purpose of generating new knowledge. A clinical trial is an experiment carried out to assess the effectiveness of a new treatment regime.

Volunteers are allocated to one of two groups, the treatment group or the control group. The control group receives either the standard treatment for the condition, as in evaluation of a new drug, or no effective treatment as in the assessment of efficacy of a therapy or treatment. Extensions of the method are the inclusion of several "different dose" groups and the simultaneous trial of several different treatment regimes. It is important to design the trial so that any observed differences between the treatment and the control groups can be attributed to a real effect of the treatment. The groups should initially be as alike as possible in all respects other than the treatment received. Furthermore, the method of handling and assessing the groups should be the same.

The opportunity for any bias to occur during the trial is minimised by the use of single or double blind design and a placebo. Random allocation of participants to the treatment and control groups is the method used to avoid any bias in selection, either by investigator or by the participants, which may lead to inherent differences between the groups. The treatment and control groups are identified by code alone, the code being held by an uninterested party until all the data have been collected. The most common clinical trial is the double blind in which neither experimenter nor subject knows who is getting treatment. In the single blind trial the person responsible for data collection is blind as to which group a subject is assigned (Payton, 1994). A suitable method of statistical analysis is used to evaluate the data collected from the subjects (Rees, 1994).

In this clinical study, the effect of *Thuja occidentalis* crude extract and fractions were investigated in the treatment of verruca pedis. A pilot study was initially carried out to evaluate the action and effectiveness of a crude extract of *Thuja occidentalis* on verruca pedis (Appendix 2). The pilot or single blind controlled study was carried out in the Marigold Clinic, Department of Homoeopathic Podiatry at the Royal London Homoeopathic Hospital NHS Trust (RLHH). A pilot study was carried out to establish the effective dose for the samples which were applied once a day for four weeks. Follow-up treatment at home was monitored after a further 1,3 and 6 months. The double-blind controlled study was carried out in the Marigold Clinic, RLHH and the Wart Clinic, Department of Dermatology, Royal London Hospital (RLH). The study compared the active crude *Thuja* extract against a placebo preparation of *Thuja*, which was prepared using *Thuja*, which was boiled until its activity was destroyed and checked. An independent assessor (IA) recorded clinical measurements of the lesion sizes. Photographs were taken as visual evidence before and after each treatment. The collected data were statistically evaluated.

3.1.1 SUBJECTS

Patients were recruited and referred from general practitioners and hospital consultants to the Royal London Homoeopathic Hospital and the Royal London Hospital.

3.1.2 INCLUSION AND EXCLUSION CRITERIA

Male and female volunteers aged between 7-40 years were included, who, having read the patients information sheet supplied, were willing to sign a consent form. Volunteers previously contra-indicated to other treatment regimes and those who had received unsuccessful treatment regimes were also included.

Exclusion criteria included diabetes or other systemic diseases requiring medication (including steroids or non-steroidal analgesics), allergy to adhesive plaster and patients unable to complete the full course of treatment.

3.1.3 SELECTION OF PATIENTS

For convenience, selection of patients from the sampling frame was carried out systematically rather than randomly, by taking individuals at regular intervals (Kirkwood, 1991, Rees, 1994). A systematic sample is equivalent to a random one provided there is no underlying pattern in the order of individuals on the list. Ninety volunteers meeting the inclusion criteria were admitted to the study and allocated to 1 group of 30 consisting of open study, all patients receiving active preparation of *Thuja*. 2 groups of 15, one of the groups received active preparation and the other group received placebo, and 3 groups of 10 each received different partitioned fraction of *Thuja*.

3.1.4 ROLE OF INDEPENDENT ASSESSOR (IA)

To minimise experimental bias, a clinician was appointed as an IA to measure and record lesion size before and after treatment.

3.1.5 ETHICAL CONSIDERATIONS

The study was approved by: The Supervisor, Head of the Centre for Pharmacognosy, School of Pharmacy, University of London; The Director of the RLHH Research and Development Committee and The Clinical Director, Department of Dermatology, RLH, to be carried out in the Marigold Clinic at the Hospital and Wart Clinic, Department of Dermatology, RLH. A ruling had been given in relation to the previous clinical research projects on *Thuja occidentalis* that, as the treatment is topical and non-invasive, Ethical Committee approval is therefore not needed, provided the nature of the study is explained to each patient and each signs a consent form before treatment commences.

Patients were assured of the confidentiality of their medical data and informed that they could withdraw from the study at any time. Examples of the patient information sheet and consent form are included in Appendices 1 and 2.

3.1.6 PILOT STUDIES

The effective dose response for each sample was obtained in pilot studies using concentrations of ethanolic crude extract and hexane, chloroform and ethyl acetate fractions of *Thuja occidentalis*. Volunteers were selected from the population waiting for treatment that met the inclusion and exclusion criteria (Section 3.1.2). Ninety volunteers were selected and divided into groups (table 8). Different concentrations of each sample were chosen, a daily dose consisting of 1ml: *Thuja occidentalis* ethanolic crude extract 300mg/ml.dose/day; hexane, chloroform and ethyl acetate fractions of *Thuja occidentalis* 60mg/ml.dose/day and 70% ethanol 1ml/dose/day as control. The samples were prepared by dissolving *Thuja occidentalis* ethanolic crude extract and fractions in 1ml of 70% ethanol using a sonicator for ten minutes. A single dose of each sample was dispensed into a glass vial with a label indicating a code.

The samples were stored in a refrigerator at 4°C before use. The pilot studies were carried out over a period of six months.

3.1.7 SAMPLES FOR CLINICAL STUDY

The samples listed below for clinical evaluation of *Thuja occidentalis* were prepared as described in Chapter 2, Figure 16 and Figure 17. A single dose of each sample was dispensed into a glass vial with a label indicating the code, which was designated for identification.

Table 10. A summary of the different clinical studies.

Different studies	**Number of volunteers**	**Procedure and Treatment**
Study 1	30 volunteers	*Thuja occidentalis* Ethanolic crude extract 300mg/ml; 1ml dose/day for a duration of 3months
Study 2	30 volunteers	*Thuja occidentalis* Ethanolic crude extract 300mg/ml; 1ml dose/day (15 patients). 70% ethanol; 1ml dose/day, for a duration of 3months (15 patients).
Study 3	30 volunteers	*Thuja occidentalis* hexane fraction 60mg/ml; 1ml dose/day (10 patients). *Thuja occidentalis* chloroform fraction 60mg/ml; 1ml dose/day (10 patients). *Thuja occidentalis* ethyl acetate fraction 60mg/ml; 1ml dose/day, for a duration of 3months (10 patients).

3.2 APPARATUS FOR MEASUREMENTS

The IA, to record the size of lesion, used a Tegaderm wound mapping chart (3M medical) consisting of a Grid 5mm^2 square. This was carried out by placing the grid over the lesion and tracing the size, then counting the number of complete boxes present.

A single auto 1.8/50 Pentacon lens camera (Nikkon C.S. Japan) with Macroflash (Cobra) and Colorama 200 ASA colour film (Agfa Ltd) was used by the researcher to photograph the verruca pedis.

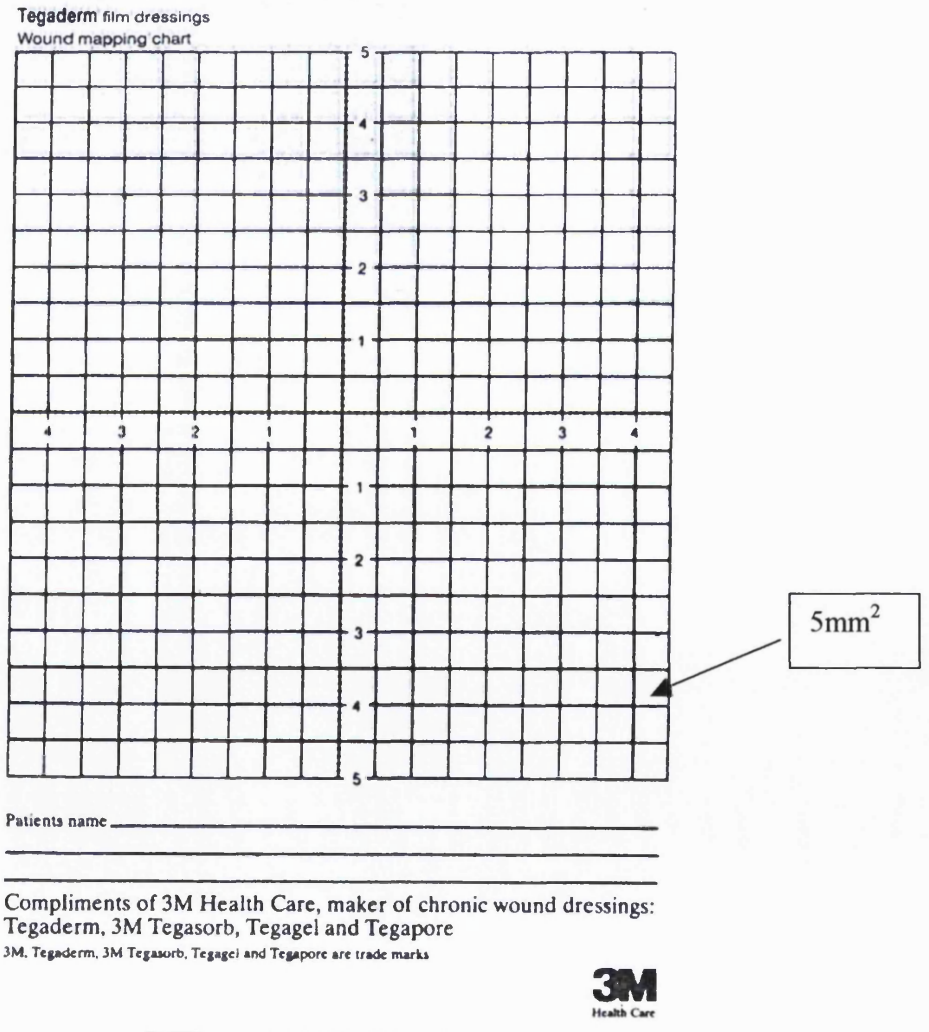


Figure 23. Apparatus for Clinical Treatment Assessment

3.2.1 VALIDITY AND RELIABILITY OF MEASUREMENTS

In previous studies, the test re-test reliability of measurements of verruca pedis were established with the use of Pearson's Product Moment Correlation Coefficient on the measurement taken by the IA on Day 1 and Day 2 (Khan 1995).

3.3 MATERIALS

A Sofsorb swab 10cmx10cm 4ply (Vernais; Vernoncarus) was used as a vehicle to apply the extracts and fractions to the lesions. Micropore 2.5cm surgical tape (Unichem Ltd) was used to cover the pad. Mefix 5cm self-adhesive fabric (Molnlycke, Sweden) was used to hold the dressing in place.

Figure 24. Materials for protective pad and strapping.
From the top, clockwise; W= Dropper, X= Mefix plaster,
Y= Gause, Z= Micropore tape.

Table 11. Methodology Schedule

Duration of Study: 12 months	**Subjects, Apparatus, Materials and Samples**	**Procedure and Treatment**	
Pre-study	Volunteers invited	Screened for inclusion & exclusion criteria	
Pre-study	Selection of patients	90 volunteers	
Pre-study	Allocation to groups by convenience	Group A-30 Group B1-15, B2-15 Group C1-10, C2-10, C3-10	
1st Week		Case taken and V.P. photographed by researcher and measured by IA. First treatment given by researcher.	Study entry
End of 1st week		Measurement taken by IA. Treatment given by researcher.	
End of 2nd Week		Procedure repeated	
End of 3rd Week		Procedure repeated	
End of 4th Week		Procedure repeated	
End of 8th Week		Procedure repeated	
End of 3 months		Observation and evaluation by researcher	End of Course of treatment
End of 6 months		Observation and evaluation by researcher	End of Study

3.4 PROCEDURE

3.4.1 PRE-TREATMENT

The verruca of each patient was measured by tracing the lesion through the Tegaderm wound mapping chart with a felt tipped marker pen.

A photograph of the foot was taken by the researcher using the same camera, lens, light source and film and at the same distance for each patient. A label with each patient's name and the date was placed beside the verruca as identification for each photograph.

3.4.2 PATIENT CHARACTERISTICS PRE-TREATMENT

Characteristics of patients' gender, age range, ethnic origin and duration of verruca pedis were calculated as a percentage and the results are presented in Tables 12-14 and Graphs 1-6.

3.4.3 PATIENT SELECTION

Due to the nature of the investigation and the small number of patients in each group, subjects were selected for convenience from a sampling frame rather than randomly (Kirkwood, 1991). In the blinded study, neither the patient nor the IA who took and recorded the measurements knew whether a patient was in active or placebo group. The IA had no access to previous data recorded except for the date when the measurements were taken. To minimise experimental bias, care was taken to treat all patients at the same time of day and the same day of the week as for their first treatment.

3.4.4 PILOT STUDIES

The effective dose response for each sample was obtained in pilot studies using different concentrations of *Thuja occidentalis* crude extract and hexane, chloroform and ethyl acetate fractions. The effective dose was 300mg/ml dose/day for ethanolic crude extract and 60mg/ml dose/day for hexane, chloroform and ethyl acetate fractions.

3.4.5 METHOD OF TREATMENT

For all groups the method of application was the same.

1. 70% ethanol was applied to the lesion as an antiseptic and followed by scraping the surface of the verruca pedis with an emery board (Figure 25a,b).
2. Extract or fraction or 70% ethanol 1ml was soaked in a piece of gauze was placed on the verruca pedis (Figure 26).
3. The gauze was covered with a piece of micropore (Figure 27).
4. Mefix Strapping plaster was placed over the gauze to hold the pad in position (Figure 28).
5. During the next six days the patient carried out the same procedure.
6. After a period of one week, the researcher examined the lesion for improvement or any adverse reactions. The IA measured and recorded the size of the lesion. The researcher then repeated the procedure 1-5.
7. Procedure 6 was then repeated twice more, then the patient was called back after one, three and six months.

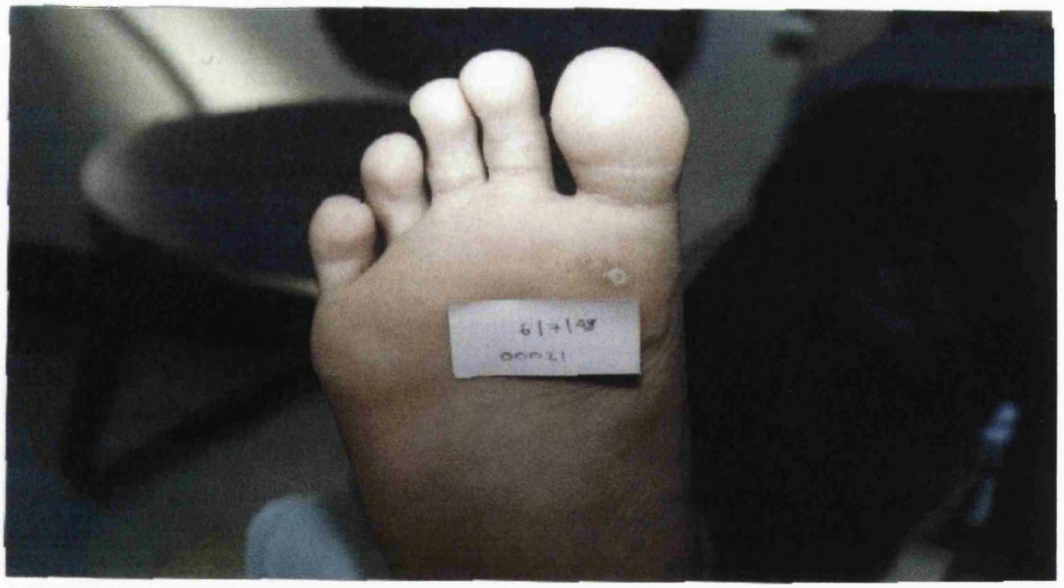


Figure 25a. Verruca before treatment

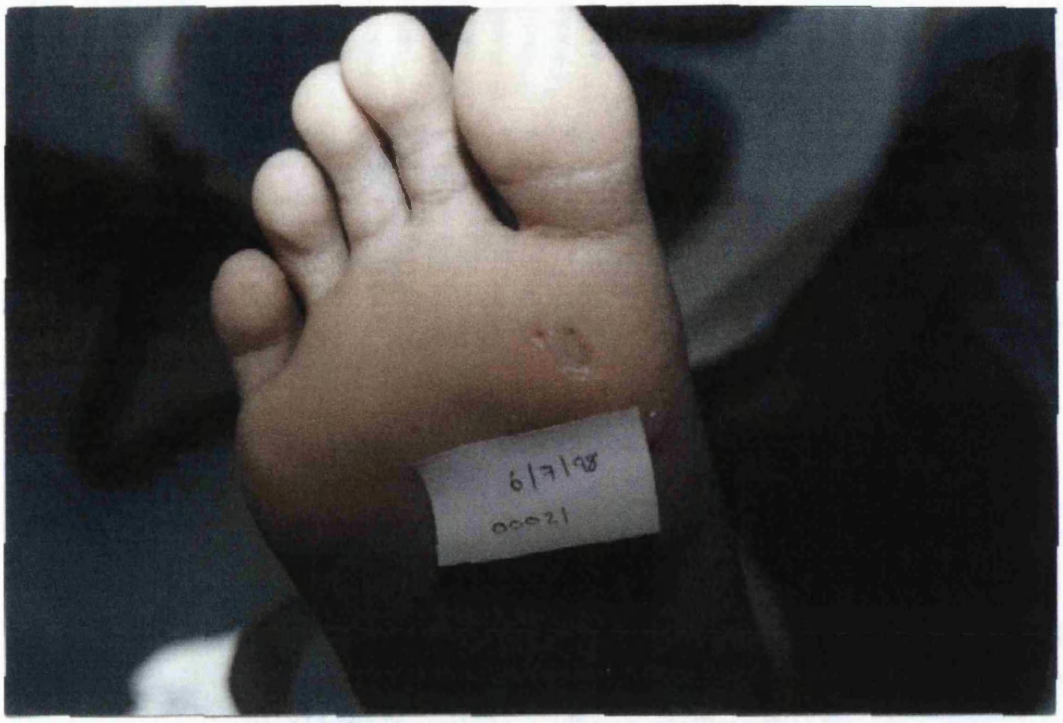


Figure 25b. Verruca after debridement

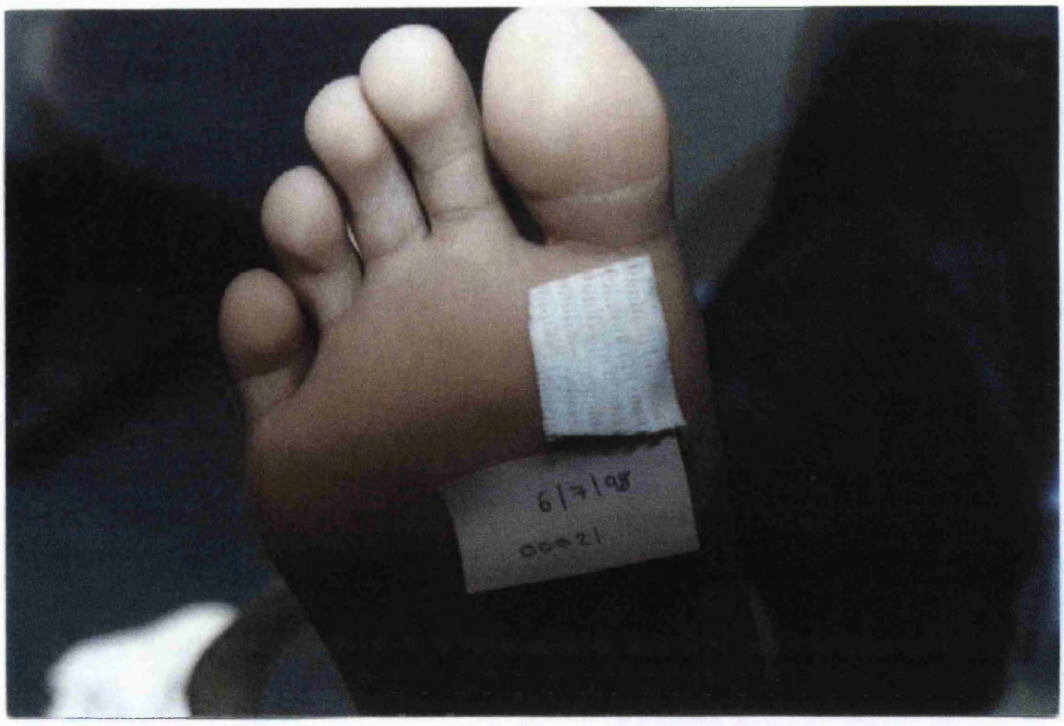


Figure 26. Gauze soaked with *Thuja occidentalis*

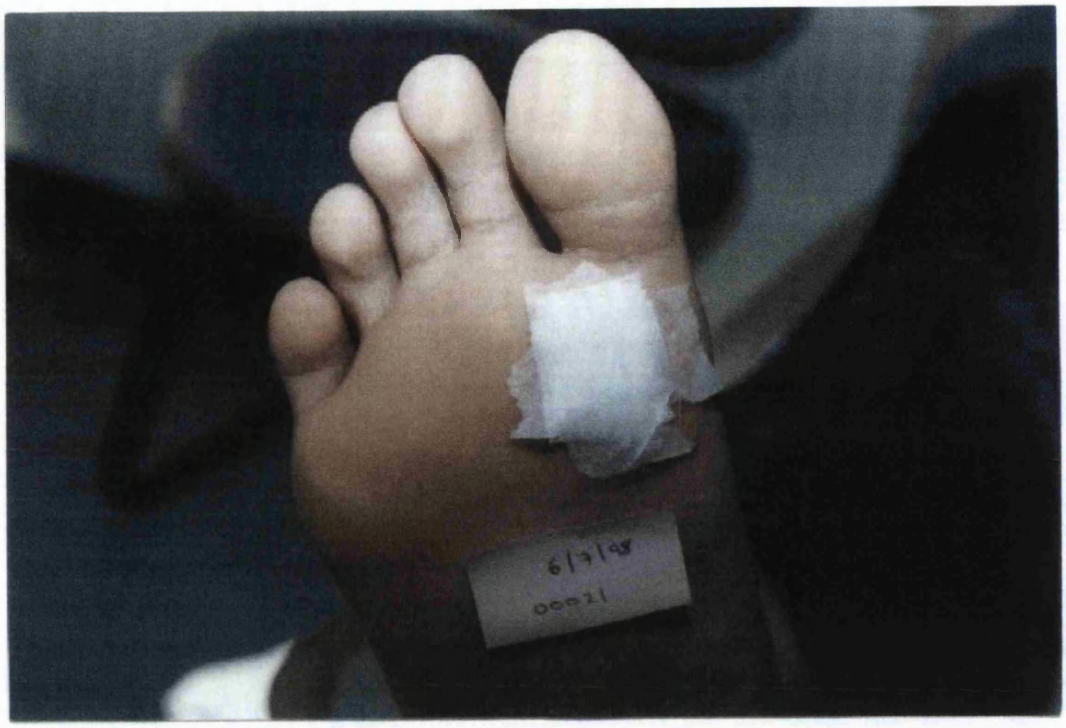


Figure 27. Micropore covering gauze

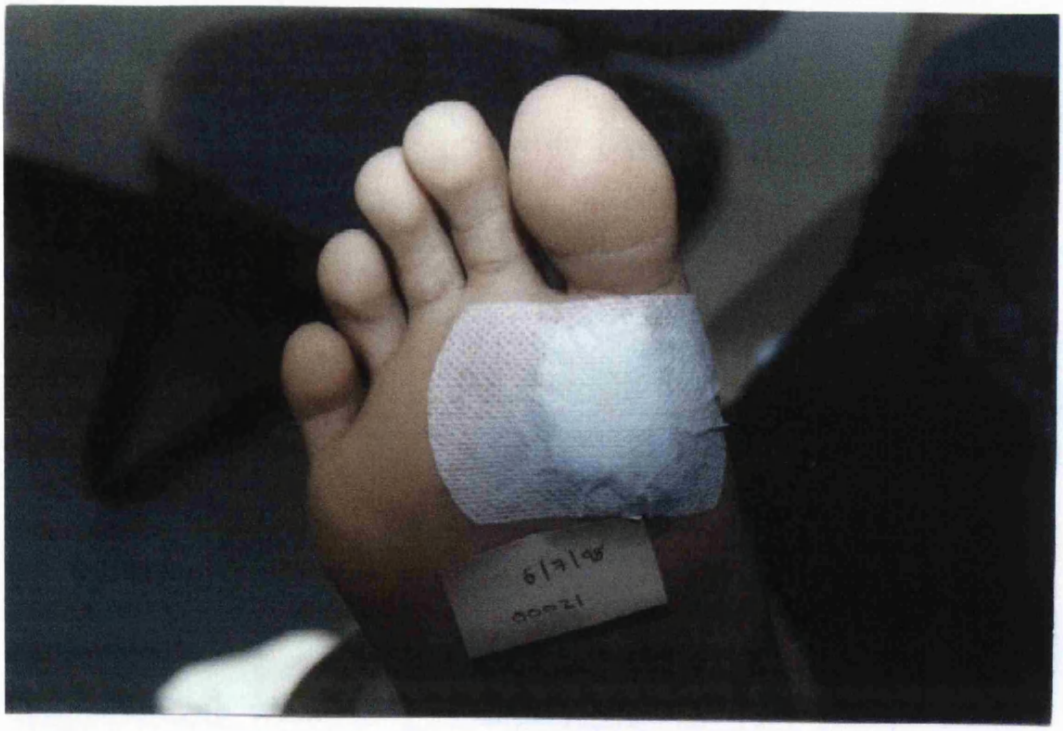


Figure 28. Meffix strapping over gauze

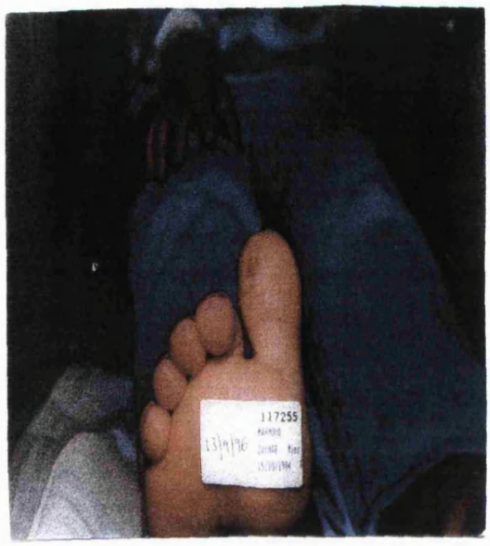
3.4.6 POST TREATMENT

At the end of 6 months verruca pedis lesions were examined and assessed by the researcher for improvement or any adverse effect of treatment. Measurements were taken and recorded by the IA.

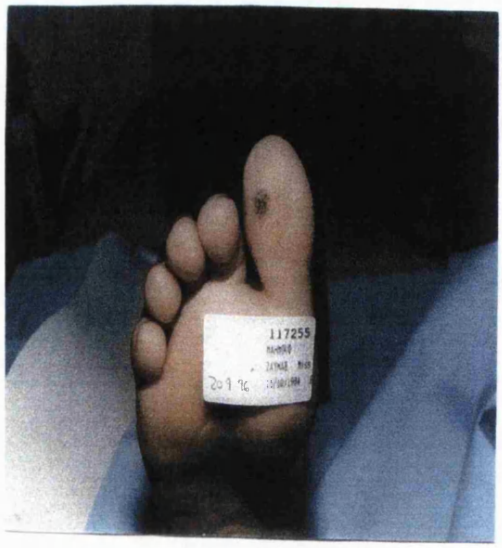
3.4.7 STATISTICAL ANALYSIS

The student's t-test was used for comparing between two groups and multiple comparisons were made using a one-way analysis of variance test (ANOVA) with equal variances at the significant level: $p < 0.05$. Where the differences were found significant, adjusted t-tests with p values corrected by the Bonferroni method were performed and only Bonferroni p values are given in the text. ANOVA is based on the assumption that not only do populations have a normal distribution but also have the same variance. In practise, data often shared differences in variances but since the sample size in each group (n=15) was consistent, the equality of variances is not considered crucial (Erickson and Nosanchuck, 1992; Ryan et al., 1985).

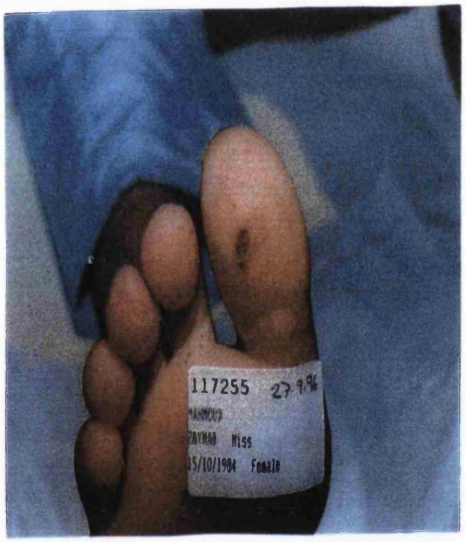
All statistical analysis were performed using Graph Pad InStat (Graph Pad Software, version 2.01, 2001).



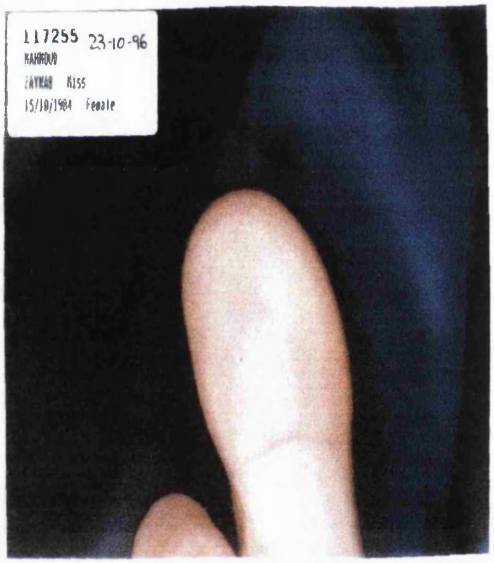
Verruca Pedis prior to treatment.



Verruca Pedis following 1 week of treatment with *Thuja occidentalis*.



Verruca Pedis following 2 weeks post-treatment with *Thuja occidentalis*.



Verruca Pedis following 1 month post-treatment with *Thuja occidentalis*.

Figure 29. Photographic presentation pre- and post-treatment

3.5 RESULTS

3.5.1 DEMOGRAPHIC DATA

Analysis of the data of pre-treatment patient characteristics showed that 81% of those entering the trial were female and 19% male. Ages ranged from 7 to 40 with the largest number being children and adults in their 20's.This can be attributed to the activities and participation in leisure pursuits at school, like swimming, physical education and fitness gyms. Ethnicity results of the study were 56% Asian population, 22% European, and a lower number of other origins (this was due to the location of the clinic, which was situated in a large Asian community). 60 % of the patients had their verruca for 1-2 years, 21% for 2-5 years and 4% greater than 5 years. This is interesting as Massing and Epstein (1963), showed that 67% of warts would regress spontaneously without treatment within 2 years period. So the verrucae present can be assessed as being over that period of natural regression. Also interesting was the types of verruca pedis seen. Most prevalent were 'common' type 48%, with mosaic verruca closely followed at 32% and smaller numbers of other verrucae: plane, flat and filiform. This indicated the HPV 'type', as common and mosaic warts are closely associated with HPV 2, 4 and 7, whilst deep plantar are associated with HPV1 and flat and plane warts with HPV3 and 10 (Bunney et al., 1992). Unfortunately biopsy were not carried out for all lesions, so it was not conclusive which HPV 'type' each verruca were.

It was interesting to note that all patients attending the trial had previously used over the counter (OTC) preparations. These included salicylic acid preparations, Formalin, Occlusol, etc. 54% of patients had received cryotherapy, whilst a few had tried homeopathic and herbal preparations.

Table 12a. Patient characteristics – Percentage difference between male and female patients represented in the study.

Gender	Number of patients	Percentage of patients
Male	17	19 %
Female	73	81 %

Graph 1.

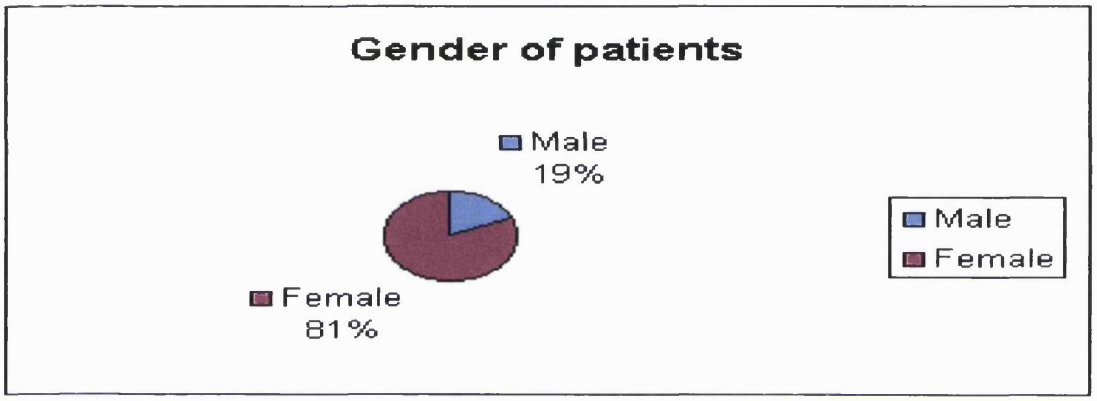


Table 2. Patient characteristics – Percentage of age groups represented in the study.

Age range	Number of patients	Percentage of patients
7-14 years	51	57 %
15-28 years	27	30 %
29-40 years	12	13 %

Graph 2.

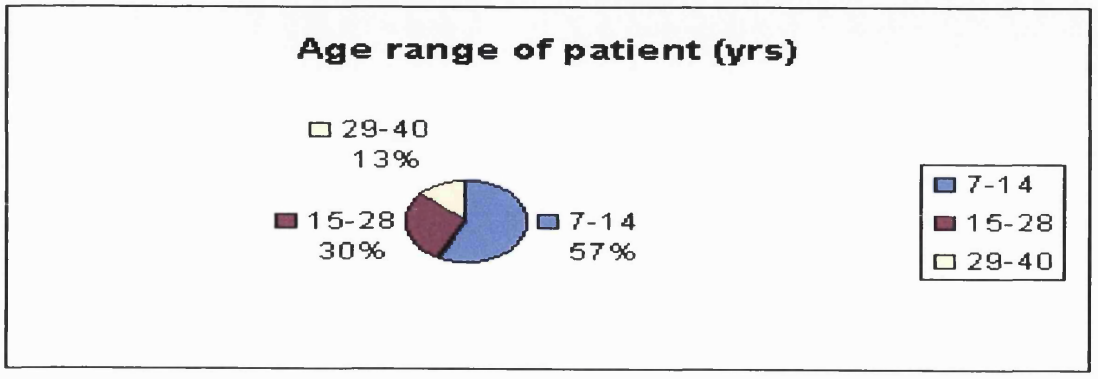


Table 12c. Patient characteristics – Percentage of ethnic origin represented by patients in the study.

Ethnic origin	Number of patients	Percentage of patients
European	13	22 %
Asian	34	56 %
Far East	2	3 %
Middle East	6	10 %
South American	1	2 %
African	4	7 %

Graph 3.

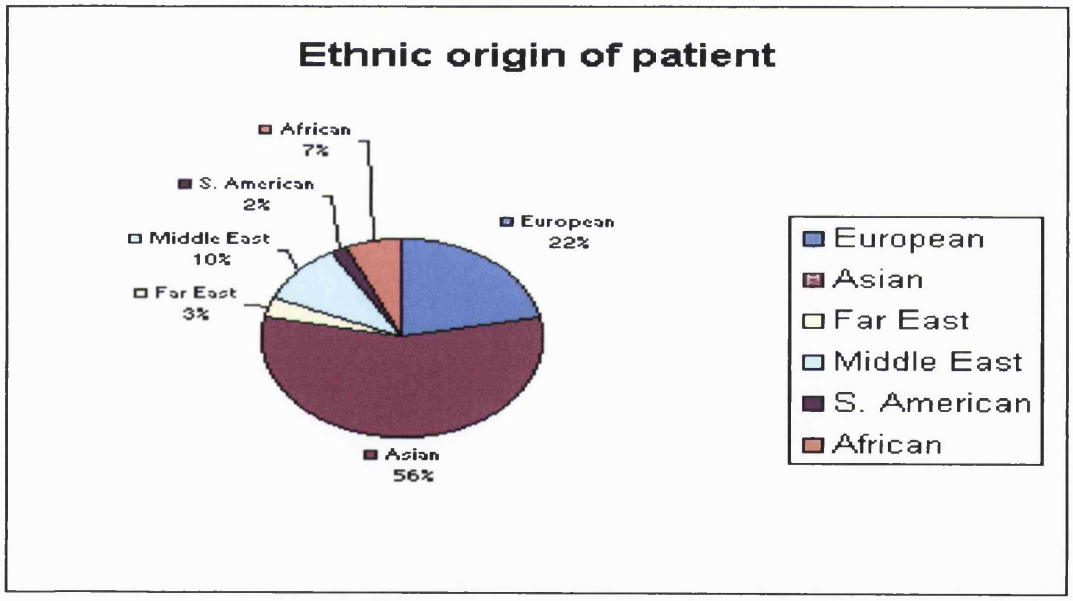


Table 13a. Patient characteristics – Percentage of patients having suffered from verruca pedis for different periods in time.

Duration of verruca pedis	Number of patients	Percentage of patients
< 6 months	5	6 %
6months – 1 year	8	9 %
1 – 2 years	54	60 %
2 – 4 years	19	21 %
> 4 years	4	4 %

Graph 4.

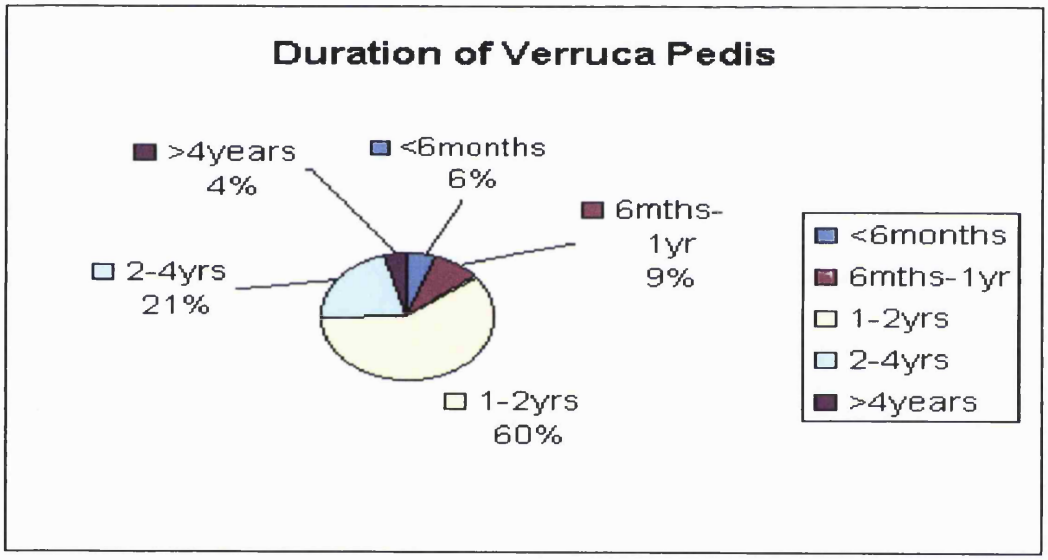


Table 13b. Percentage of patients having suffered from different types of verruca pedis.

Type of verruca pedis	Number of patients	Percentage of patients
Common	43	48 %
Mosaic	29	32 %
Filiform	6	7 %
Flat	8	9 %
Plane	4	4 %

Graph 5.

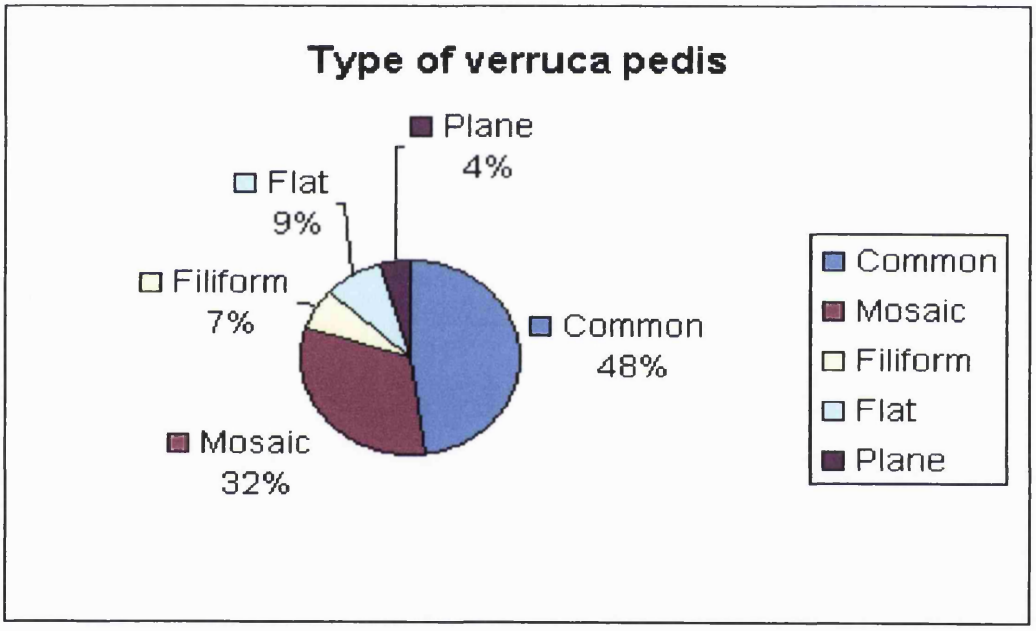
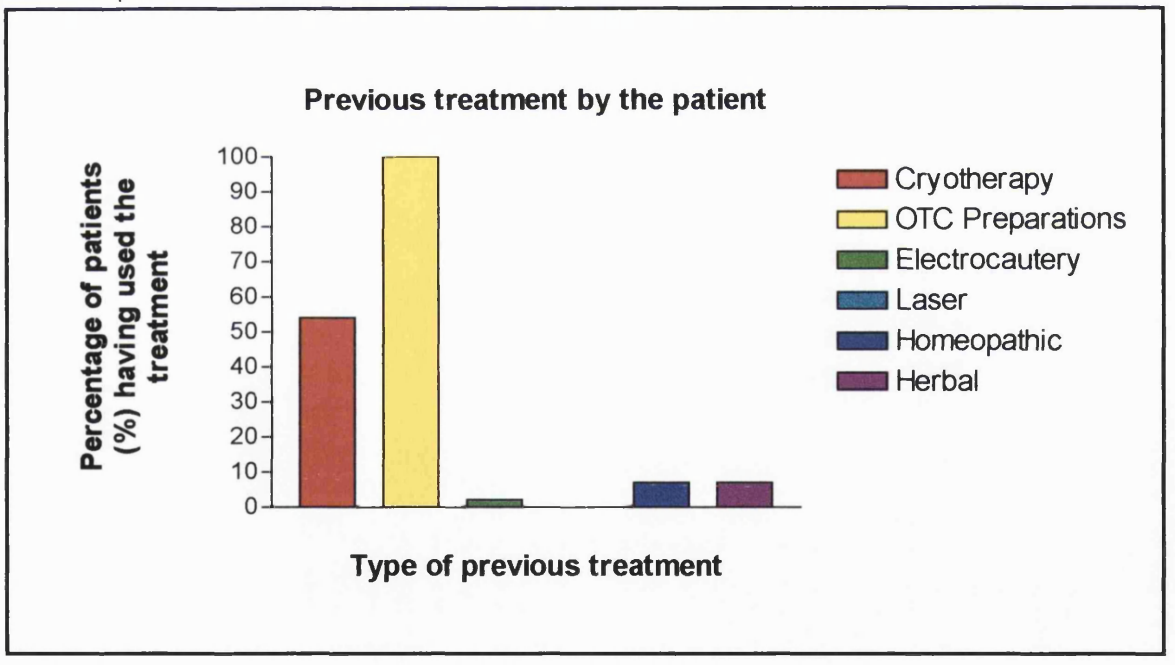


Table 14. **Pre-treatment - Percentage of patients having used various treatments.**

Type of treatments	Number of patients	Percentage of patients
OTC preparations	90	100 %
Cryotherapy	49	54 %
Electrocautery	2	2 %
Laser	0	0 %
Homoeopathic	6	7 %
Herbal	6	7 %

Graph 6.

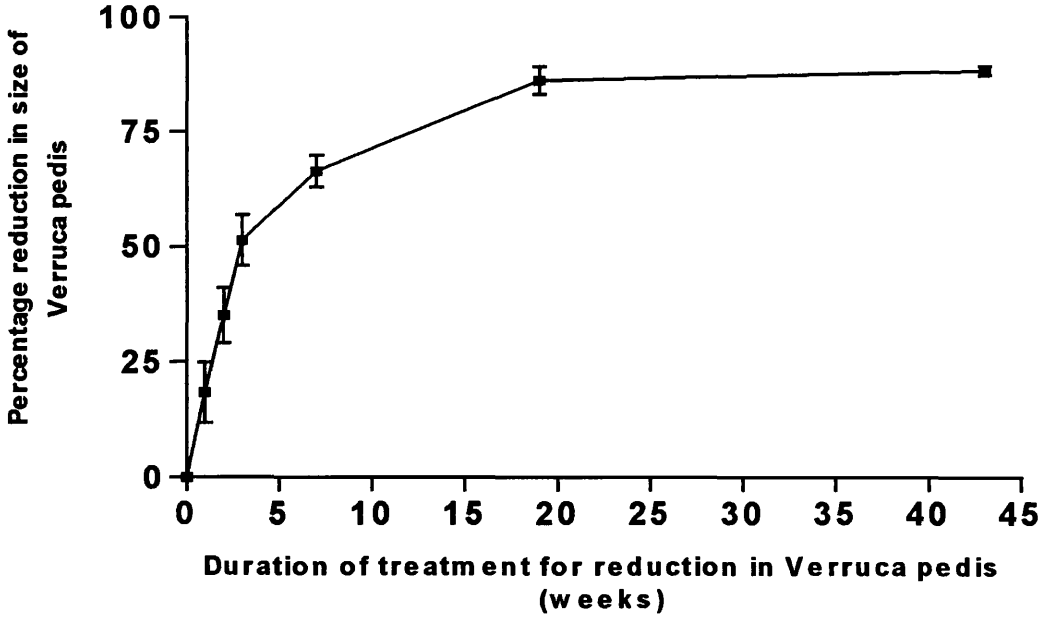


3.5.2 RESULTS TABLE STUDY 1, NUMBER OF PATIENTS = 30

Table 15a. Mean reduction in size of Verruca pedis lesion.

Percentage reduction in size of Verruca pedis.	
Time (weeks)	**Means ± SD**
1	18.3 ± 6.5
2	35.0 ± 6.0
3	51.3 ± 5.5
7	66.3 ± 3.5
19	86.0 ± 3.0
43	88.0 ± 1.0

Graph 7. Percentage reduction in size of Verruca Pedis.



A graphical representation of the mean reduction in size of Verruca Pedis during treatment with Thuja occidentalis extract. Values are a mean ± SD, where n = 30.

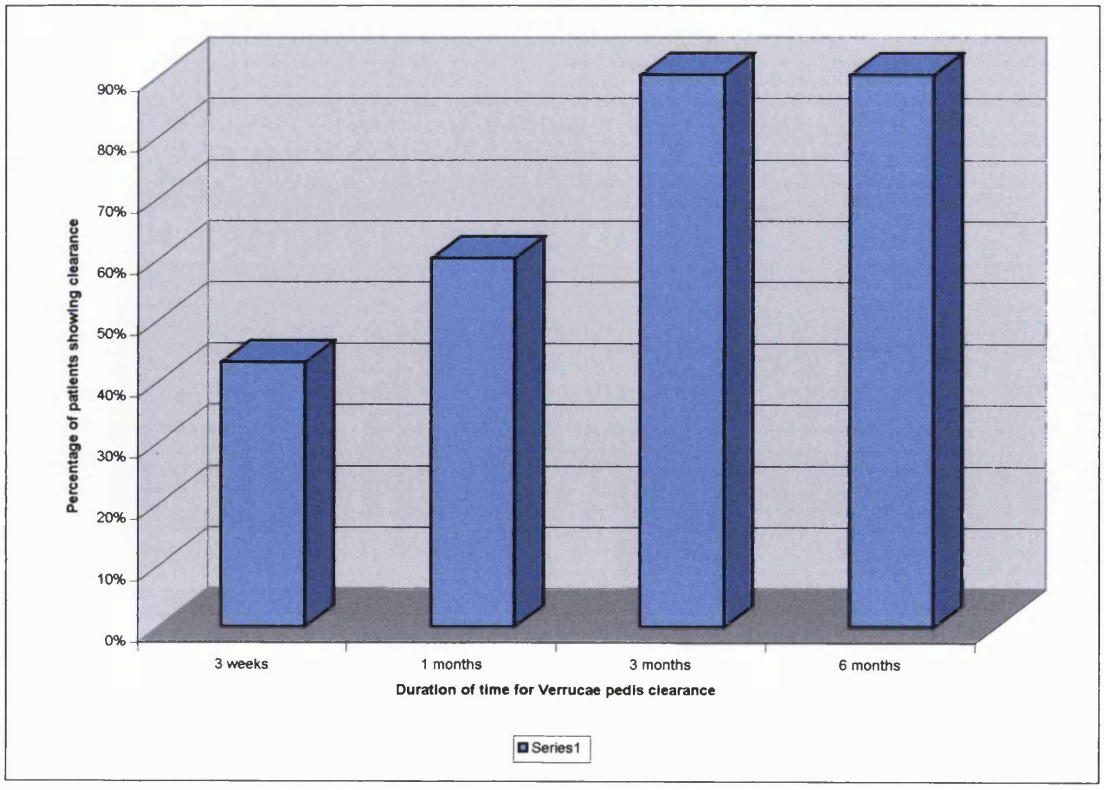
■ = *Reduction in Verruca size.*

Table 15b. Rate of verruca pedis clearance (number of patients).

Duration of time for Verruca pedis clearance.	
Length of time (weeks)	**Percentage cumulative clearance of verruca pedis**
3	43 (13 out of 30 patients)
7 (1 month)	60 (5 out of 30 patients)
19 (3 months)	90 (9 out of 30 patients)
43 (6 months)	90 (27 out of 30 patients)

The results from Table 15a show a linear reduction in the size of the verruca lesion on a weekly basis. In Table 15b, there is clearance of verruca pedis in 43% of the patients within the first 3 weeks, with a further 17% clearance in the following 4 weeks and 30% clearance in the next 3 months. 27 out of 30 patients had complete clearance of verruca pedis by the end of the study giving a total of 90% clearance.

Graph 8. Rate of Verruca clearance with *Thuja* extract.

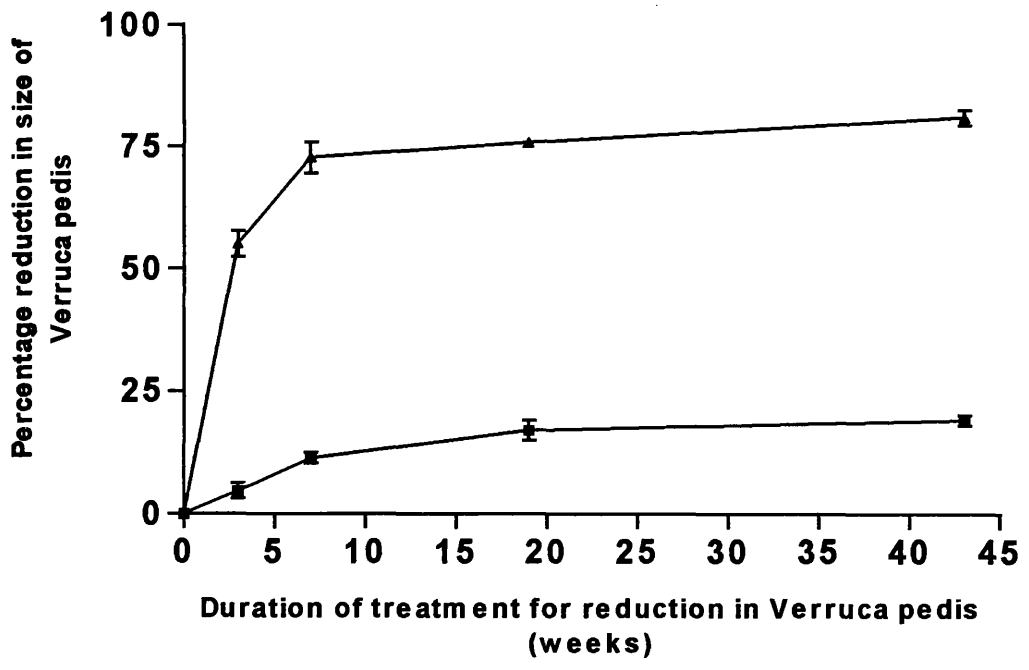


3.5.3 STUDY 2, DOUBLE BLIND CONTROLLED, 30 PATIENTS-2 GROUPS OF FIFTEEN

Table 16a. *Thuja occidentalis* compared to a placebo preparation of *Thuja occidentalis.*

Percentage reduction in size of Verruca pedis		
Time (weeks)	**Piacebo ± SD**	**Active ± SD**
3	4.6 ± 1.5	55.0 ± 2.6
7	12.0 ± 1.2	72.7 ± 3.2
19	17.0 ± 2.0	75.7 ± 0.6
43	19.0 ± 1.0	81.0 ± 1.5

Graph 9. Rate of Verruca pedis reduction in size with *Thuja* extract in comparison to Placebo.



A comparison between patients treated with Thuja and Placebo after 43 weeks, treatment revealed that the reduction in size of Verruca pedis was significantly (t-test; P<0.001) reduced. Values are a mean ± SD, where n = 15. ▲ = Active; ■ = Placebo.

Table 16b and 17. Results of comparison between active and placebo groups tested in the double blind controlled study.

Cumulative percentage rate of verruca pedis clearance (number of patients)		
Time (weeks)	**Active**	**Piacebo**
3	20 (3 patients)	0 (0 patients)
7	67 (7 patients)	13 (2 patients)
19	80 (2 patients)	33 (3 patients)
43	80 (0 patients)	33 (0 patients)

Table 17.

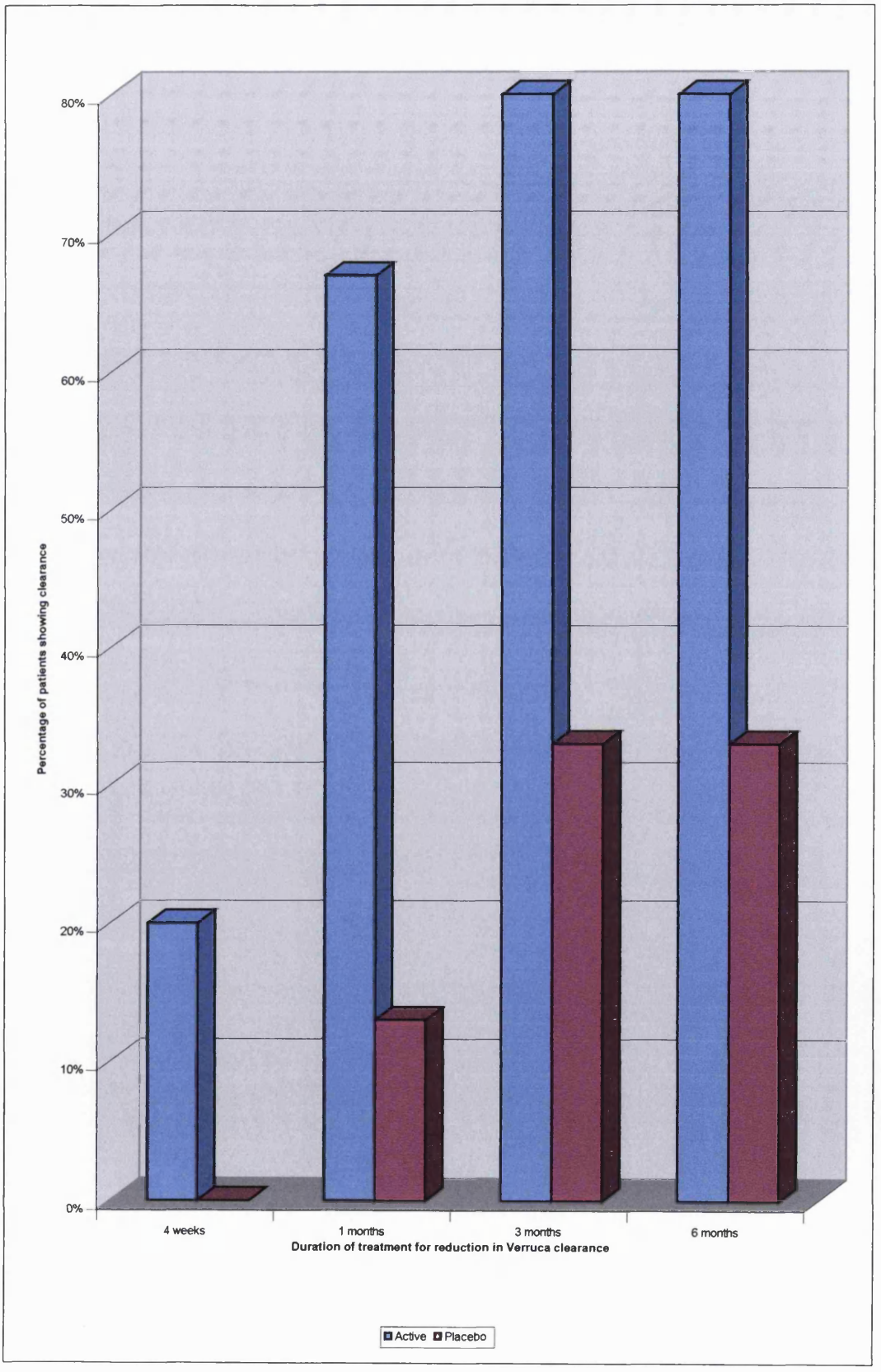
Rate of verruca pedis clearance after 11 months			
Group	**No of patients**	**No cleared**	**% Cleared**
Active	15	12	80%
Placebo	15	5	33%

The results from Table 16a show, a progressive reduction in verruca pedis size. Tables 16b and 17 show clearance of verruca pedis in the active groups, there is 20% clearance the first 4 weeks, with a further 47% reduction in the next 8 weeks and a further 13% in the following 3 months, giving an overall clearance of 80%.

In the placebo group, there was no clearance during the first four weeks, 13% clearance during the next four weeks and a further 20% clearance during the next 3 months, giving an overall clearance of 33%.

In conclusion, there is a marked difference between the active treatment and placebo and the active treatment is superior to placebo.

Graph 10. Rate of Verruca clearance with *Thuja* extract in comparison to Placebo.

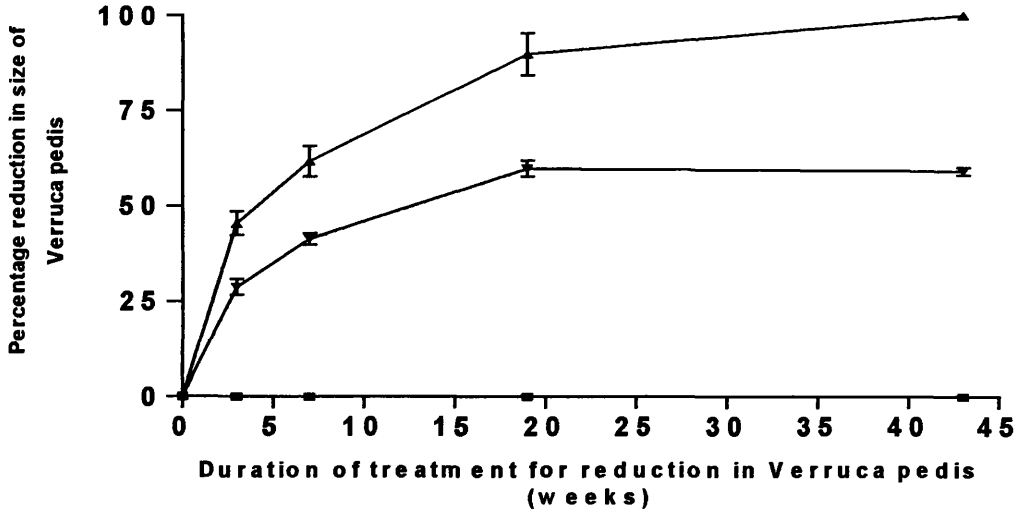


3.5.4 STUDY 3, EVALUATION OF *THUJA* FRACTIONS ON VERRUCA PEDIS.

Table 18. The percentage reduction in size of verruca lesion over time, on treatment with various *Thuja* fractions.

Percentage mean reduction in size of Verruca pedis lesion			
Time (weeks)	**Hexane ± SD**	**Chloroform ± SD**	**Ethyl acetate ± SD**
3	0.0 ± 0.0	45.3 ± 3.1	28.7 ± 2.1
7	0.0 ± 0.0	61.7 ± 4.0	41.3 ± 1.5
19	0.0 ± 0.0	89.7 ± 5.5	59.7 ± 2.1
43	0.0 ± 0.0	99.7 ± 0.6	59.0 ± 1.0

Graph 11. Rate of reduction of Verruca size with various *Thuja* fractions.



A comparison between patients treated with various Thuja fractions after 43 weeks, treatment revealed that the reduction in size of Verruca pedis was significantly (t-test; P<0.001) reduced. Values are a mean ± SD, where n = 10.

▲ = Chloroform; ▼ = Ethyl acetate; ■ = Hexane.

Table 19. The percentage clearance of verruca lesion over time, during treatment with various *Thuja* fractions.

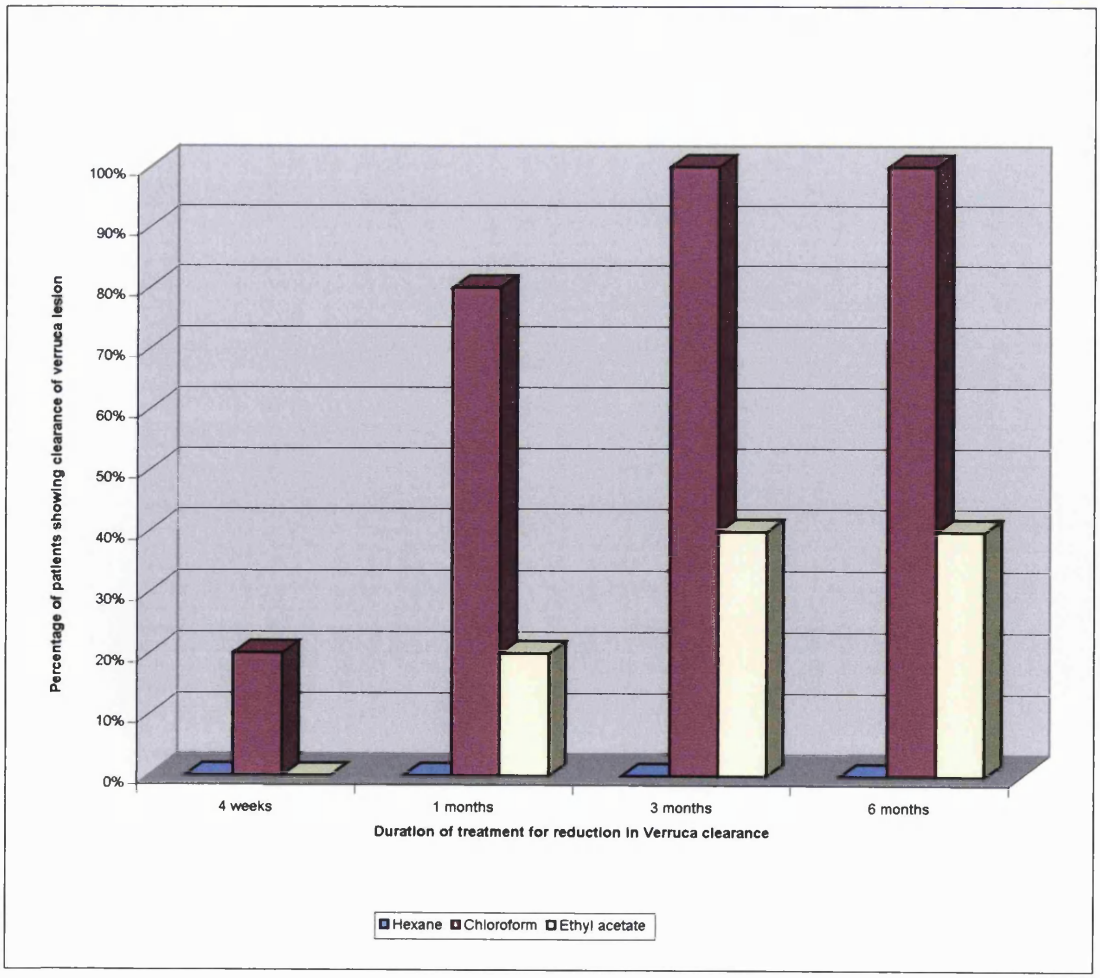
Cumulative percentage rate of verruca pedis clearance (number of patients)				
Fraction	**1 – 4 weeks**	**2 months**	**5 months**	**11 months**
Hexane	0	0	0	0
Chloroform	2 (20%)	8 (80%)	10 (100%)	10 (100%)
Ethyl acetate	0	2 (20%)	4 (40%)	4 (40%)

Hexane 0% clearance

Chloroform 100% clearance

Ethyl acetate 40% clearance

Graph 12. Rate of Verruca clearance with various *Thuja* fractions.



The evaluation of fractions indicated that the hexane fraction showed no change throughout the study. The chloroform fraction showed progressive reduction in size throughout the study. There was 20% clearance of verrucae within the first four weeks with a further 60% in the next four weeks and another 20% in the following 3 months, giving 100% clearance by the end of the study.

With the ethyl acetate fraction, there was no clearance in the first 4 weeks and a further 20% clearance in the next 3 months giving an overall 40% clearance of verruca pedis.

In comparison of the 3 fractions, the chloroform fraction was the most effective with all patients showing clearance of verrucae, the ethyl acetate fraction seemed to show some activity with some clearance, but in no way was activity superior to the chloroform fraction. The hexane fraction showed no activity throughout.

This concludes that the activity, which has an action on verruca pedis, is mainly present in the chloroform fraction, which has been confirmed with phytochemical and biological studies (Chapter 2).

3.5.5 REDUCTION OF VERRUCA PEDIS IN SIZE

The size of reduction in verruca pedis was 88% in study 1, 81% active group and 19% placebo group in study 2, whilst the fractions had 0% in hexane, 100% chloroform and 59% ethyl acetate. These indicate that all active preparations caused a steady reduction in verruca size.

3.5.6 CLEARANCE OF VERRUCA PEDIS

Patients in study 1 showed 90% clearance by the end of trial, In study 2 the active group showed 88% clearance to 33% clearance in placebo group, whilst in study 3 hexane group showed 0% clearance, chloroform showed 100% clearance and ethyl acetate showed 59% clearance.

3.6 DISCUSSION

3.6.1 PATIENT COMPLIANCE

No problems were encountered in carrying out the trial in accordance with the protocol. Care was taken to ensure that all patients were able to commit themselves to keeping clinic appointments and no patient withdrew from the trial. Care was also given to facilitate patient compliance by simplifying as much as possible the information, guidance and home treatment sheets issued to the patients.

There is evidence to suggest that painful treatment may affect patient compliance. However, in this study, compliance was enhanced due to the painless, non-invasive nature of the treatment. Patients also welcomed the ability to wear their usual footwear throughout the trial and to take a bath as normal.

3.6.2 ADVERSE REACTIONS

No adverse effects of the treatment with ethanolic crude extract and chloroform fraction of *Thuja occidentalis* were observed clinically or reported by patients.

3.6.3 OUTCOME OF CLINICAL STUDY

The outcome of the clinical trials was very impressive. The size of each group gave a significant statistical measurement of data (Kirkwood, 1991; Rees, 1994). Study one gave an indication to the value of *Thuja* as a treatment for verruca pedis. The results of this study concluded that *Thuja* was effective, it also showed that lesions would regress uniformly through out the trial. Clearance of lesions, were seen in a high proportion of patients during the first 3 weeks. From 1 to 3 months, the number of patients with lesions continued to clear at the same steady rates as at three weeks. There is no apparent reason for this except the possibility of each lesion not being at the same stage of its cycle and duration of infection, therefore regression would be taking place at different times.

Study 2, investigated how *Thuja* would be compared to a placebo preparation. 30 patients were included in the trial so that a statistically significant number was used in evaluation and analysis. Like the first study there was clear linear regression in size of the lesion, with a significant difference between active and placebo groups in clearance with $p > 0.001$. 88% clearance was achieved in the active group, where only 33% clearance achieved in placebo. The possibility that verruca regression could be explained by every therapeutic procedure implies a suggestive component (Vollmer, 1946), therefore patients being treated in the placebo group whose lesions had regressed could be the result of the researcher contact and application of dressing.

Study 3 was carried out to determine which group of compounds present in *Thuja* was directly acting on the verruca. *Thuja* was partitioned in solvents of increasing polarity hexane, being less polar than chloroform and ethyl acetate. The results seem to be clear indication of the chloroform fraction having the best effect when compared to hexane, resulting in $p<0.001$. This could be due to Lignan and Flavonoid compounds, present in the fraction. Wagner (1993), Beusher and Kopanski (1986) demonstrated that these fractions contained these families of compounds. In the hexane fraction the monoterpenoids were present, as were flavonoids in the ethyl acetate. Overall this concluded that ethyl acetate fraction had some activity against verruca pedis, but hexane fraction had no activity, which showed that the monoterpenoids were ineffective on verruca pedis.

Ethanolic crude extracts of *Thuja occidentalis* and chloroform fraction were shown to be the most effective of the samples tested. These results confirm the antiviral activity of *Thuja occidentalis* and support the findings of Wagner (1993) , Beuscher and Kopanski (1986).

Chapter 4 -

Biological Investigation of *Thuja occidentalis* on Human Keratinocytes

4.1 INTRODUCTION

The relatively simple structure of viruses may suggest that they should be easy targets for chemotherapy (Kitchington et al., 1995). However, the problems associated with specifically inhibiting viral action have proved more intractable than one might have first envisaged (Kitchington et al., 1995). As the replication cycle of a virus is intimately associated with the host cells' biochemical pathways, good selectivity to inhibit replication is hard to achieve (Blair et al., 1998). Viruses can be either clinical or persist, as a latent infection, whereupon no morphological abnormalities are seen and no virus particles are produced (Bunney et al., 1992). In this case antiviral drugs are less likely to be effective, as the virus has the ability to persist in this state, where it may be more likely to evade the host immune response (Blair et al., 1998). The virus may persist in the latent state for the lifetime of the host and the extent of latency has obvious clinical implications with respect to antiviral therapy. However, increased understanding of the molecular events of virus infections has meant that the search for antiviral drugs, against specific targets, can be conducted on a more rational basis. Most attention has been given to herpes simplex virus (HSV), human immunodeficiency virus (HIV), varicella-zoster virus (VSV), cytomegalovirus (CMV) and influenza virus, but not to the human papilloma virus (HPV). Only recently has work been carried out to unlock and understand possible mechanisms of the human papilloma virus (Blair et al., 1998). This approach has led to the discovery of a number of compounds active against herpes virus enzymes involved in DNA replication (Kitchington et al., 1995) and to the synthesis of proteinase inhibitors to combat HIV (Roberts et al., 1990). With more detailed information of how drugs act at the molecular level, it may be possible to improve efficacy further and to combat the increasing problems of drug resistance in patients on long-term treatment.

Papillomaviruses have a specific tropism for squamous epithelial cells, which is illustrated by the restriction of the viral replication functions to the most terminally differentiated keratinocytes (zur Hausen et al., 1996). By the use of

in situ hybridisation techniques it has been shown that viral DNA is present within basal and parabasal cells of a papilloma (Stoler et al., 1989). Analysis of early gene expression has revealed the presence of papillomavirus activity in the basal cells of the epidermis, while late gene expression, the synthesis of capsid proteins, vegetative viral DNA synthesis and the assembly of virions takes place only in the terminally differentiated cells, which are the most superficial within the wart (Blair et al., 1998). It is thought that this absolute requirement for terminal differentiation of squamous epithelial cells for the expression of the late viral functions is the reason why papillomaviruses have been so recalcitrant to culture *in vitro*, and it is only in recent years that progress has been made on this aspect of papilloma virology by the development of the raft tissue culture system technology (Frattini et al., 1996; Flores et al., 1999). However, Ruesch et al. (1998) demonstrated that a simple system for epithelium differentiation to study HPV-late formation could be used. A suspension of HPV-infected keratinocytes in semi solid media containing 1.6% methylcellulose for 24h was sufficient for the activation of the late promoter, transcription of late genes, and amplification of viral DNA (Ruesch et al., 1998).

The development of antiviral therapies against the human papilloma viruses requires appropriate *in vitro* and *in vivo* models and the restricted species and tissue specificity of these viruses has made this difficult (Blair et al, 1998). Study models are currently restricted to *in vitro* organotypic 'raft' cultures, and heterologous and homologous *in vivo* animal system (Blair et al, 1998). Heterologous animal models include the severe-combined immunodeficient (SCID) and athymic mouse xenograft system, a mouse model grafted with syngeneic keratinocyte expressing human viral proteins (Blair et al, 1998, Sundberg et al., 1987a), and more recently the transgenic mouse model where transgene expression is restricted to the epithelium by use of the human keratin 14 gene promoter (Blair et al, 1998). Homologous systems (surrogate models) employ animal papillomaviruses in their natural hosts and include bovine papillomavirus models, cottontail rabbit papilloma virus (CRPV) model and canine oral papillomavirus (COPV) model (Brandsma 1996, Nicholls et al., 1999). Nicholls et al. (1999) demonstrated that recurrent

lesions seen in some human papillomavirus infections, such as recurrent laryngeal papillomatosis, are associated with specific defects in host immunity rather than variations in viral pathogenicity.

The difficulty associated with recreating the three-dimensional structure of the epithelium on which the virus depends to complete its life cycle led to establishing the HPV type 16 (HPV-16) life cycle in an immortalised human foreskin keratinocyte (HFK) cell line (Flores et al., 1999). This cell line exhibited many of the characteristics of the early passage HFKs including the ability to stratify and terminally differentiate in an organotypic raft culture system (Flores et al 2000). Because of their similarity to early-passage HFKs, these cells were tested for their ability to support the HPV-16 life cycle (Flores et al., 1999). It was found that these cells were able to stably maintain two HPV genotypes, HPV-16 and HPV-31b, episomally (Flores et al., 1999). In addition to this when this cell line was transfected with HPV-16 and cultured using the organotypic raft culture system (rafts), it sustained the HPV-16 life cycle (Flores et al., 1999).

Two different approaches are available for investigating compounds that may be active against any particular virus. The first involves random screening of compounds, such as those isolated from plant extracts or from collections of chemically synthesised compounds, which may have been produced for other purposes. Those compounds, which show activity, may then be used to synthesize new analogues in an attempt to improve their antiviral activity. The second approach is to synthesise compounds, which are designed to act on a specific viral target (Kitchington et al., 1995). The subsequent discovery of an active drug may involve a detailed analysis of structure-activity relationships of a series of derivatives, together with an assessment of their toxicity in the target cells. The relationship between the antiviral activity and toxicity of a compound is given as its selectivity index (SI).

$$SI = \frac{\text{Drug concentration required to inhibit cell proliferation (or DNA synthesis)}}{\text{Drug concentration required inhibiting virus replication}}$$

Usually a compound should have an SI of > 100 *in vitro* before it would be considered for further development (Kitchington et al. 1995).

The preliminary evaluation of new drugs often starts with screening for toxicity in the culture system to be used. This ensures that the drugs will eventually be tested in the appropriate concentration range when tested against the virus. A crude measure of toxicity can be obtained simply by including a series of dilutions of the drug in maintenance medium added to cell cultures in clusters plates and observing the cells for evidence of toxicity (Kitchington et al., 1995). Staining of the cells with a vital stain after exposure to the drug can be used and is most suitable for assessing toxicity in cultures of non-adherent cells. However, repeated passage of cells in the presence of the compound, accompanied by total and viable cell counts (Formazan method, which is based on the fact that the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is converted to dark blue formazan by living cells but not by dead cells or culture medium.), provides more discriminating method of detecting cytotoxicity (Mossman 1983). Many compounds can be excluded from further studies at this stage. Compounds that are not soluble in water are usually dissolved in dimethylsulphoxide (DMSO), and the final concentration of DMSO in cell culture medium must be no more than 1% (Kitchington et al., 1995).

The activity of compounds on viruses is generally assessed by dose-response experiments. A standard virus preparation is incubated with indicator cells in the presence of increasing concentrations of compound to determine the concentration that will inhibit virus replication. The assays can be made quantitative by a number of methods: plaque counting, dye uptake, or virus antigen detection. The activity of the drug or the sensitivity of the virus isolate

to a given drug is usually expressed as the concentration of drug required inhibiting virus replication by 50% (IC_{50}: 50% inhibitory concentration).

It has been well documented that Thuja *occidentalis* has an activity against a host of different viruses. Beuscher and Kopanski (1986) demonstrated that an extract of *Thuja occidentalis* had an antiviral effect on herpes simplex 1 virus (HSV-1) *in vitro*. Using a test model, the plaque reducing assay with HSV-1, showed that 90% plaque reduction was achieved between the ranges of 50μg/ml to 1000μg/ml. The 50% cytotoxic dose in the highly purified fraction was 400μg/ml and the antiviral dose was smaller than 50μg/ml. This indicates that *Thuja occidentalis* had an antiviral activity against herpes simplex virus and a good therapeutic potency *in vitro*. Roth (1993), isolated the lignan deoxypodophyllotoxin from *Thuja occidentalis* and investigated its antiviral effect on a strain of the influenza virus *in vitro*. The results demonstrated that the compound was successful in arresting viral growth. Hassan et al. (1996) showed that a polysaccharide of *Thuja occidentalis*, *Thuja* polysaccharide g (TPSg), an anti-human immunodeficiency virus (HIV-1) agent, isolated from *Thuja occidentalis* enhances *in vivo* haemopoetic progenitor cells recovery in sublethally eradicated mice (*in vitro*). Gohla et al. (1992) had earlier reported the effect of TPSg and was shown to be an inducer of the CD4+ fraction of the human peripheral blood T-cell subset. Furthermore, it could be demonstrated that TPSg is a potent inhibitor of the expression of HIV-specific antigens and of the HIV-1 specific reverse transcriptase.

As described in previous chapters, *Thuja occidentalis* has been clinically investigated *in vivo* and phytochemically. The results from both investigations have given an introduction to the action of this plant on the wart/verruca and shows that it is therapeutically beneficial as a treatment for verruca pedis. From the results of the phytochemical investigation, the compounds deoxypodophyllotoxin and kaempferol 3-methyl ether, were identified in the chloroform sample, which were significantly active against verruca pedis *in vivo*. Such experiments can provide insights into the potential mode of action of these compounds.

As a first step towards gaining a better understanding of *Thuja occidentalis* on keratinocytes, the aim of this chapter will be to determine how *Thuja occidentalis* affects keratinocytes which are infected with human papilloma virus compared with non-HPV (cells that excessively proliferate without HPV present) containing keratinocytes. As reported previously, the difficulties experienced testing HPV viruses is such that the following investigations will be carried out: Toxicity testing, using the Formazan method, measuring compound efficacy in cell culture by dose-response experiments and bivariate staining for analysis of cell cycle.

4.1.1 THE CELL CYCLE (FACS Labs, Cancer Research UK 2003)

A eukaryotic cell cannot divide into two, the two into four, etc. unless two processes alternate:

- Doubling of its genome (DNA) in S Phase (synthesis phase) of the cell cycle
- Halving of that genome during mitosis (M Phase)

The period between M and S is called G_1; that between S and M is G_2

So, the cell cycle consists of:

- G_1 = growth and preparation of the chromosomes for replication
- S = synthesis of DNA (and centrioles)
- G_2 = preparation for
- M = mitosis

When a cell is in any phase of the cell cycle other than mitosis, it is often said to be in interphase.

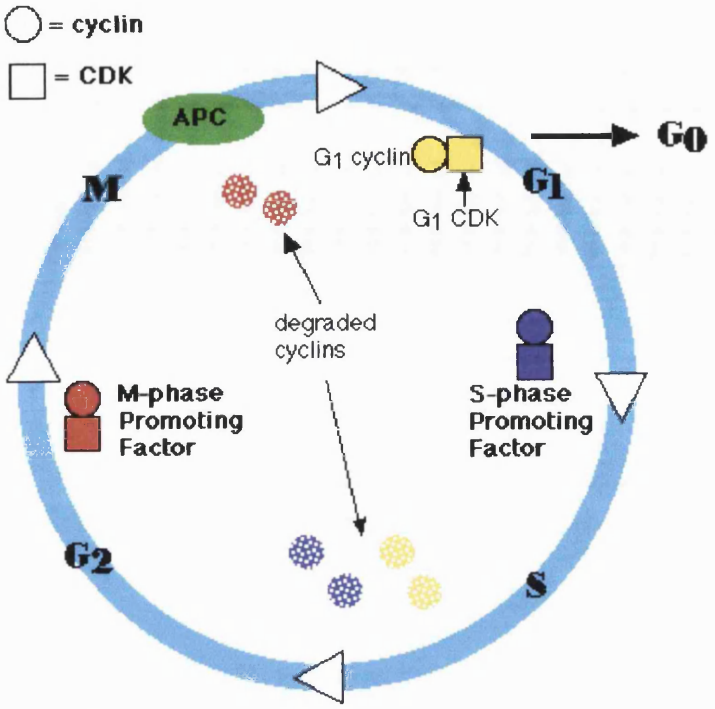


Figure 30. Cell Cycle

4.1.2 CONTROL OF THE CELL CYCLE (FACS Labs, Cancer Research UK 2003)

Proteins in the cytoplasm control the passage of a cell through the cell cycle. Among the main players in animal cells are:

Cyclins

- G_1 cyclins
- G_1/S- and S-phase cyclins
- M-phase cyclins

Their levels in the cell rise and fall with the stages of the cell cycle.

Cyclin-dependent kinases (CDKs)

- G_1 CDKs
- A CDK shared by both G_1/S- and S- cyclins
- M-phase CDK

Their levels in the cell remain fairly stable, but each must bind the appropriate cyclin (whose levels fluctuate) in order to be activated. They add phosphate groups to a variety of protein substrates that control processes in the cell cycle.

The anaphase-promoting complex (APC) and other proteolytic enzymes. The APC:

- Triggers the events leading to destruction of the cohesions and thus allowing the sister chromatids to separate.
- Degrades the mitotic (M-phase cyclins)

4.1.3 STEPS IN THE CYCLE (FACS Labs, Cancer Research UK 2003)

- A rising level of G_1 cyclins bind to their CDKs and signal the cell to prepare the chromosomes for replication.
- A rising level of S-phase promoting factor (SPF), which contains both G_1/S prepares the cell to enter S-phase and duplicate its DNA (and its centrioles).
- As DNA replication continues, the G_1/S-phase cyclins (cyclin E) are destroyed and the level of M-phase cyclins begins to rise (in G_2).
- M-phase promoting factor (the complex of M-phase cyclins with M-phase CDK) initiates
    - assembly of the mitotic spindle
    - breakdown of the nuclear envelope
    - condensation of the chromosomes
- these events take the cell to metaphase of mitosis
- at this point the M-phase promoting factor activates the anaphase promoting complex (APC) which
    - separates the sister chromatids at the metaphase plate to separate and move to the poles(= anaphase), completing mitosis
    - destroys the M-phase cyclins. It does this by conjugating them with protein ubuquitin which targets them for destruction by proteasomes.
    - turns on synthesis of G_1 cyclins for the next turn of the cycle.
    - Degrades geminin, a protein that has kept the freshly-synthesised DNA in S phase from being re-replicated before mitosis.

4.1.4 G ZERO (FACS Labs, Cancer Research UK 2003)

A cell will leave the cell cycle, many times, temporarily or permanently. It exits the cycle at G_1 and enters a stage designated G_0 (G zero). A G_0 cell is often called "quiescent", but that is probably more a reflection of the interests of the scientists studying the cell cycle than the cell itself. Many G_0 cells are anything but quiescent. They are busy carrying out their functions in the organism, e.g. secretion, conducting nerve impulses, attacking pathogens.

Often G_0 cells are terminally differentiated: they will never reenter the cell cycle but instead will carry out their function in the organism until they die.

For other cells, G_0 can be followed by reentry into the cell cycle. Most of the lymphocytes in human blood are in G_0. However, with proper stimulation, such as encountering the appropriate antigen, they can be stimulated to re-enter the cell cycle (at G_1) and proceed on to new rounds of alternating S phases and mitosis.

4.2 MATERIALS AND METHODS

4.2.1 TISSUE CULTURE MEDIA AND REAGENTS

All tissue culture reagents were supplied by Sigma unless otherwise stated.

4.2.1.1 TISSUE COLLECTION MEDIUM

This was prepared by adding the following components to Dulbecco's modified Eagle's medium (DMEM, Gibco):

Penicillin G (sodium salt)	100units/ml
Streptomycin sulphate	100μg/ml
Amphotericin B	2.5μg/ml

This was supplemented with either 10% donor calf serum (DCS, Gibco) to prepare media for the growth of NIH3T3 cells or with 10% foetal calf serum (FCS, Gibco) for the maintenance of all other cell lines.

4.2.1.2 KERATINOCYTE BASE MEDIUM

This was made up to 440ml as follows:

DMEM (Dulbecco's modified Eagle's medium)	75% (v/v)
Ham's F12 Medium (ICN, Thame)	25% (v/v)
To which was added	45ml FCS

4.2.1.3 RM+ CONCENTRATE

Stock solutions of the mitogens comprising RM+ concentrate (keratinocyte culture medium) were prepared as follows:
All stocks were stored at -20^0 C.

- Adenine: 2.43g was dissolved in distilled water and adjusted to pH 9.0 by the drop-wise addition of 1.0 M sodium hydroxide. The solution was made up to a total volume of 10 ml and stored as 1ml aliquots.

- Cholera Toxin: 1ml of distilled water was added to a 1mg vial and the solution was stored as 100µl aliquots.
- Epidermal growth factor: 1mg of EGF was dissolved in 1 ml of distilled water and stored as100µl aliquots.
- Hydrocortisone: 100 mg of hydrocortisone were dissolved in 25 ml of distilled water and stored in 1 ml aliquots.
- Insulin: 500 mg of bovine pancreatic insulin were dissolved in 10 ml 0.05M HCl and stored in 1 ml aliquots.
- Liothyronine (Tri-iodo-thyronine): 0.13 mg liothyronine were dissolved in 6 ml 1M HCl / absolute ethanol (1:2), made up to 100ml in distilled water and stored in 100µl aliquots.
- Transferrin: 500 mg of human transferring were dissolved in 10ml distilled water and stored as 1 ml aliquots.

The stock RM+ concentrate was made up as follows, sterilised by passage through a 0.2 µm filter (Acrodisc, Gelman Sciences) and stored as 4ml aliquots at -20^0C:

Adenine stock solution	1 ml
Cholera toxin stock	100µl
EGF stock solution	100µl
Hydrocortisone	1 ml
Insulin stock solution	1 ml
Liothyronine stock solution	100µl
Transferrin stock solution	1 ml
Distilled water	to 100 ml

4.2.1.4 RM + MEDIUM

This was prepared by the addition of 4ml of RM+ concentrate to 440ml keratinocyte base medium to give final concentrations of:

Hydrocortisone	0.4μg/ml
Cholera toxin	10^{-10}M
Transferrin	5μg/ml
Liothyronine	2×10^{-11}M
Adenine	1.8×10^{-4}M
Insulin	5μg/ml
Epidermal growth factor (EGF)	10ng/ml

Reason for contents of medium:

- Hydrocortisone has a potent anti-inflammatory effect.
- Cholera toxin is a multimeric protein toxin from *vibrio cholerae*. The A subunit activates adenyl cyclase irreversibly. The B subunit has 5 identical monomers, binds to GM1 ganglioside and facilitates passage of the A subunit across the cell membrane.
- Transferrin is the iron storage protein, found in mammalian serum. It is an important constituent of growth media.
- Liothyronine influences growth and metabolism for cell growth.
- Adenine is one of the bases found in nucleic acids and nucleotides. In DNA, it pairs with thymidine.
- Insulin is a polypeptide hormone, which induces hypoglycaemia. It is also a mitogen and has sequence homologies with other growth factors.
- Epidermal growth factor (EGF) is active on a variety of cell types, especially but not exclusively epithelial. A family of similar growth factors is now recognized. It is the human equivalent originally named urogastrone owing to its hormone activity.

Trypsin 100mls (a) commercially available as 2.5% (10x) (Gibco #610.5090) sterile solution or (b) by adding 25g trypsin powder (DIFCO, 1:250) per litre of normal (0.85%) saline

Versene 0.02% EDTA in PBS containing 0.5% phenol red

Phosphate Buffered Saline (PBS)

NaCl	8g/l
KCl	0.2g/l
Na_2HPO_4	1.44g/l
KH_2PO_4	0.24g/l

Adjusted to pH 7.4 using HCl

4.2.1.5 CELL CULTURE

The Centre for Cutaneous Research, Cancer Research UK, Whitechapel, provided cell lines used for this study.

Two cell lines were used, RTS3b cells (Rapp et al 1997) and Dt16 cells (Sampson et al 2001). RTS3b cells were a spontaneously immortalised renal transplant recipient line derived from a patient with a squamous cell carcinoma. The other lines used, Dt16 cells, were derived from normal skin immortalised with HPV 16 DNA (Sampson et al 2001). The need to assess different cell lines was due to investigate the mode of action of *Thuja occidentalis*, in evaluating whether the activity is cytotoxic or cytostatic. RTS3b cells are a line of cells, spontaneously immortalised whereas DT16 cell line has been immortalised with transfected HPV 16 DNA, which gives us an understanding whether *Thuja occidentalis* is affecting the cell directly or the virus.

All procedures using human tissue were carried out routinely in a Class II laminar flow hood under aseptic conditions. Disposal of waste cultures and

medium through the normal procedures of the ICRF was achieved by autoclaving or decontaminating with sodium hypochlorite solution (0.5M).

4.2.1.6 CRYO-PRESERVATION OF CELL LINES

Trypsinized cells were counted and resuspended in freezing medium containing a mixture of 10% DMSO and 90% foetal calf serum. The final concentration of cells was 2 x 10^6/ml. The cell suspension was placed in a 2ml screw-top ampoule and left at 4°C for three hours. Cells were then placed in a biological freezer unit and frozen at -70°C at a rate of -1°C per minute. To defrost cells, the ampoule was removed from the liquid nitrogen and rapidly thawed for a few minutes in a water bath at 37°C. The contents of the ampoule were then placed in a Falcon flask with 10ml of growth medium and 10% fetal calf serum to dilute out the DMSO. Cells were spun at 800 rpm for 5 minutes before replating the following day and the cells were sub-cultured as normal.

4.2.1.7 RECOVERY

The medium was decanted from the culture flask and the monolayer washed with sufficient trypsinizing solution to cover the cells. The flasks were left to incubate at room temperature for approximately 15 minutes until the cells became detached. The resulting cell suspension was transferred to the sterile centrifuge tubes (10ml) and spun at 1000 rpm in a IEC 215A rotor for approximately 5 minutes. The supernatant was discarded and the cells resuspended in culture medium. The cells were counted using a haemocytometer and the appropriate number of cells added to the fresh culture vessels containing culture medium to give a final concentration of 2.5 x10^3 cell/ml. The flasks were placed in the incubator with 5% CO_2 air at 37°C until the medium became pale orange in colour as suggested by Barlow and Pye (1990). After a sample of the cells had been subjected to between 10 and 15 passages, it was replaced by another batch taken from liquid nitrogen storage while wearing gloves and a facemask. A liquid nitrogen frozen ampoule was removed from the cylinder and thawed in a water bath at 37°C. The outside of the tube was sterilised with 70% ethanol and allowed to dry

before the contents were aseptically transferred to a culture flask. Sufficient culture medium was added to dilute DMSO at least tenfold. The flask was then placed in an incubator as described previously. The cells were left to settle for 24 hours and the culture medium was replaced and then incubated until the culture was 90% confluent.

4.3 TECHNIQUES

4.3.1 CELL PROLIFERATION ASSAYS

MTT is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes (Slater et al., 1963). This water insoluble formazan can be solubilized using isopropanol or other solvents and the dissolved material is measured spectrophotometrically, as the absorbance brings forward a concentration of converted dye.

The cleavage and conversion of the soluble dye to the insoluble purple formazan has been used to develop an assay system alternative to the conventional ^{3}H-thymidine uptake and other assays for measurement of cell proliferation. Active mitochondrial dehydrogenases of living cells will cause this conversion. Dead cells do not cause this change. This has been applied in measurement of interleukin-2 activity in a multiwell assay (Mossman, 1983). Modification has improved the sensitivity (Denizot and Lang, 1986).

4.3.2 BIVARIATE STAINING

The use of the bromodeoxyuridine (BrdU) incorporation for the analysis of the cell cycle has proved very useful in the study of cell cycle kinetics. Bromodeoxyuridine is an analogue of the DNA base thymidine and competes with that base for uptake during the synthesis of DNA. Only those cells that have been actively synthesising DNA during that time that BrdU is present will stain positively. However, unless DNA is undergoing repair, synthesis will usually follow after damage. It can be detected by a monoclonal antibody and

by simultaneously staining for DNA content with propidium iodide, the percentage of cells in G_0/G_1, S and G_2/M can be determined. By altering the time that BrdU is present cell cycle times can also be assessed. Or by pulse labelling with BrdU for a short time, a labelled cohort of cells can be followed round the cell cycle (Lackie and Dow, 1999).

4.4 METHODOLOGY

4.4.1 PLANT MATERIALS

Thuja occidentalis plant material (400g) yielded 74.38g of *Thuja* crude extract. *Thuja* extract (2g) was dissolved in 50% ethanol (1mg/ml) and stored in a freezer at -20°C. Prior to testing, the extract was thawed and filtered through a sterile filter of size 0.2μm.

3mls of *Thuja occidentalis* extract was mixed with 27mls of DMEM, indicating the first serial dilution 1:10 (0.01%) of 50% ethanol. 3ml was further taken from this dilution and added to another 27mls of DMEM representing 1:100 (0.01%) dilution. This process was continued until a 0.00001% (1:100000) dilution was reached. The same process was carried out for the known antiviral/cytotoxic drug podophyllotoxin (1mg/ml) in 50% ethanol.

In order for the *Thuja* extract to be dissolved for biological studies, it first had to be dissolved into a solvent, water alone was not able to completely dissolve it. Thus, a mixture of ethanol and water was used. At a concentration of 50:50 the *Thuja* extract was completely dissolved. The next important concern was the effect this solvent would have on the results of the studies by having action on the cells. A 50:50 concentration of water-ethanol mixture was acceptable as this showed no affect on growth patterns of the cell lines tested. This was measured using the Formazan Method (MTT).

4.4.2 PREPARATION OF FRACTIONS

The method of evaluation of the crude extract of *Thuja occidentalis* was most effective. So similar methodology was implemented when evaluating separated partitions of *Thuja*. The crude extract of *Thuja* was separated in hexane, chloroform and ethyl acetate fractions. This was carried out to evaluate the activity of the compounds present in the *Thuja occidentalis* and to identify the active compounds.

2g of the original *Thuja* fraction were serially diluted, in 50ml of a 50% concentrate of ethanol. The same methodology was carried out for the preparation of Podophyllotoxin.

Table 20. Concentration of *Thuja* Extract, Fraction and Podophyllotoxin on Cell Cultures.

Dilution factor	Agent = x*
	1.0 mg/ml
1:10	0.1 mg/ml
1:100	10.0 µg/ml
1:1000	1.0 µg/ml
1:10000	0.1 µg/ml
1:100000	10.0 ng/ml

* x = *Thuja* crude, hexane fraction, chloroform fraction, ethyl acetate fraction, podophyllotoxin

The Table 20 shows the amount of *Thuja* extract, fractions and podophyllotoxin at different serial dilutions.

Table 21. The concentration of *Thuja* extract with the comparison of quantities of fractions at each concentration.

The dilutions used of fraction from *Thuja* crude extract					
Dilution	*Thuja*	Hexane	Chloroform	Ethyl Acetate	Podophyllotoxin
	1.0 mg/ml	0.27 mg/ml	0.21 mg/ml	0.15 mg/ml	1.0 mg/ml
1:10	0.1 mg/ml	.027 mg/ml	.021 mg/ml	0.015 mg/ml	0.1 mg/ml
1:100	10.0 µg/ml	2.7 µg/ml	2.1 µg/ml	1.5 µg/ml	10.0 µg/ml
1:1000	1.0 µg/ml	0.27 µg/ml	0.21 µg/ml	0.15 µg/ml	1.0 µg/ml
1:10000	0.1 µg/ml	0.027µg/ml	0.021µg/ml	0.015 µg/ml	0.1 µg/ml
1:100000	10.0 ng/ml	2.7 ng/ml	2.1 ng/ml	1.5 ng/ml	10.0 ng/ml
1:1000000	1.0 ng/ml	0.27 ng/ml	0.21 ng/ml	0.15 ng/ml	1.0 ng/ml

Table 21 shows the dilutions used of each fraction. The quantities reflect the amounts present in the original *Thuja* crude extract. For example, 1.0 mg/ml of *Thuja* extract has the equivalent of 0.27 mg/ml of Hexane, 0.21mg/ml of Chloroform, and 0.15 mg/ml of Ethyl acetate. This indicates that the yields of compounds extracted in each fraction from *Thuja* crude extract are as follows: 27%: Hexane, 21%: Chloroform, 15%: Ethyl acetate. This should show the results to the precise relationship of extract activity to fraction activity.

4.4.3 CELL CULTURE OF RTS3B AND DT16 CELLS

RTS3b (Rapp et al., 1997) and Dt16 keratinocyte cells are fast growing in culture with a passage time period of 2-3 days. Cells were cultured under conditions of 10% CO_2 at 37°C in RM+. When confluent, the cells were washed with magnesium and calcium free PBS. The PBS was decanted and 5mls of trypsin (0.05%) in EDTA (0.02%) (Sigma) was added. The cells were incubated for 10 minutes until detached from the plastic surface at which point the cell suspension was poured into a centrifuge tube and PBS/ 10%FCS was added to a volume of 50mls. The cells and medium were centrifuged at 1000 rpm in a IEC 215A rotor for 5 minutes. The supernatant was decanted, the pellet was re-suspended in 20mls of PBS/FCS and cell counts were determined using a haemocytometer.

4.4.4 MICROTITRE ASSAYS

A density of 5000 cells/ well (well size $2ml^2$) in a volume of 200µl media was required for the *in vitro* assay. Once the cells were plated on twenty four well-plates, with each group having 6 wells, they remained in an incubator at 37°C, 10% CO_2 for 24 hours in order to allow the cells to adhere to the microtitre plate well surface. The outer edge wells of the plate contained 200µl of PBS containing 10% FCS only. A single row (n=6) contained cells and medium only to determine the 100% growth control. A control experiment was also carried out to determine the tolerance of the keratinocytes to ethanol solvent, as used for the preparation of crude extracts and fraction, to confirm they were not causing any interference at the concentrations used in the assay. For all assay plates, the plating medium (DMEM/FCS only) was removed by aspiration and 200µl of the medium containing test material was added to each well. For each extract, three plates were set up, each containing 6 replicated wells at each concentration.

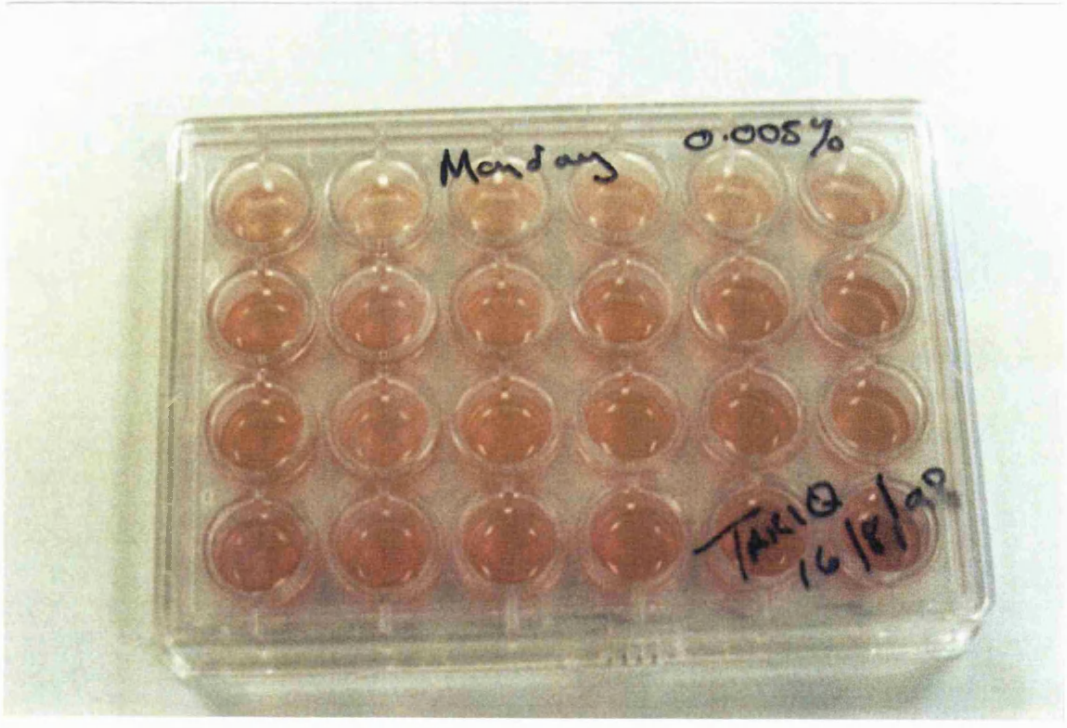


Figure 31. Microtitre assay containing cultured cells with different concentrations of *Thuja occidentalis*, media, solvent and podophyllotoxin.

4.4.5 INCUBATION OF PLANT EXTRACTS AND COMPOUNDS WITH RTS3B AND DT16 KERATINOCYTES

All plant extracts and control compounds, once added to the plate, were left in contact with the cells for a seven day period at 37°C, 8% CO_2. Readings were taken at two, four and seven days to collect better data of understanding the effect the drug has over 24 hours as oppose to a longer period of time.

4.4.6 FORMAZAN (MTT) ASSAY

MTT Stock (Sigma) 5mg/ml stored frozen at -20°C and kept in the dark, thawed and diluted 1:10 in PBS to make 0.5mg/ml.

RM+ was aspirated and 1ml of MTT (0.5mg/ml) is added to each well. This was then incubated at 37°C, 10% CO_2 for two hours in the dark. The MTT solution was removed and the cells are washed with PBS and then removed. 300µl of 10% DMSO/90% isopropanol is added and allowed to dissolve dye for 5-10 minutes. The cells are then aliquoted into centrifuge tubes and spun for 5 minutes in a centrifuge, rpm (12-14K). Then 200µl are pipetted into each well of a 96 well reader plate and read at 570nm (measure filter) and 620nm (reference filter) using a Titertek Multiscan MCC/340 II plate reader.

4.4.7 DATA ANALYSIS

Mean optical density (OD) ± SD was calculated for each concentration from the six replicate wells in a single plate. The data was used to plot a dose response curve from which IC_{50} value was obtained. For each substance, the mean IC_{50} value ± SD from the 3 plates is given in Table 24.

4.4.8 CELL CYCLE ANALYSIS

To evaluate the effect of *Thuja* crude extract, chloroform fraction and podophyllotoxin on cell cycle:

RTS3b and Dt16 p30 cells were prepared as in Section 4.2.1.5 (cell culture) and plated out at a density 5000 cells/well (well size $2ml^2$) (in a volume of

200µl media required for the *in vitro* assay. Once the cells were plated out, they remained in an incubator at 37°C, 10% CO_2 for 24 hours in order to allow the cells to adhere to the plate surface. As measurements were to be taken at 3 time points, 3 plates were prepared with each cell line and drug, which also included a control of media only.

All plant extracts and control compounds were left in contact with cells for 24 hours at 37°C, 8% CO_2. Once added to the plates, readings were taken at 8,16 and 24 hours.

4.4.9 BrdU

2ml BrdU (bromodeoxyuridine) (0.1M) was added to each plate and incubated for 30minutes at 37°C, 8% CO_2.

The BrdU was then removed and cells were cleared with versene, then 2mls of trypsin/versene mixture was added, the plates were then incubated for 5mins, 37°C, 8% CO_2. Once the cells had detached from the surface of the plate, they were aliquoted into centrifuge tubes and spun for 5 minutes at 13,000 rpm. The supernatant was aspirated and the pellet then washed with PBS and respun for 5 minutes. The pellet was then resuspended in 200µl PBS and 1ml 70% ethanol was added, this was placed in ice for 15 minutes. The cells were then spun down at 5000rpm for 5 minutes and 1 ml of 70% ethanol was added, then was stored at -20°C until it was sent to the FACS labs at Cancer Research UK for flow cytometric analysis, where they were analysed using various cytometers, which measure the light intensity of the cells using either scattered laser light or fluorescence. The results were then returned after analysis.

4.5 RESULTS

The evaluation of the plant extracts *Thuja occidentalis* (TO) and its sub fractions: hexane, chloroform and ethyl acetate was carried out alongside the control, Podophyllotoxin (PD), a standard cytotoxic agent used for treatment of warts.

As *Thuja* and its extracts have limited solubility in water they were prepared as solutions in an appropriate concentration of ethanol. *Thuja* was extracted into the ethanol and water mixture, the extract containing 70% ethanol. It was important to know if ethanol solvent would make any difference to the outcome of the experiments, therefore ethanol and water mixtures were tested at different concentrations on RTS3b cells (as described in Section 4.4.1) using Formazan methods. The outcome indicated that over a period of 7 days, at concentrations of up to 50% of ethanol/water, change did not occur in the growth of cells. In comparison with cells, which were being investigated in media at concentrations of 60% and above of water ethanol mixture, the effect of showed inhibited proliferation rate of cell growth during the 7 days so for all experiments where *Thuja* and its subfractions and podophyllotoxin were used a concentration of 50% ethanol water mixture was used to dissolve them before they underwent dilutions with media for experiments (Section 4.4.1).

Thuja occidentalis was dissolved in 50% ethanol water mixtures, as at this starting concentration followed by dilution solvent would not have any influence on cell growth. Extracts were prepared to 1.0 mg/ml as the starting point and were diluted 10 fold, with medium in each case (Section 4.4.1). At dilution factor 10^{-1} the concentration was 0.1 mg/ml and at the dilution factor 10^{-6} the concentration was 1.0ng/ml. These were used always at equivalent dilution to the sample under treatment. These were then used in growth rate experiments.

4.5.1 GROWTH OF CELLS EXPOSED TO *THUJA OCCIDENTALIS* EXTRACT

The first investigation was carried out to determine the effect of *Thuja occidentalis* extracts on RTS3b cell lines. It was carried out using a range of concentrations from 1.0ng/ml (10^{-6}) to 0.1mg/ml (10^{-1}) per well. Optical density reading (ODR), were measured for each test material, the results of which can be seen in Graphs 13-15 and Tables 22-24. Throughout the investigations it was quite clear that cell growth in both media and solvent were not in any way compromised. The growth rate of RTS3b cells progresses to a confluent state by 7 days. It is evident that from the Graphs 13-15 and Tables 22-24, both media and solvent curves followed a pattern expected of RTS3b cell growth over a 7 day period. With the other test materials there was slowing of cell growth during the 7 day period. In Graph 13 and Table 26, *Thuja* extract at dilution 10^{-1} to 10^{-3} has a direct effect on the number of viable cells. The curves clearly show that the *Thuja occidentalis* had a marked effect, diminishing the number of cells at days 4 and 7 at concentrations of 0.1mg/ml to 10.0µg/ml. At dilutions 10^{-3} the effect of *Thuja occidentalis* on viable cells diminished. At 10^{-4} and 10^{-5} dilutions, in comparison to higher concentrations of *Thuja occidentalis*, cell growth was affected however, at a slower rate of cell proliferation than the control group. Finally at dilution 10^{-6}, there was no effect on viable cells and the growth rate of cells was judged to be not significant from that of the control groups. These results indicate that the higher concentration of *Thuja* extract have a direct effect on cell growth rate. This was dose dependent, lower doses having less effect on cell growth and is such that growth rate increases relatively, showing limited action by *Thuja.*

The podophyllotoxin was a reference marker to evaluate how toxic it was on cell growth compared with *Thuja.* Podophyllotoxin exerted a direct effect on cell growth and showed that no viable cells were present even up to 7 days: The effect of dilutions of podophyllotoxin on cell growth was minimal at all concentrations from 10.0ng/ml to 0.1mg/ml. However at 1ng/ml the concentration of podophyllotoxin was so dilute that there was an incremental increase in cell growth, but the proliferation rate remained slow. Only

concentrations of 0.1ng/ml showed a diminished effect on cell growth but clearly slowed the proliferation rate. It was decided that a concentration of 0.1μg/ml (10^{-4}) of *Thuja* extract would be investigated further on cell lines. This figure corresponds to the IC_{50} of *Thuja*.

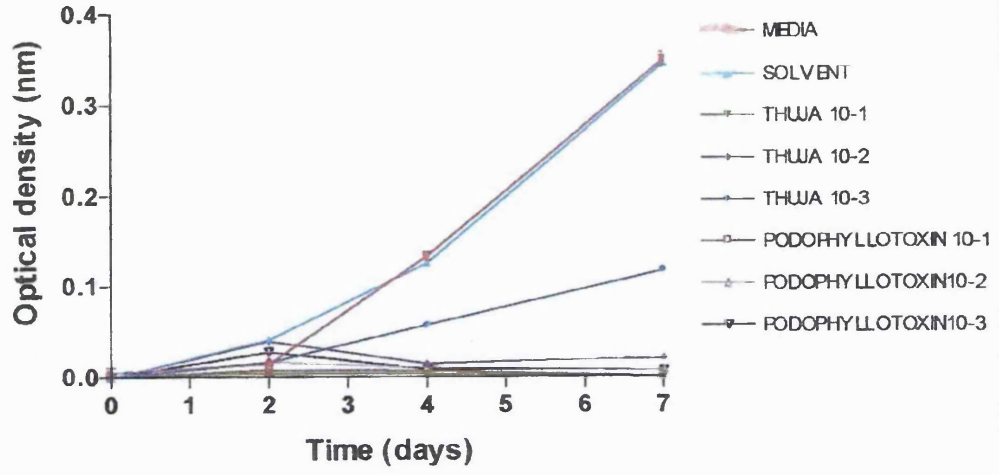


Figure13 Graph showing Formazan assay results of *Thuja* extract and controls on the growth of RTS3b cells at dilutions (10^{-1} to 10^{-3}), values are a mean ± SD, where n=6.

Table 22. Formazan assay results of *Thuja* extract and controls of RTS3b cells at dilutions (10^{-1} to 10^{-3})

FORMULATION	TIME (Days)			
	0	2	4	7
MEDIA	0.0057 ± 0.010	0.0153 ± 0.021	0.1337 ± 0.008	0.3487 ± 0.014
SOLVENT	0.0000 ± 0.000	0.0450 ± 0.006	0.1270 ± 0.006	0.3800 ± 0.061
THUJA x 10^{-1}	0.0000 ± 0.000	0.0060 ± 0.001	0.0040 ± 0.001	0.0000 ± 0.000
THUJA x 10^{-2}	0.0000 ± 0.000	0.0400 ± 0.005	0.0170 ± 0.002	0.0250 ± 0.006
THUJA x 10^{-3}	0.0000 ± 0.000	0.0170 ± 0.003	0.0520 ± 0.017	0.1170 ± 0.007
PODOPHYLLOTOXIN x 10^{-1}	0.0000 ± 0.000	0.0090 ± 0.002	0.0070 ± 0.002	0.0000 ± 0.000
PODOPHYLLOTOXIN x 10^{-2}	0.0000 ± 0.000	0.0190 ± 0.001	0.0120 ± 0.002	0.0060 ± 0.002
PODOPHYLLOTOXIN x 10^{-3}	0.0000 ± 0.000	0.0270 ± 0.003	0.0090 ± 0.002	0.0060 ± 0.001

Optical density means calculated in nm. Column values are mean ± SD, where n=6

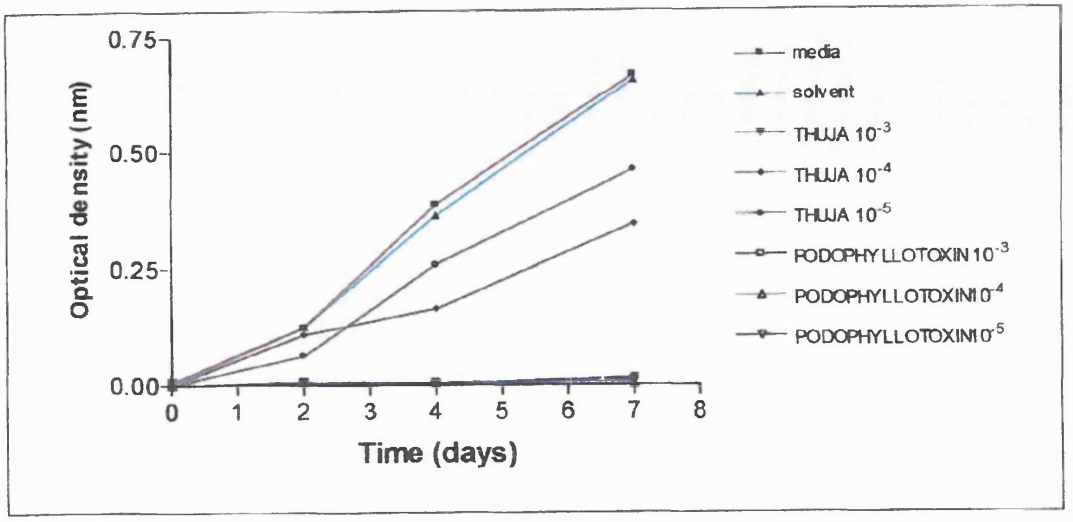


Figure 14. Graph showing Formazan assay results of *Thuja* extract and controls on the growth of RTS3b cells at dilutions (10^{-3} to 10^{-5}), values are a mean ± SD, where n=6.

Table 23. Formazan assay results of *Thuja* extract and controls on the growth of RTS3b cells at dilutions (10^{-3} to 10^{-5})

FORMULATION	TIME (Days)			
	0	2	4	7
MEDIA	0.0057 ± 0.010	0.1257 ± 0.005	0.3883 ± 0.003	0.6687 ± 0.004
SOLVENT	0.0000 ± 0.000	0.1270 ± 0.003	0.3650 ± 0.005	0.657 ± 0.001
THUJA x 10^{-3}	0.0000 ± 0.000	0.0070 ± 0.002	0.0030 ± 0.001	0.0160 ± 0.003
THUJA x 10^{-4}	0.0000 ± 0.000	0.0110 ± 0.003	0.1650 ± 0.004	0.3460 ± 0.004
THUJA x 10^{-5}	0.0000 ± 0.000	0.0630 ± 0.001	0.2600 ± 0.002	0.4610 ± 0.003
PODOPHYLLOTOXIN x 10^{-3}	0.0000 ± 0.000	0.0050 ± 0.003	0.0030 ± 0.002	0.0140 ± 0.002
PODOPHYLLOTOXIN x 10^{-4}	0.0000 ± 0.000	0.0060 ± 0.002	0.0030 ± 0.002	0.0030 ± 0.002
PODOPHYLLOTOXIN x 10^{-5}	0.0000 ± 0.000	0.0080 ± 0.003	0.0030 ± 0.001	0.0080 ± 0.001

Optical density means calculated in nm. Column values are mean ± SD, where n=6

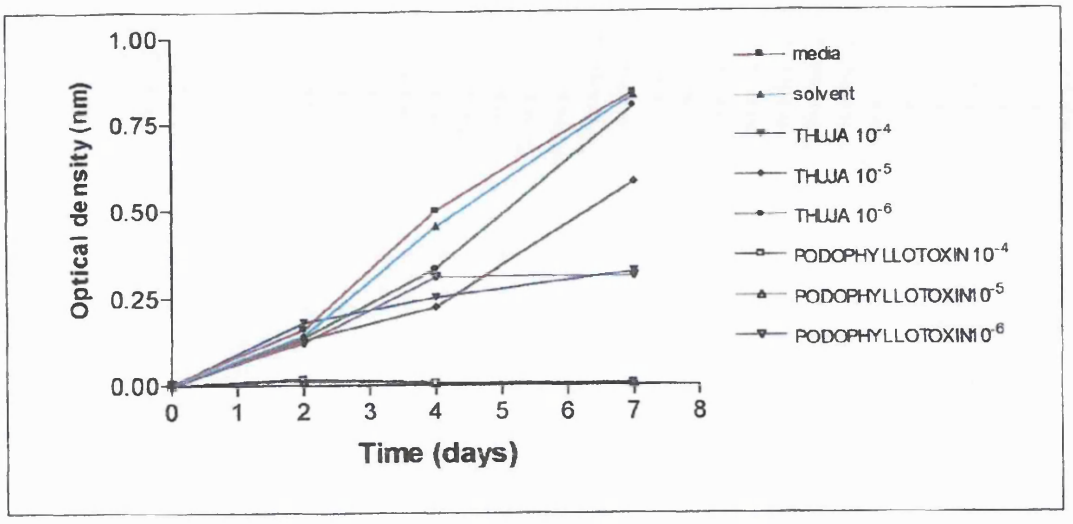


Figure 15. Graph showing Formazan assay results of *Thuja* extract and controls on the growth of RTS3b cells at dilutions (10^{-4} to 10^{-6}), values are a mean ± SD, where n=6.

Table 24. Formazan assay results of *Thuja* extract and controls on the growth of RTS3b cells at dilutions (10^{-4} to 10^{-6})

FORMULATION	TIME (Days)			
	0	2	4	7
MEDIA	0.0057 ± 0.010	0.1623± 0.002	0.5020 ± 0.002	0.8453 ± 0.004
SOLVENT	0.0000 ± 0.000	0.1440 ± 0.002	0.4560 ± 0.002	0.8360 ± 0.002
THUJA x 10^{-4}	0.0000 ± 0.000	0.1810 ± 0.002	0.2540 ± 0.003	0.3260 ± 0.003
THUJA x 10^{-5}	0.0000 ± 0.000	0.1320 ± 0.002	0.2250 ± 0.001	0.5850 ± 0.003
THUJA x 10^{-6}	0.0000 ± 0.000	0.1390 ± 0.003	0.3350 ± 0.002	0.8030 ± 0.002
PODOPHYLLOTOXIN x 10^{-4}	0.0000 ± 0.000	0.0170 ± 0.000	0.0060 ± 0.001	0.0050 ± 0.001
PODOPHYLLOTOXIN x 10^{-5}	0.0000 ± 0.000	0.0120 ± 0.002	0.0060 ± 0.002	0.0040 ± 0.002
PODOPHYLLOTOXIN x 10^{-6}	0.0000 ± 0.000	0.123 ± 0.003	0.3110 ± 0.005	0.3150 ± 0.001

Optical density means calculated in nm. Column values are mean ± SD, where n=6

4.5.2 THE COMPARISON OF *THUJA OCCIDENTALIS* ON RTS3B AND DT16 CELL LINES

From previous results on RTS3b cells, it then became important to further the results obtained and evaluate them on keratinocytes immortalised by HPV. Looking at the study from a clinical starting point, particular concentrations were needed as they have been used throughout and produce the necessary guidelines.

Dt16 cells were used which were derived from normal skin cultures transfected with HPV 16 (E6 & E7) DNA. The investigation was carried out to evaluate the effect of *Thuja occidentalis* on cell growth at the concentration 0.1μg/ml. Cells were plated out into 24 wells plates, each well containing 5000 cells. Readings were taken at 2, 4 and 7 days.

The IC_{50} best described will be the concentration of the inhibitor (*Thuja*) at which 50% inhibition of growth rate occurs. To calculate the IC_{50} for *Thuja*, 50% inhibition of cell growth was measured at 0.1μg/ml.

The results from tables 25 showed that there was steady growth of cells during 2-7 days, which clearly reflected a normal growth curve for RTS3b cells. Solvent (ethanol dilution) followed a similar profile to the media with an ODR of 0.326 at 2 days, becoming 0.954 at 4 days and 1.389 at 7 days. The rate of cell proliferation was only slightly lower than the control. The conclusion is that there is minimal effect at this concentration on cell proliferation. *Thuja occidentalis* extract seemed to have a marked effect on cell growth at 2 days, the ODR was 0.264, slightly lower to the control but at 4 days *Thuja occidentalis* was slightly lower at 0.573 and at 7 days was 0.986. These results show that *Thuja occidentalis* extract was directly affecting cell growth. The dilution of podophyllotoxin used was 1.0ng, this concentration allowed some cell viability. There was a small rate of growth in cell numbers from 0-7 days, but when compared with the control, this was minimal as seen in the graph due to the very cytotoxic nature of the podophyllotoxin.

In comparison, the results of DT16 cell lines:

A marked effect of *Thuja occidentalis* was also observed on this cell line. The control showed an ODR of 0.076 at 2 days, which increased, to 0.186 and then 0.416 at 7 days. This was taken to reflect the normal cell growth over that period. With the solvent, it was observed that the ODR at 2 days was 0.067, which increased to 0.179 and was 0.397 at 7 days, a growth pattern, which was very similar to the control. The *Thuja occidentalis* extract had an ODR of 0.043, slightly lower than the control at 2 days and 0.156 at 4 days and 0.284 at 7 days. *Thuja occidentalis* caused a decrease in the number of viable cells at day 4 compared to control.

The podophyllotoxin showed an ODR of 0.031 at 2 days, 0.141 at 4 days and 0.247 at 7 days. This was lower than the number of viable cells at day 4 compared to *Thuja occidentalis* and the control.

Figure 16 shows podophyllotoxin cell growth plateau at four days when used on RTS3b cells, wheras early growth rates up to four days are the same for podophyllotoxin and the controls and *Thuja*. This could suggest different modes of action are present within the podophyllotoxin, possibly due to delayed arrest of cell growth, or cell death occurring. Looking at figure 17 where Dt16 cells are used, podophyllotoxin seems to follow the same pattern as the controls and *Thuja*.

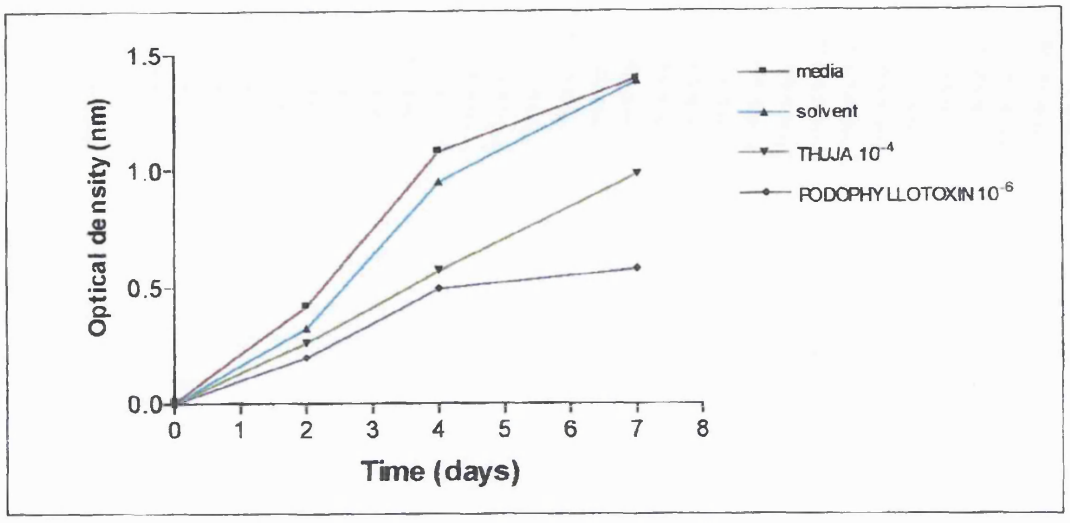


Figure 16. Formazan assay results of *Thuja* extract and controls on the growth of RTS3b cells), values are a mean ± SD, where n=6.

Table 25. Formazan assay results of *Thuja* extract and controls on the growth of RTS3b cells

FORMULATION	TIME (Days)			
	0	2	4	7
MEDIA	0.0057 ± 0.010	0.4203± 0.002	1.0830 ± 0.003	1.4007 ± 0.002
SOLVENT	0.0000 ± 0.000	0.3260 ± 0.002	0.9540 ± 0.001	1.3890 ± 0.002
THUJA x 10^{-4}	0.0000 ± 0.000	0.2640 ± 0.001	0.5730 ± 0.003	0.9860 ± 0.005
PODOPHYLLOTOXIN x 10^{-6}	0.0000 ± 0.000	0.1970 ± 0.002	0.4980 ± 0.001	0.5800 ± 0.001

Optical density means calculated in nm. Column values are mean ± SD, where n=6

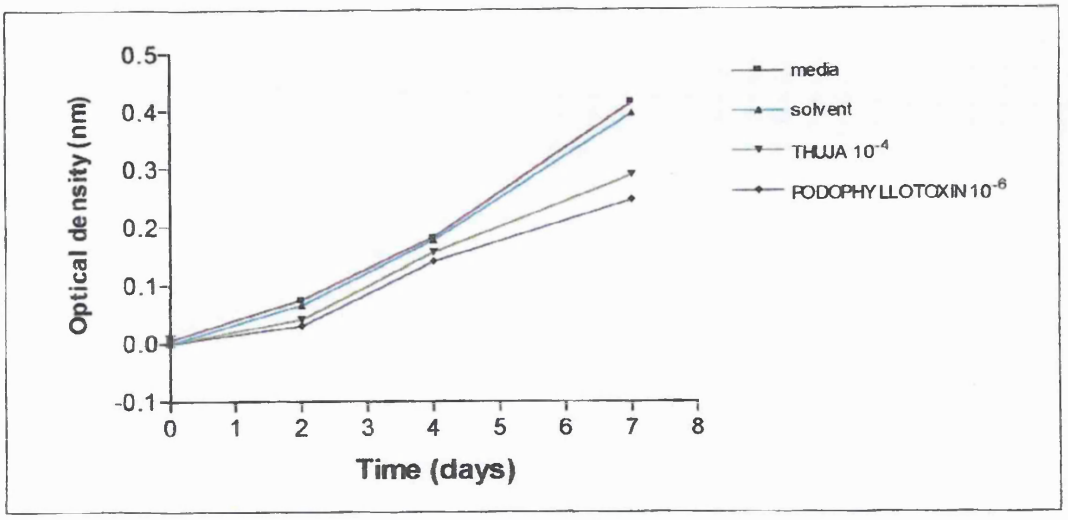


Figure 17. Formazan assay results of Thuja extract and controls on the growth of DT16 Keratinocytes, values are a mean ± SD, where n=6.

Table 26. Formazan assay results of Thuja extract and controls on the growth of DT16 Keratinocytes

FORMULATION	TIME (Days)			
	0	2	4	7
MEDIA	0.0036 ± 0.010	0.0760 ± 0.002	0.1860 ± 0.003	0.4163 ± 0.002
SOLVENT	0.0000 ± 0.000	0.0670 ± 0.001	0.1790 ± 0.002	0.3970 ± 0.003
THUJA x 10^{-4}	0.0000 ± 0.000	0.0430 ± 0.002	0.1560 ± 0.001	0.2840 ± 0.002
PODOPHYLLOTOXIN x 10^{-6}	0.0000 ± 0.000	0.0310 ± 0.001	0.1410 ± 0.002	0.2470 ± 0.001

Optical density means calculated in nm. Column values are mean ± SD, where n=6

4.5.3 SEPARATED FRACTIONS FROM *THUJA OCCIDENTALIS* TO INVESTIGATE AND DETERMINE THE EFFECTS ON RTS3B CELLS AND DT16 CELLS

(Tables 27,28 and Graphs 18,19)

As noted previously, the *Thuja occidentalis* extract can be separated into various fractions using chemical separation techniques (Chapter 2.3.3). The effect of each of these on keratinocyte growth was investigated in order to determine whether the growth inhibited proportions of the *Thuja occidentalis* extract used was present within one or more fractions.

The fractions were prepared and dissolved in 50% ethanol the concentrations used were determined by the amount separated from the *Thuja occidentalis* extract. The hexane fraction amounted to 0.27mg/ml when separated, the chloroform fraction amounted to 0.21mg/ml and the ethyl acetate fraction amounted to 0.15mg/ml. These were then diluted as outlined in Table 25 Section 4.4.2. Extracts of 0.27μg/ml hexane fraction, 0.21μg/ml chloroform and 0.15μg/ml ethyl acetate, were used to investigate the effect of RTS3b and DT16 cell lines. The results of the control group on both RTS3b and DT16 cell lines shown in table 27 and 28 demonstrated ODRs of 0.253 at 2 days, at 4 days 0.876 and 1.153 at 7 days for RTS3b cells and ODRs of 0.082 at 2 days, 0.609 at 4 days and 1.114 at 7 days for DT16 cells reflecting normal growth curves for that period.

The hexane fraction showed an ODR of 0.238 at 2 days, 0.842 at 4 days and 1.147 at 7 days, which showed that hexane, had minimal effect on cell growth. The effects of the chloroform fraction was much more significant. At 2 days an ODR of 0.065 was observed which increased to 0.214 at 4 days and was 0.309 at 7 days. These results showed direct action on the function of cell growth, with a clear indication of inhibited proliferation rate of cell growth over 7 days in comparison to the control. There was a very small number of viable cells present. The ethyl acetate fraction showed an ODR of 0.095 at 2 days, 0.788 at 4 days and 1.126 at 7 days, which also reflected minimal effect on

the growth curve and was similar to the control. Notably between the graphs the chloroform fraction visibly seems to inhibit proliferation of cell growth.

In comparison with the solvent control, cells exposed to the hexane fraction proliferated normally. The chloroform fraction had a marked effect on growth rate at 2 to 4 days, a very small number of viable cells were present which slowly increased over the next 3 days. In comparison to the control, a significant difference was seen in the number of viable cells present during that period. In the ethyl acetate fraction, the ODR at 2 days rose at 4 days, slightly lower to the control and rose again at 7 days, again lower to the control. It was demonstrated that the ethyl acetate fraction lowers proliferation rate, possibly affecting re-entry of cells into the cycle after plating, but not as much as the chloroform fraction. It was noted that chloroform fraction significantly inhibited proliferation of cell growth.

Comparing the results of the RTS3b cells to the DT16 cells, it can be observed that ethyl acetate fraction shows a more marked activity on cell proliferation in figure 19 as compared with figure 18. The proliferation rate slows with the ethyl acetate fraction when used on Dt16 cells as compared with RTS3b cells.

It was concluded that activity present on the *Thuja occidentalis* extract is primarily in the chloroform fraction with some activity in ethyl acetate on HPV infected cells.

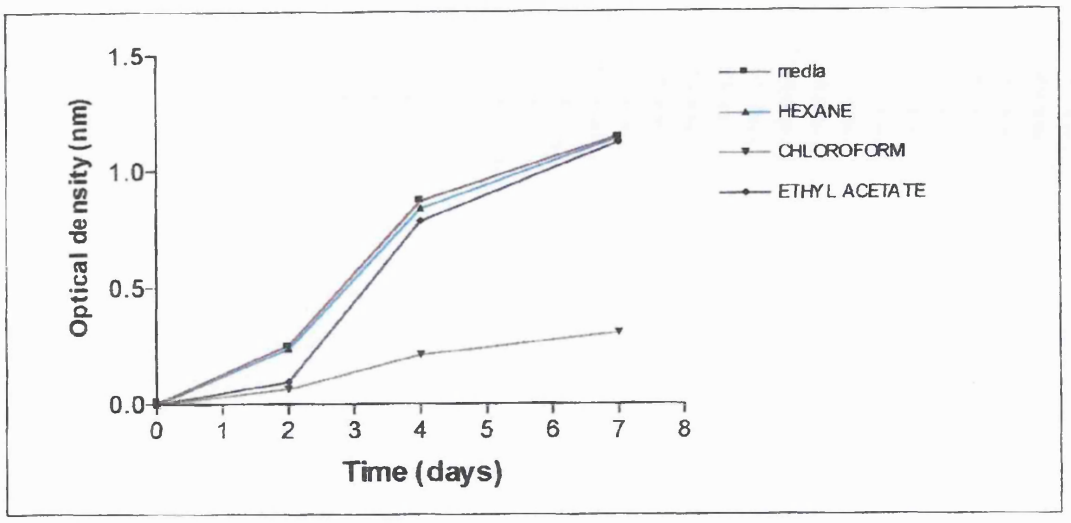


Figure 18. Formazan assay results of subfractions of *Thuja* extract and controls on the growth of RTS3b cells, values are a mean ± SD, where n=6.

Table 27. Formazan assay results of subfractions of *Thuja* extract and controls on the growth of RTS3b cells

FORMULATION	TIME (Days)			
	0	2	4	7
MEDIA	0.1100 ± 0.010	0.2533± 0.002	0.8763 ± 0.003	1.1533 ± 0.002
HEXANE	0.0000 ± 0.000	0.2380± 0.002	0.8420 ± 0.001	1.1470 ± 0.002
CHLOROFORM	0.0000 ± 0.000	0.0650 ± 0.003	0.2140 ± 0.003	0.3090 ± 0.001
ETHYL ACETATE	0.0000 ± 0.000	0.0950 ± 0.003	0.7880 ± 0.001	1.1260 ± 0.002

Optical density means calculated in nm. Column values are mean ± SD, where n=6

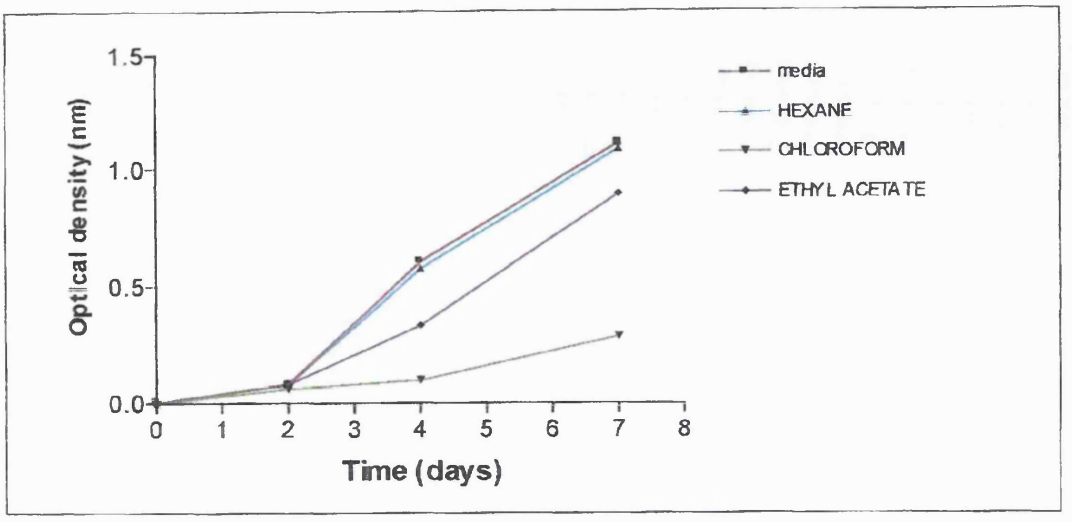


Figure 19. Formazan assay results of subfractions of *Thuja* extract and controls on the growth of Dt16 Keratinocytes, values are a mean ± SD, where n=6.

Table 28. Formazan assay results of subfractions of *Thuja* extract and controls on the growth of Dt16 Keratinocytes

FORMULATION	TIME (Days)			
	0	2	4	7
MEDIA	0.0208 ± 0.010	0.0820± 0.001	0.6087 ± 0.003	1.1137 ± 0.003
HEXANE	0.0000 ± 0.000	0.0800± 0.002	0.5770 ± 0.003	1.0880 ± 0.002
CHLOROFORM	0.0000 ± 0.000	0.0610 ± 0.004	0.0980 ± 0.002	0.2870 ± 0.002
ETHYL ACETATE	0.0000 ± 0.000	0.0810 ± 0.005	0.3360 ± 0.003	0.896 ± 0.004

Optical density means calculated in nm. Column values are mean ± SD, where n=6

4.5.4 EFFECTS OF *THUJA OCCIDENTALIS*, CHLOROFORM FRACTION AND PODOPHYLLOTOXIN ON THE CELL CYCLE USING FACS ANALYSIS ON DT16 CELLS

Figure 31 and 32 show analysis carried out using BrdU incorporation, a minimal inhibitory concentration (MIC) incorporated during DNA synthesis, which acts as a good marker for S phase cells. The effects of the agents on cell cycles following Propidium Iodide (PI) are summarised in Figure 31. The cells used in this experiment were DT16 cells. PI staining was used to determine the proportion of cells in G_1 S phase and G2/M.

4.5.4.1 PROPIDIUM IODIDE (PI) STAINING RESULTS

Panel A shows asynchronous populations of exponentially growing cells which were subjected to FACS analysis. This shows that only a small fraction of cells have sub G_1 content. Most cells are in G1 as might be expected.

Panel B shows the profile of the cell cycle distribution when the cells were exposed to the *Thuja* extract at a concentration of 0.1μg/ml. This resulted in a greater fraction of the cells to have a sub G_1 content. A substantial decrease in the G_1 fraction was apparent in the *Thuja* extract with a large increase in the proportion of the cells in G_2.

Panel C shows the profile of the cell cycle distribution when the cells were exposed to the chloroform fraction. This resulted in a greater fraction of the cells 28% compared to the control at sub G_1. Similar to *Thuja* extract, a substantial decrease in the G_1 fraction was also apparent in the chloroform fraction with a large proportion of the cells in G_2.

Panel D shows the profile of the cell cycle distribution when the cells were exposed to the podophyllotoxin. This resulted in a greater fraction of the cells 27% compared to the control and *Thuja* extract at sub G_1 Similar to *Thuja* extract, a substantial decrease in the G_1 fraction was also apparent in the podophyllotoxin with a large proportion of the cells in G_2.

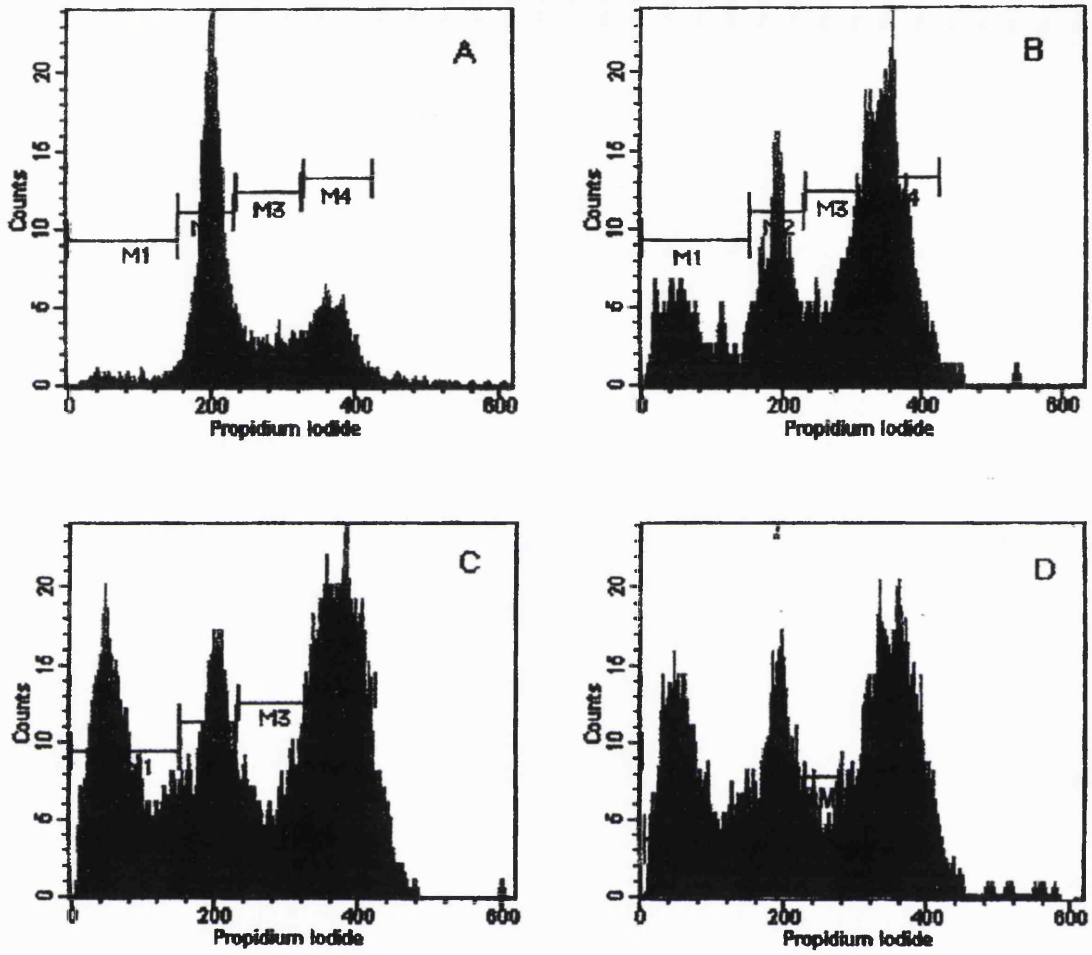


Figure 32. Propidium Iodide staining results

A- Media

Marker % Total

ALL	100.00	
M1	2.61	-subG$_1$
M2	56.19	-G$_1$
M3	16.62	-S
M4	22.21	-G$_2$/M

B-*Thuja* Extract

Marker % Total

ALL	100.00	
M1	16.31	-subG$_1$
M2	20.62	-G$_1$
M3	24.59	-S
M4	35.87	-G$_2$/M

C-Chloroform fraction of *Thuja occidentalis*

Marker % Total

ALL	100.00	
M1	28.64	-subG$_1$
M2	18.59	-G$_1$
M3	12.94	-S
M4	35.56	-G$_2$/M

D-Podophyllotoxin

Marker % Total

ALL	100.00	
M1	27.48	-subG$_1$
M2	19.25	-G$_1$
M3	13.94	-S
M4	36.46	-G$_2$/M

4.5.4.2 BIVARIATE STAINING RESULTS

The use of bromodeoxyuridine (BrdU) incorporation of the analysis of the cell cycle has proved very useful in the study of cell kinetics. The limitation of looking at a single fluorochrome is that no kinetic information is gained. To assess this bromodeoxyuridine can be used. Bromodeoxyuridine is an analogue of the DNA base thymidine and competes with that base during the synthesis of DNA of cycling cells. Detection involves unwinding of the DNA and then using an antibody against BrdU. The technique is a simple and quick method to determine DNA synthesis during the S phase of the cycle and G_1, S and G2 cells can be separated. It is a more accurate method than using P.I. for example, and therefore a more detailed examination of results can be carried out (Burke and Balkwill 2000).

Panel A (Figure 32) shows asynchronous populations of exponentially growing cells which were subjected to Fluorescence-activated cell sorter (FACS) analysis. This shows that a large fraction (27.26%) was present during S phase, the point of synthesising DNA, represented a normal/expected profile for these cells. In comparison to the PI result for S phase were 16.62% a lower proportion of cells than the BrdU result.

Panel B shows the profile of cell cycle distribution when the cells were exposed to the *Thuja* extract and resulted in a lower fraction (17.76%) of the cell present at S phase and therefore less synthesis and less DNA present. In comparison the PI was 24.59% a higher proportion of cells than BrdU.

Panel C shows the profile of cell cycle distribution when the cells were exposed to the chloroform fraction and resulted in a lower fraction (13.04%) of the cell present at S phase and therefore less synthesising DNA present. In comparison the PI was 12.94% a higher proportion of cells than BrdU.

Panel D shows the profile of cell cycle distribution when the cells were exposed to the hexane fraction and resulted in a lower fraction (12.16%) of

the cell present at S phase and therefore less synthesising DNA present. In comparison the PI was 13.94% a higher proportion of cells than BrdU.

The results of these investigations show that both *Thuja occidentalis* and chloroform have a direct effect on cell cycling. Profiles C and D show similar results, leading to a selective effect on DNA synthesis at these concentrations. These results will be discussed further in detail later.

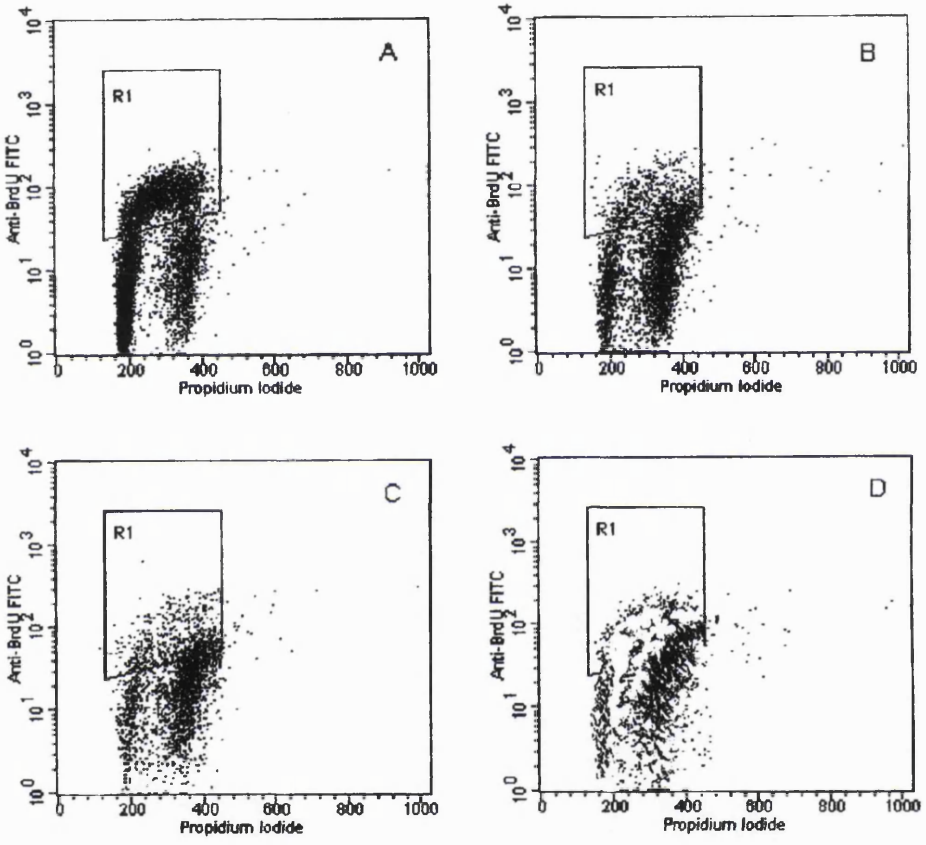


Figure 32. Bivariate staining results

A - Media

Region % Total
R1 27.26

B – *Thuja* Extract

Region % Total
R1 17.76

C – Chloroform fraction of *Thuja occidentalis*

Region % Total
R1 13.04

D – Podophylloxin

Region % Total
R1 12.16

4.6 DISCUSSION

The study of human papilloma viruses in tissue culture has been difficult because of the lack of a cell culture system that stably maintains HPVs episomally, coupled with the difficulty in recreating the 3-dimensional structure of the epithelium to induce the full HPV life cycle. Within the past decade, great progress has been made in facilitating the study of HPVs in tissue culture. Several cell lines have been derived from HPV-infected patients that stably maintain HPV genomes episomally, for example, W12E cells harbouring HPV-16 (Stanley et al., 1989; Jeon et al., 1995) and CIN612-9E cells harbouring HPV-31b (Bedell et al., 1991; Hummel et al., 1992). These cells have proven to be excellent tools for the study of the full HPV life cycle (Bedell et al., 1991; Hummel et al., 1992; Meyers et al., 1992, Flores and Lambert 1997; Ozbun and Meyers 1997). Although these cells have provided a great means to study HPV in tissue culture, they cannot be used to determine the consequence of mutations in the viral genome on the HPV life cycle. To achieve this goal, a method using transfected HPV genomes in human foreskin keratinocytes (HFKs) was developed. (Frattini et al., 1996; Meyers et al., 1997). The power of this method is that it allows genetic manipulation of the viral genome and analysis of genetic effects on the HPV life cycle (Flores et al., 1999). This method however is difficult to set up and reproduce accurately, therefore it is difficult to test drugs and more simpler methods were used initially.

In this study the effects of *Thuja* extract were studied. The results showed that at concentrations of 0.1μg/ml, *Thuja* extract was not cytotoxic to RTS3b and DT16 cells, but the separated fraction chloroform showed most activity on the cell lines tested. The findings of the study showed *Thuja* extract was cytotoxic at certain concentrations above 10μg/ml and at others it was more inhibitory to cell proliferation i.e. cytostatic at concentrations below 1.0μg/ml.

Thuja extract in comparison to PD was significantly less cytotoxic, at equivalent concentration. PD seems to have an effect on the proliferation rate

at concentration 10.0ng/ml, but on higher concentration cell proliferation was significantly inhibited. *Thuja* extract had similar effect on both RTS3b and DT16 cells, by slowing the rate of proliferating cells. The *Thuja* extract sub fraction were tested on RTS3b and DT16 cells, clear action was seen in chloroform fraction whilst some was seen in ethyl acetate on DT16 cell. This part of the investigation was carried out to identify whether *Thuja* extract activity was located in one, or more parts of the extract.

Further cell cycle investigations were carried out using Propidium iodide staining (PI) and bivariate staining (BrdU). Both studies were executed using flow cytometry.

The PI stain evaluates the peak at the sub G_1 phase. These phases have cells less than G_1. This is commonly used as a tool to look for apoptotic cells (FACS Labs, Cancer Research UK 2003), however other methods are also used to confirm that the cells are apoptotic. The DNA of the cell begins to break up into small molecular weight fragments, which are left free in the nucleus. Following PI staining, organelles such as the cell wall showed a reduced fluorescence and a sub G_1 peak which is a good indicator of the number of cells which are apoptotic. The results showed clear indication that *Thuja* extract, chloroform fraction and PD result in a greater fraction of cells with sub G_1 content, with substantial decrease in G_1 fraction an large fraction of cell in G_2. These results obtained indicate that the test materials may directly affect the cell cycle, possibly by interrupting the cell division during its natural progression. As there are two cell cycle checkpoints at G_1/S and G_2/M, these could be activated to be overridden due to the presence of the test material and cause programmed cell death.

The BrdU staining allowed us to study the effect of *Thuja* extract on DNA synthesis. The results clearly indicated that *Thuja* extract, the chloroform fraction and PD had a marked effect on S phase, each cytopathic dose reducing the number of cells compared to controls. This could be explained because cells were killed in G_1 phase and therefore less cells would enter S phase with the majority of sub G_1 peaks derived from the G_1 peak. The

compound present in *Thuja* extract and chloroform earlier identified in chapter 2 is a derivative of PD and is deoxypodophyllotoxin (DPD). DPD drastically inhibits thymidine transport into a cell but does not directly bind to and inhibit DNA synthesis (Markkanen et al., 1983a). This indirectly stops the viral DNA synthesis, as thymidine is one of the required bases among guanidine, cytosine and adenosine. By this method it is possible to imply that the mechanism of action of *Thuja occidentalis* and the chloroform subfraction may be attributed to inhibition of thymidine transport into the cell (Markkanen et al., 1983a).

In relation to the mechanism of actions described so far, many studies have been conducted in order to assess and confirm the activity of DPD and PD. *In vivo* toxicity (subcutaneous injections on rats) of DPD showed a LD50 dose of >200mg/kg compared to LD50 of 14mg/kg for PD. Such a large difference is not however always apparent with alternative routes of administration used for *in vitro* studies where IC_{50} of PD are 5nM and 12nM respectively. Thus, DPD is less toxic than PD. Anti-microtubules (anti-MT) activity on human amnion cells exposed to herpes simplex virus type-1 (HSV-1) showed IC_{50} of PD and DPD as 0.6μm and 0.5μm respectively; thus, both are compatible at MT assembly inhibition. However, there are significant differences in thymidine transport where IC_{50} of PD is 9μm compared to 0.7μm of DPD. This suggests DPD is 13-fold more potent than PD inhibiting thymidine transport (Markkanen et al., 1983a).

In studies carried out by Markkanen et al (1983b), Minimum Inhibitory Concentration (MIC) of PD and DPD are 0.04μM and 0.035μM respectively and CI of PD and DPD are 6000 and 650. MIC data suggest little difference between antiviral effect of PD and DPD but data suggest PD is about 10-fold more active than DPD against HSV-1 [indicating MIC of PD is small since its CI data (a ratio) is so large]. DNA breakage of 5% by DPD, compared to 75% by VP-16/VM-26 compounds and IC_{50} (MT assembly inhibition) of 0.5μm by DPD, compared to> 10μm by VP-16/VM-26 compounds indicate the action of VP-16/VM-26 causes significant DNA breakage as main cytotoxic effect,

whereas DPDs cytotoxicity is mainly by MT assembly inhibition (Markkanen et al., 1983b).

It is clear from the results that *Thuja* extract is effective on HPV infected cells and is substantially less toxic compared to PD so its use as verruca pedis treatment seems to be justified compared to a known wart preparation.

Iwashita et al. (2000) investigated the effect of flavonoids at concentration 100μm and 200μm by adding kaempferol. The study showed that the number of viable cells did not increase to the control level as measured after 24h of incubation. The flavonoids inhibited the proliferation of B16 cells, but the number of viable cells did not decrease. These flavonoids seemed to have a little effect in terms of causing cell death.

Quercetin induces apoptosis in some kinds of cell lines (Shen et al 2003). But, in the case of B16 cells, kaempferol inhibited cell proliferation but they seemed to induce little cell death. No particular structural features of flavonoids associated with the effects on B16 cells, like desaturation of a bond or hydroxyl group position, were observed among the flavonoids tested. The relationship between the growth inhibitory activity and the structural features of the flavonoids remains unclear.

From the results further investigations are required to evaluate points, which have arisen from the study, and were unable to be completed due to time restraints. The actions or influence of the check points at the G_1/S and G_2/M borders of the cell cycle could be involved whether either one or both are affected by the *Thuja* extracts and its sub fractions. *Thuja* induces dose-dependent cytostatic and cytotoxic effects in two different cell lines. In order to test whether checkpoint controls are activated levels p53, an important tumour suppressor protein, activated by DNA damaging agents and in stress responses, could be investigated. P53 plays an important part in signalling either growth arrest, chiefly by activating the cyclin-dependent kinase inhibitor p21, or alternatively apoptosis. From the current study, the RTS3b cell lines

have no p53 content. Therefore any cell death is not due to the activation of p53. Whether the increase in sub G_1 cells seen here as a result of *Thuja* extract exposure was the result of apoptosis induced by p53 or another means remains to be determined.

Future development include investigation of evaluating plant extracts like raft cultures, which contain HPV genes. These could be used if *Thuja* was included in the media and it would be possible to evaluate pathways which affect growth. Further analysis may be carried out to evaluate the effect of RTS3b cells transfected with HPV genes with comparative effects of *Thuja*, and investigations of HPV DNA associated with verruca pedis like HPV 1-4, while comparing the effect on normal growing keratinocytes to evaluating skin toxicity.

CHAPTER 5 -
GENERAL DISCUSSION AND CONCLUSION

5 GENERAL DISCUSION AND CONCLUSION

The phytochemical investigation of *Thuja occidentalis* resulted in the isolation, purification and characterisation of one flavonoid, and one lignan deoxypodophyllotoxin. In the clinical trial, *Thuja occidentalis* ethanolic crude extract and chloroform fraction was found to give 100% clearance in the treatment of verruca pedis. The ethanolic crude extract and chloroform fraction was shown to be the most effective in reduction of verruca pedis size. These results confirm the therapeutic effectiveness of the *Thuja occidentalis* demonstrated in earlier studies.

Thuja extract was cytotoxic to cells *in vitro* at a concentration of 1.0mg/ml. At a concentration of 1.0μg/ml *Thuja* was found to be cytostatic to cell growth. The chloroform fraction was found to be cytotoxic at a concentration of above 2.1μg/ml but was not cytotoxic at a concentration of 0.21μg/ml. In comparison, podophyllotoxin was cytotoxic down to 10.0ng/ml with diminished toxicity seen at 1.0ng/ml.

The activity of *Thuja occidentalis* and fractions on cell lines was attributed to the identified compound deoxypodophyllotoxin which has been investigated and reported to inhibit thymidine transport into a cell but not by directly binding to or inhibiting DNA synthesis (Markkanen et al., 1983a). This compound indirectly stops viral DNA synthesis and is the likely mechanism of action of extracts of *Thuja occidentalis*.

It has been shown that, for the treatment of verruca pedis, *Thuja occidentalis* provides a gentle, non-invasive alternative to traditional treatments, which have been shown to have limitations and disadvantages.

Economically, the demonstrated effectiveness of the treatment provides an opportunity for significant savings in the NHS. The treatment is a much less costlier option than surgery and reduces the number of patients needing to attend podiatry and dermatology clinics. This, together with consideration of

long waiting lists for surgery in the NHS, is a factor, which may commend itself to purchasing authorities.

The phytochemical, biological and clinical study of *Thuja occidentalis* was undertaken to further the investigation of a species which according to the available literature had not previously been the subject of much clinical study. It is a logical and essential progression after many years' clinical use of *Thuja occidentalis* and other species of the genus *Thuja* in the field of medicine.

The flavonoids, kaempferol, 3-methyl ether (isokaempferide), quercetrin, mycertrin and deoxypodophyllotoxin were previously isolated from the crude extract of *Thuja occidentalis*. In the present study, a flavonoid and the lignan deoxypodophyllotoxin were isolated from *Thuja occidentalis*.

Samples for the clinical study were prepared on the basis of the effective dose established in pilot studies of *Thuja occidentalis* at 300mg/ml.dose/week for ethanolic crude extract and 60mg/ml.dose/week for fractions. Ninety subjects (divided into three groups: 1 group of 30, 2 groups of 15 and 3 groups of 10). The method of application was the same as that used in previous studies. Measurements were recorded before application of each sample dose and again after five days.

Test / re-test reliability of measurement of verruca pedis size was established in previous studies with the use of Pearson's Product Moment Correlation Coefficient performed by the Independent Assessor on Day 1 and Day 2. The test was not therefore repeated in this study (Chapter 3). Care was taken to ensure that the wound-mapping chart was read from the same angle each time to avoid the possibility of misreading (Payton, 1994).

Assessment of the effect of treatment was by the standard method of evaluating change in verruca pedis: clinical measurement of verruca pedis size. Additionally in this study, evaluation included observation of the effect of treatment in terms of presence of viral tissue recorded photographically by the researcher pre- and post treatment (Figure 35 a-d) and analysis of the data of

clinical measurement were statistically analysed and compared for significant differences between the treatment and control groups.

All patients in the active groups experienced total clearance of verruca tissue reduction of verruca pedis size after the first treatment and throughout the trial period 0-72 weeks. In the other groups patients experienced mild relief of clearance and minor reduction of verruca pedis size but verruca tissue was present at the end of the study.

No problems were encountered in carrying out the trial in accordance with the protocol. Care was taken to ensure that all patients were able to commit themselves to keep the clinic appointments. There is evidence to suggest that painful treatment may affect patient compliance. In this study, compliance was enhanced due to the painless, non-invasive nature of the treatment. Patients also welcomed the ability to wear their usual footwear throughout the trial and to take a bath as normal. In the event, no patient withdrew from the study and only one patient was unable to complete the trial. Dermatological preparations may often confer advantages in terms of ease of application and patient compliance (Schaefer and Redelmeier, 1996).

The stratum corneum has been shown to be a major barrier to penetration of compounds into the skin (Schaefer and Redelmeier, 1996). The human epidermis and especially the stratum corneum represent an effective permeability barrier to water loss and to the transfer of water soluble compounds although lipid soluble drugs are able to cross the barrier. This is a route whereby potent non-irritant drugs are effective. The flavonoid and Lignan extracted from *Thuja occidentalis* used in this study are soluble in water and have been shown to be effective in the treatment of verruca pedis.

Rougier and Lotte (1987) observed that the transcutaneous delivery of medication was the object of unfavourable prejudice in the medical world and dermatological preparations were considered to have only local effects. However, it is now well accepted that penetration of substances is sometimes desirable for both local and systemic effects and considerable work has been

done to elucidate skin structure, physiology, barrier properties and mechanisms by which substances enter and cross the skin (Rougier and Lotte, 1987). A major problem in the study of skin permeability has been posed by the interpretation of results. Scientists from the different disciplines involved in the research use different methodology to find the way to assess the penetration potential of a drug. The stratum corneum has been shown to be the major barrier to penetration of compounds to the skin. Schaefer and Redelmeier (1996) appreciated the "extraordinary nature of the stratum corneum, the composition, structure and material properties of which are not only unique compared to other structures within the body but also particularly suited for their prime role of maintaining barrier function" (Khan 1999).

This barrier function presents the pharmacological scientist and the clinician with the task of ensuring that a therapeutic substance permeates the stratum corneum in the required amount necessary to achieve the desired rate of bioavailability. According to Schaefer et al. (1987), the plasma level of compounds is usually extremely low following topical application, often below assay detection level. They describe the method by which the kinetics of liberation and subsequent penetration into normal skin can be studied at a very early stage in the development of a topical product using *in vitro* methods which have essentially two different goals. This method consists of measuring the facility of a substance to be liberated from its vehicle and, at the same time, its capacity to penetrate through the skin (Khan 1999).

Percutaneous absorption of compounds has been well defined by a study of relationships between the permeability co-efficient of the stratum corneum and the structural features of the penetrant. Schaefer and Redelmeier (1996) reported that the available evidence in the case of most low molecular weight uncharged molecules is that the primary penetration pathway is thought to be the intercellular lipids. This is consistent with morphological studies which indicate that this is the only continuous pathway across the stratum corneum (Kastigs and Robinson, 1993). Although there is evidence that the level of penetration through hair follicles and sweat glands for some of these compounds is perhaps greater than had been originally acknowledged,

penetration through the inter-cellular lipid spaces appears to predominate especially after long term exposure.

Ethanolic crude extract and chloroform fraction of *Thuja occidentalis* was in sustained contact with the skin over the verruca for 36 weeks. Each time a patient bathed or showered briefly, as advised, the extract or fraction was moistened. The flavonoid compound and deoxypodophyllotoxin present in *Thuja occidentalis* are phenolic substances which tend to be water-soluble since they most frequently occur combined with sugar as glycosides (Harborne, 1973). This suggests that the effect of the treatment may be enhanced by brief regular immersion.

Anti viral activities of Flavonoids have been researched over many years, but no clear mechanism has been found. The antiviral activity appears to be associated with nonglycosidic compounds, and hydroxylation at the 3-position is apparently a prerequisite for antiviral activity (Middleton, 1991). Possible theories have been put forward, the ability of flavonoids to increase cyclic AMP synthesis in cells seem to exist in high concentrations. These observations suggest a relationship between cyclic nucleotide metabolism, cyclic nucleotide-dependent protein kinase activity, and virus infection (Middleton, 1991).

The effect of flavonoids on infectivity and replication of viruses have been studied in cell culture monolayers, by using the technique of viral plaque reduction. Kaul et al. (1985) observed that a flavonoid quercetin caused a concentration-dependent reduction in the infectivity of each virus and, in addition, that intracellular replication of the viruses was reduced when monolayers were infected and subsequently cultured in medium containing quercetin. Another flavonoid hesperetin had no effect on infectivity, but reduced intracellular replication of virus.

Flavonoids are reported to have cytostatic activity *in vitro* and *in vivo*. Analysed using flow cytometry to study each phase of the cell cycle, it creates a histogram where the first peak illustrates double strands of DNA and the

second shows tetra strands of DNA. Chalone (known as the precursor of all flavonoids) seems to block the passage from S to G2 and M phases. Flavones, which transform with chalone *in vivo,* show similar patterns. Among the anthocyanins most effective in suppressing the tumour cell growth, cyanin and pelargonidin made a mild rise between the 1st and 2nd peak, suggesting block between G1 and S phase. Delphinidin gave a high percentage of cells in G1 phase, suggesting prolongation of G1 phase. Kaempferol or quercetin, both of which are flavonols, were added to the culture, the second peak rose significantly indicating a block between G2 and M phases (Koide et. al, 1997). Comparison of the action on the cell cycle by 5-FU, Cis-platinum and flavonoids have given us an indication of these effects. 5-FU (cytostatic agent) results in a significant rise in the region heavier than the diploid peak and mainly blocks cells in the S phase of the cell cycle. Cis-platinum (cytotoxic agent) which showed an elevation in the number of the diploid and heavier than the tetraploid peak. Quercetin showed a significant rise in the tetraploid peak. Kaempferol also lead to an elevated tetraploid peak, although the increase was not significant like that seen with quercetin. With flavonoids, the percent of G1 phase decreases and S phase increases. Since the doubling time generally was elongated more than 1.5 times by the flavonoids at the dose 100μg/ml, the G_1 phase was seemingly elongated by most flavonoids, even though the percent of G1 phase decreased. Thus, depending on the kind of flavonoid, the site of cell cycle is different (Koide et al 1997).

Petersen and Weismann (1995) investigated whether quercetin and kaempferol would be more effective than podophyllin as a treatment for warts. The study found that kaempferol makes up 67% of dry substance of Podophyllin with 13% podophyllotoxin.

The effect of Lignans has been well documented for their anti viral and cytotoxic activity (Ayres and Loike 1990). Lignans are chemically related to the polymeric lignans of the plant cell wall and are found mainly in the woody tissues. They have one or more free phenolic groups and a great majority contain methoxy groups in their structures. They are relatively non-polar and

may be extracted from plant tissues with other lipophilic constituents. Majority of 200 or more lignans occurs in free state in heartwood tissue. Some from root, leaf and flower (they may be found in glycosidic combination) (Ayres and Loike 2000).

Podophyllotoxin has antiviral activity against the DNA viruses, herpes simplex virus (HSV-1) and murine cytomegalovirus (CMV) but not against the RNA viruses. As mentioned earlier the antiviral activity of PD is thought to be mediated at an early stage of replication involving disruption of microtubules, cellular transport processes essential for virion assembly at the cell membrane (Hammonds et al., 1996).

The signs of virus proliferation in nuclei of infected cells were not recognised in the presence of Deoxypodophyllotoxin (DPD), suggesting the virus genome has not reached the nucleus of the cell or the processes (e.g. mRNA transcription, DNA replication and DNA packaging to produce viral capsid protein) coded by the viral genome are inhibited by DPD (Markkanen 1983b). Microtubules (MTs) at the nucleus may act as a biological 'anchor' for viral proteins to allow these processes to take place. This explains the inactivity of PD towards RNA viruses because the replication of these viruses does not occur in the nucleus, negating the need for MT involvement in replication. But typical signs of virus infection - margination of chromatin, production of nuclear capsids and emergence of mature virions in the cytoplasm and extracellular space - were seen under electron microscopic pictures taken after removal of DPD.

DPD also drastically inhibits thymidine transport into a cell but does not directly bind to and inhibits DNA synthesis as thymidine is one of the required bases among guanidine, cytosine and adenosine (Markkanen 1983a).

The 3H-thymidine incorporation level of amnion cell cultures is suppressed to about 1/10 of the normal. There is a prompt increase of incorporation to over 50-fold in two days, where it decreases gradually later due to cell destruction

by virus. In uninfected cells, the normal level is restored when DPD is removed from the culture (Markkanen 1983a).

In conclusion *Thuja occidentalis* is an effective treatment for verruca pedis, and it has been identified that the active principle to its action is a lignan deoxypodophyllotoxin. The action of *Thuja* seems to have a cytostatic effect by slowing down the rate of cell proliferation. It was shown that DPD in the chloroform fraction was cytotoxic at higher concentrations, but when compared with the reference compound PD which is a derivative of DPD and known wart treatment it showed less cytotoxicity and equal and better clinical effect. This lead to a positive outcome for suffers with warts and verruca pedis.

Throughout this study novel and interesting techniques have been assessed, A comprehensive literature search has confirmed the originality of the research into the therapeutic use of *Thuja occidentalis*, in particular *Thuja occidentalis* in podiatric medicine and the subsequent development over the past 5 years of its clinical use in the treatment of foot disorders.

The majority of studies have concentrated on Homeopathic *Thuja*, which is potentised (serially diluted in water) and administered orally. The approach, which was taken to analysis and evaluates *Thuja occidentalis* on verruca pedis, was uniquely carried out. The use of *Thuja* extracts and subfractions on verruca pedis and the method of application was produced by the author and the test carried out on cell lines and keratinocytes was the first reported case especially on HPV transfected keratinocytes. This methodology of both clinical and biological work has shown to be most advantages as procedure for examining plant extracts.

The work and results have shown a clear relationship between previous work carried out by Roth (1993) on the isolation of DPD from *Thuja occidentalis,* which has shown an antiviral effect, on the influenza virus. In the same light Beuscher and Kopanski (1986) reported that *Thuja occidentalis* had an antiviral action on herpes simplex virus. These both testified to the antiviral

activity of *Thuja occidentalis,* Beuscher and Kopanski (1986) using plaque reduction assay with HSV1, demonstrated the effect of *Thuja occidentalis* on the DNA metabolism of Hela cells by measuring the incorporation of (6-^{3}H) thymidine and cellular kinetics of subcultures after application against controls. Results of which was 50% cytotoxic dose of 400 μg/ml and minimal antiviral dose was smaller than 50 μg/ml. In the study we carried out it was interesting to note that cell growth was slowed during the presence of *Thuja* and subfractions but cells did continue to grow at a inhibited rate this give us an indication that the extract and subfractions are effecting the cell and not the virus directly, but by slowing the proliferation rate of cell the kinetic of the cell alter and the virus present is unable to function and lead to an antiviral effect.

The outcome of this work has shown great promise for the treatment of verruca pedis. Future development of this work could be beneficial if taken to a larger scale especially in the number of subjects the extracts and subfractions were applied to, this would give significant data for statistical analysis. Other principal areas for development would be cell line culture and keratinocytes with HPV DNA from types 1-4 as these are most commonly present in verruca pedis. Further studies using isolated DPD against purified DPD would give interesting structural relationship of activity. Especially when comparative biological assays and *in vivo* studies may show impurities that influence the outcome. A clear indication to the worth of *Thuja occidentalis* as a mainstream therapeutic agent would be, to go through a vigorous process of comparative trials with known verruca agents like Salicylic acid preparations, cryotherapy and anti-mitotic drugs. The measure of the outcome would be conclusive. Work into the mechanism that the *Thuja* and subfractions interact with HPV DNA should be studied further, to unlock the site of action, and evaluate cell cycle checkpoints and apoptosis. The uses of Polymerase chain reaction (PCR) and other microbiological techniques would prove to be beneficial.

In the area of pharmacological development, this and earlier studies have shown the therapeutic effect of species of the genus *Thuja* which hitherto has received little attention in clinical research. Investigation of other *Thuja* species and their mode of action may yield further beneficial results for healthcare. As Houghton (1995) has said: "Plants have an important role in both traditional and orthodox medicine and this warrants increased activity and funding in scientific investigation".

APPENDICES

Appendix 1

Department of Homoeopathic Podiatry (Marigold Clinic)
Royal London Homoeopathic Hospital
NHS Trust

CLINICAL TRIAL
VOLUNTEER INFORMATION SHEET

The aim of this study is to investigate the effect of extract and subfractions of a plant preparation in the treatment of verruca pedis.

VOLUNTEERS INVITED

Volunteers may be male or female aged 7 – 40 years. They will have verruca pedis on one or both feet and will not be taking medication for their verruca or any other form of regular medication. They will not have diabetes, osteo- or rheumatoid arthritis or any other systemic disease. They will not previously have had Marigold treatment for their verruca. If female, they will not be pregnant.

Volunteers will be free to withdraw at any time. However, once you have undertaken to participate, it is essential that you keep to the prescribed procedure. All participants will be examined and diagnosed. All information collected will be treated in strict confidence and remain the property of the researcher.

Participants are asked to:

- Attend the Wart Clinic / Marigold Clinic for treatment by appointment every Monday / Tuesday for four weeks and then return for observation after one, three and six months.

M. Tariq Khan
Chief Podiatrist and Specialist in Homoeopathic Podiatry
Honorary Research Fellow in Dermatology

Appendix 2

Department of Homoeopathic Podiatry (Marigold Clinic) Royal London Homoeopathic Hospital NHS Trust

CONSENT FORM

I ,

Hereby consent to participate in the research trial for the treatment of verruca pedis and I support the clinical trial at this clinic.

The procedure and my commitment to the study as a patient have been explained to me by the researcher.

I understand that I may withdraw from the trial at any time. Date Signature of patient

I confirm that I have explained the procedure and direction of the treatment to the patient.

Date Signature of researcher

Appendix 3

Wart & Verruca Clinic

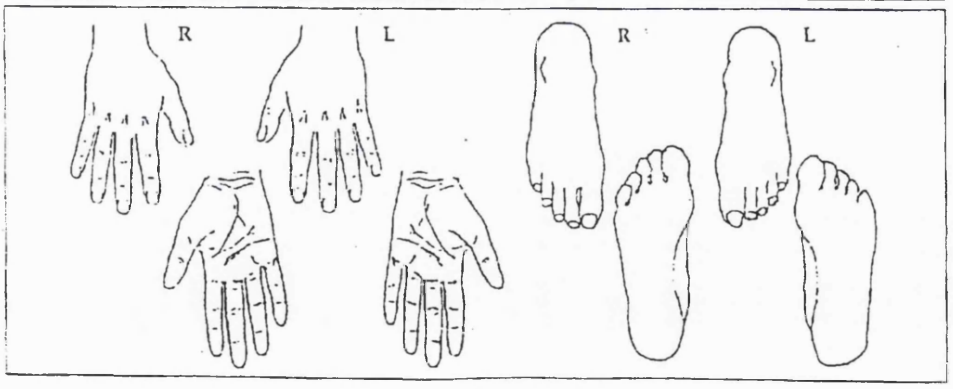
Patient Information

Name:	
Address:	
Tel No:	
Next of kin:	
GP details:	
Date of birth:	
Sex:	Ethnic Group:

Medical History and Drug History

Wart Assessment

Site:	
Type:	
Duration:	
Number:	
Previous Treatment:	



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	Initial	Week 1	Week 2	Week 3	1 Month	3 Months	6 Months
Date							
Wart Score							
Size (mm^2)							
Appearance							

Notes

M Tariq Khan, BSc Hons, MChS, LFHom, FLS
Honorary Research Fellow in Dermatology

Appendix 4

TOOLS AND APPARATUS

Apparatus

TLC plates:

Silica Gel:
DC-Alufolien, Kieselsgel 60 F_{254}
20 x 20 centimetres, 0.2 millimetres thick
Merck

Sephadex LH-20:

Pharmacia

Solvents used:

1. Acetone-General Purpose Reagent (GPR), BDH.
2. Toluene-GPR, BDH.
3. Chloroform (Analytical Grade)-Super Purity Solvent, Romil.
4. Chloroform-GPR, BDH.
5. Ethanol-Absolute, BDH.
6. Ethyl Acetate-GPR, BDH.
7. 90% Formic Acid-Laboratory Chemical, May & Baker Ltd.
8. Glacial Acetic Acid-Specified Laboratory Reagent, Fisions.
9. Methanol-General Laboratory Reagent, Pharmacos Ltd.
10. Methanol-HiPerSolv, BDH.
11. Sulphuric Acid-sp.gr.1.84, BDH.

Size of columns used:

Column Chromatography- 4.5 x 100 centimetres.
Sephadex- 1.5 x 40 centimetres.
All supplied by BDH.

Soxhlet:

Has a turnover point at approximately 2 Litres.
Supplied by BDH.

Rotary evaporator:

Model-Rotavapor R.
Manufacturer-Buchi.

Nuclear magnetic resonance spectrometer:

Model-FT-NMR 500 MHz.
Manufacturer-Bruker.

Mass spectrometer:
Model-VG MALDE Tofspec.

Appendix 5

Table A. Solvent systems and spray reagents

Solvent Number	Solvent System	Ratios
	Soxhlet extraction of plant material	
	Soxhlet:	
S-1	Ethanol: water (redistilled)	(70:30)
	Partition of crude extract:	
S-2	Hexane:water	(50:50)
S-3	Chloroform:water	(50:50)
S-4	Ethyl acetate:water	(50:50)
	Thin layer chromatography	
	Prepared silica gel & Analytical Analysis	
S-8	Chloroform: MeOH→5cm then toluene: acetone 5cm→ solvent front	(90:10) (65:35)
S-9	Ethyl acetate: formic acid: glacial acetic acid: water	(100:11:11:27)
	Column chromatography	
	Siilca gel	
S-10	Chloroform	(100%)
S-11	Chloroform: methanol	(50:50)
S-12	Methanol: water	(70:30)
	Distilled water	(100%)
	Sephadex LH-20	
S-13	Methanol HPLC grade	(100%)
	Spray Reagents	
R-1	Natural products-polyethylene glycol	
	NP: methanol	1:99
	PEG 4000: ethanol	5:95

R-2	Sulphuric acid: water	(80:20)
R-3	Ammonia (vapour)	(100%)

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Homeopathic treatment of common foot conditions

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Podiatric disorders seen in foot clinics are often only temporarily relieved by traditional treatments, necessitating regular attendance every few weeks; some chronic conditions do not respond at all. The chemical and surgical treatments commonly used in traditional podiatric practice are invasive and can cause pain or discomfort, especially the postoperative complications of surgery. Certain treatments are contraindicated for patients who are at risk; for example, the elderly and those with diabetes.

While numerous homeopathic medicines are indicated for conditions affecting the skin, bones and nails, the prognosis in weight-bearing areas is influenced by the effects of friction and pressure on the lesion and by biomechanical factors, which may cause or contribute to the problem.

Homoeopathic podiatry is a treatment method for skin, bone and nail conditions that combines homeopathy with podiatric practice. An integral part of the specialty is marigold therapy – a painless, non-invasive form of topical treatment which has been researched and developed by combining homoeopathic medicinal plants, such as *Symphytum officinalis, Thuja occidentalis, Ruta graveolens, Rosmarinus officinalis, Bellis perennis, Hypericum perforatum* and *Calendula officinalis,* with different species of *Tagetes.* Homoeopathic and biochemical medicines are prescribed orally when indicated.

Podiatric disorders seen in foot clinics are often only temporarily relieved by traditional treatments

Formulations of the topical preparations include pastes, tinctures, oils, ointments and creams. Marigold Footcare Ltd prepares and manufactures these preparations according to the *Homoeopathic Pharmacopoeia for Podologists,*[1] the *European Pharmacopoeia*[2] and the *British Homoeopathic Pharmacopoeia.*[3] Their effectiveness and safety in the treatment of foot disorders have been demonstrated clinically, as well as in double-blind placebo-controlled trials.

Methodology of marigold therapy

Marigold therapy preparations consisting of tincture, oil and paste are used with a felt protective pad. Three clinic treatments are given once a week. One advantage to this treatment is that normal hygiene procedures can be followed, although for the first three days after each treatment, baths or showers should be limited to three minutes and a pop sock or piece of tubular bandage should be placed over the foot to keep the dressing firmly in place while it is immersed.

After the three treatments, the patient is given marigold therapy tincture and oil to apply at home at prescribed intervals for a further period, which varies according to the nature of the lesion – in some cases, it may be for just one week. Advice is given on appropriate footwear. If necessary, following clinic treatment, a simple insole or an orthotic appliance may be prescribed.

Corn and callosity

The most common aetiological factors in corn and callosity (para- and hyperkeratotic lesion) are mechanical stresses (compressional, tensile, shearing or torsional) as a result of wearing inappropriate footwear. The traumatised skin becomes thickened and painful, which results in a corn which may be of hard, soft or seed type. If not treated at an early stage, the condition may become chronic, septic or even ulcerated.

Traditional treatment includes surgery, cryosurgery or caustics, keratolytic or astringent chemicals to remove overlying callosity and corns, biomechanical treatment and the use of protective pads or orthotics.

Case history

A male patient aged 75 suffered with dry, hard, painful corns and callosity for 20 years, despite routine chiropody treatment at the local hospital (see Figure 1a opposite). He was treated with marigold therapy preparations with a protective pad once a week for four weeks. *Antimonium crudum 30* was then prescribed once daily for one week. He reported pain relief within 48 hours of his first treatment and the corn and callosity were resolved within four weeks (see Figure 1b opposite).

Verruca pedis

A contagious viral infection, verruca pedis is a very common dermatological condition that is often contracted through exposure to the virus at swimming pools and sports centres. It is one of the most difficult and stubborn conditions that the podiatrist has to deal with – some health districts have special verruca clinics because of the difficulties.

The lesion may be single or multiple and can occasionally be very painful. It may be inflamed, dry or moist and it may also bleed easily (the blood supply to the virus being contained within a capsule which is covered by callosity).

Most traditional treatments for verrucae and warts are destructive and invasive, causing discomfort or pain. This is a particular disadvantage when treating children, the most frequent sufferers. The topical applications used contain salicylic acid, podophyllum or formaldehyde. Alternatively, cryosurgery or electrosurgery may be used.

Case history

A male patient aged 45 suffered with verruca pedis on the plantar of the big toe for about ten years during which he received all available treatments, including homoeopathy, without success. He had four treatments once weekly with marigold therapy preparations topically. He was then prescribed *Thuja occidentalis 30* once daily for one week and one dose of *Calcarea carbonica 200*. The patient experienced pain relief within 24 hours of the first treatment and, after four treatments, the verruca had disappeared. The patient was followed up for five years and there was no recurrence of the verruca.

Fungal infections

Onychomycosis is a dermatophyte fungal infection of the nails. It is characterised by discoloured, dry, hard or brittle nail plates that can sometimes be painful. Athlete's foot is a fungal infection of the skin, characterised by intense itching between the toes. The skin may become inflamed and/or macerated between the toes and have an unpleasant odour. Excessive sweating is one of the main pathological features and may – in rare cases – be associated with systemic disease. Both forms of infection can be stubborn and difficult to treat because of cross infection from shoes and hosiery.

Traditional treatment of onychomycosis is surgical or chemical removal of the nail plate. While this may shorten the treatment time considerably, recurrence of the problem is common. Most antifungal agents used in conventional medicine have adverse effects.

For athlete's foot, marigold therapy preparations consisting of tincture and oil are used in each case with four clinic treatments for six to nine months. Disinfection of shoes and hosiery is essential. During the treatment period, homoeopathic or biochemical medicines are prescribed according to the totality of symptoms.

Case history

A male patient aged 22 suffered 15 years with onychomycosis of the fifth toenail. He experienced pain with intense itching all around the nail. Marigold therapy tincture was applied to the nail, which was then reduced using an electric file. The cavity was covered with a two-milimetre-thick pad, which was placed over the nail and the cavity filled with paste. The cavity was covered with Micropore® (3M, UK) and the pad dressed with Mefix® (Mölnlycke, UK) strapping plaster. The patient was treated once a week for four weeks.

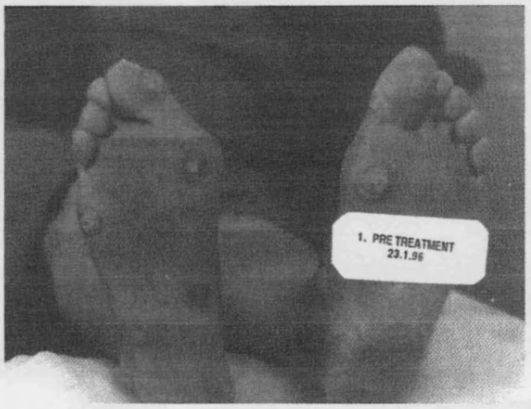

Figure 1a. A male patient aged 75 suffering with dry, hard, painful corns and callosity

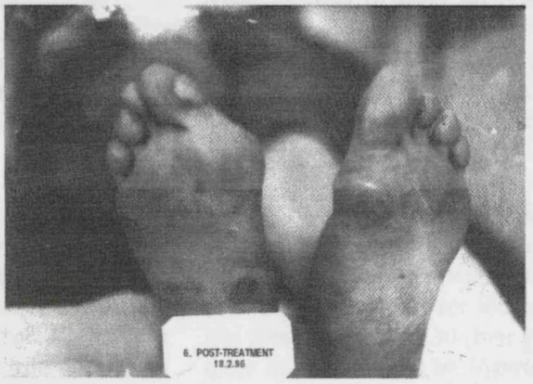

Figure 1b. After marigold therapy, the corn and callosity were resolved within four weeks

After four weeks, he received tincture and oil to continue with home treatment for six months according to a prescribed regimen to complete the course of treatment and as a preventive measure. He was prescribed one dose of *Silicea 200*, followed by *Antimonium Crudum 30* (four pills daily for one week), and then K combination New Era Biochemic Remedy (four tablets daily for three months). The patient was asked to disinfect all footwear and hosiery once only before commencing treatment. The nail was completely normal within five months and, when the patient was examined again in 1992 after six years, he reported there had been no recurrence.

Diabetic ulcer

Diabetic ulcers are a common condition that are difficult to manage with traditional treatments

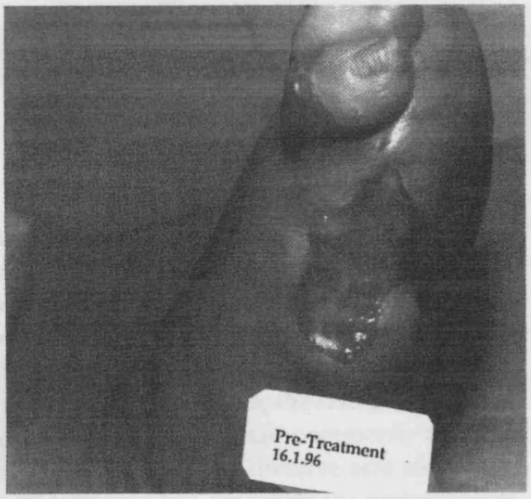

Figure 2a. A patient aged 69 with diabetic ulcers

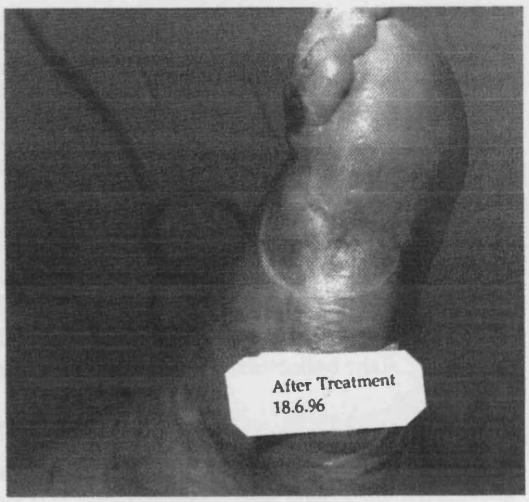

Figure 2b. After 18 weeks, all ulcers had healed

as healing depends on the patient's state of health and dietetic control of their sugar level. Diabetic ulcers take longer to resolve than non-diabetic ulcers.

Case report

A female patient aged 69, a diabetic for 40 years, had suffered with ulcers in both feet for two years (see Figure 2a). As a result of gangrene, the fifth toe on the right foot had been surgically removed. The right foot showed four ulcers: on the heel (3 cm x 3 cm); at the medial aspect of the fifth toe (4 cm x 3 cm); at the apex of the second toe (8 mm x 8 mm); and on the plantar area (5 cm x 3 cm).

The patient had been treated by a district community nurse two or three times a week for the past 20 years and had been told by her hospital consultant that it would take as much as two years to cure the ulcers.

She was referred by her GP to the marigold clinic at the Royal London Homeopathic Hospital for homoeopathic podiatry. The homoeopathic medicine was selected according to the symptomatology and treated using marigold therapy. The ulcers were cleaned with a composition mother tincture of *Calendula arvensis, Bellis perennis, Hypericum perforatum* and *Symphytum officinalis*. Cavity pads were placed over the ulcers and the cavities filled with marigold therapy healing ointment. The ulcers were treated twice a week for six weeks, then once a week for seven weeks and then every two weeks for four weeks. The patient was prescribed *Silicea 30* (four pills twice daily for three days), followed by *Hypericum perforatum 30* (once daily for two weeks).

The ulcer on the apex of the second toe healed in six weeks, the ulcers on the plantar area and on the medial aspect of the fifth toe in 17 weeks and the ulcer on the heel in 18 weeks (see Figure 2b).

Key points

- Podiatric disorders seen in foot clinics are often only temporarily relieved by traditional treatments and some chronic conditions do not respond at all.
- The effectiveness and safety of homeopathic medicines in treating foot disorders have been demonstrated both clinically and in double-blind placebo-controlled trials.
- Homeopathic podiatry is a non-invasive method requiring less frequent follow-up consultations compared with conventional treatment.
- The success of the homeopathic products, together with the painless, infrequent treatment encourages good patient compliance.

Conclusion

Homeopathic podiatry is a non-invasive method requiring less frequent follow-up patient consultations when compared with conventional treatment, leading to improved patient satisfaction and a low 'did not attend' rate, especially among adolescents. The success rate shown by the products, together with the painless, infrequent treatment encourages good patient compliance. The lack of demand for regular ongoing treatments allows for economic savings, through fewer staff and saving of NHS resources ■

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Marigold treatment of foot conditions

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A number of the podiatric disorders seen in foot clinics are relieved only in the short term by traditional treatments, necessitating repeated clinic attendance every few weeks. Some chronic conditions may not respond at all to traditional treatments. Furthermore, the chemical and surgical treatments that are commonly used in podiatric practice are invasive and can cause pain or discomfort, especially the postoperative complications of surgery. Certain treatments are contraindicated for 'at-risk' patients – for example, diabetic patients and the elderly.

Homeopathic podiatry

While numerous homeopathic medicines are indicated for conditions affecting the skin, bones and nails, the prognosis in weight-bearing areas is influenced by the effects of friction and pressure on the lesion, and by biomechanical factors, which may predispose or contribute to the problem.

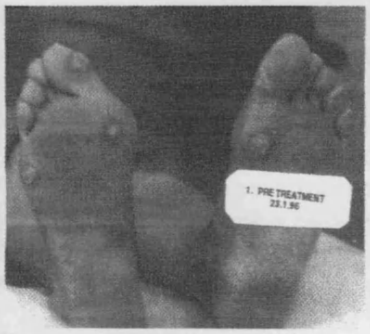

***Figure 1a.** Corns and callosity of the feet of a 75-year-old patient*

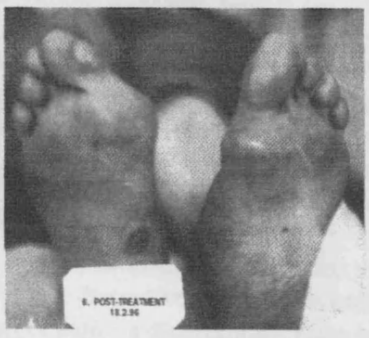

***Figure 1b.** Improvement of corns and callosity after four weeks' treatment with marigold therapy*

Homeopathic podiatry was developed as a method of treatment for conditions of the skin, bones and nails. It combines homeopathy with standard podiatric practice. An integral part of the specialty is marigold therapy, a painless, non-invasive form of topical treatment, which has been researched and developed by combining homeopathic medicinal plants, such as *Symphytum officinalis, Thuja occidentalis, Ruta graveolens, Rosmarinus officinalis, Bellis perennis, Hypericum perforatum* and *Calendula officinalis*, with different species of *Tagetes*. Homeopathic and biochemical medicines are prescribed orally when indicated.

Formulations of the topical preparations include pastes, tinctures, oils, ointments and creams. These are prepared according to the *Homeopathic Pharmacopoeia for Podologists* 1979, the *European Homeopathic Pharmacopoeia* 1993 and the *British Homeopathic Pharmacopoeia* 1995. Their effectiveness and safety in the treatment of foot disorders has been demonstrated clinically and in double-blind, placebo-controlled trials.

Methodology

Marigold therapy preparations consist of tincture, oil and paste and are used with a felt protective pad in three once-weekly clinic treatments. An advantage of this treatment is that normal hygiene procedures can be followed. The only exception is that, for the first three days after a treatment, baths or showers should be limited to three minutes and a pop sock or tubular bandage should be placed over the foot to keep the dressing firmly in place while the foot is immersed. Additional homeopathic medication may also be prescribed. Following the three clinic treatments, the patient receives marigold therapy tincture and oil to apply at home at prescribed intervals for a further period, which varies according to the nature of the lesion.

Para- and hyperkeratotic lesions (corns and callosity)

The most common aetiological factors in corns and callosity are mechanical stresses – for example, compressional, tensile, shearing or torsional stresses – as a result of wearing inappropriate footwear. The traumatised skin becomes painful and thickened, resulting in a corn which may be hard, soft or a seed. If this is not treated at an early stage, the condition may become chronic, septic or ulcerated.

Traditional treatment includes surgery; cryosurgery or caustics; keratolytic or astringent chemicals to remove overlying callosity and corns; biomechanical treatment; the use of protective pads or orthotics; and advice about footwear.

The first trial of marigold therapy preparations was a clinical study to investigate their effect in the treatment of hyperkeratotic plantar lesions.[1] Thirty randomly selected patients were divided into three groups of ten. Group A was treated with the active sample with a protective pad, Group B with placebo with a protective pad, and Group C with the active sample without a protective pad.

Measurements of lesions were made by an independent assessor. The coded active and placebo samples used in the double-blind trial were prepared by a pharmacist. The results of the study showed a high level of significance ($p<0.001$), suggesting that marigold therapy preparations with a protective pad were effective in reducing the size of the lesion and in relieving pain in the treatment of hyperkeratotic plantar lesions in comparison with marigold preparations without a protective pad and placebo with a protective pad Figures 1a and 1b.

Hallux valgus and bunions

A double-blind trial investigated the effect of marigold therapy preparations in the treatment of hallux abducto valgus (HAV) and its associated condition, bunion.[2] Twenty patients with *bilateral* HAV and bunion were randomly selected. One foot was treated with the active sample, the other with placebo, and both with a protective pad. A further 40 randomly selected patients with *unilateral* HAV and bunion were divided into two groups of 20. One group was treated with the active sample, the other with placebo, and all with a protective pad. All measurements of the lesion – the swollen soft tissue, the angle of the valgus deformity and the level of pain – were recorded by an independent assessor.

In both trials, treatment was for four weeks. The coded active and placebo samples used in both trials were prepared by a pharmacist.

The results of both studies showed a high level of significance ($p<0.001$), suggesting that marigold therapy preparations with a protective pad were effective in reducing inflammation and in relieving pain in the treatment of HAV and bunion.

Verruca pedis

Verruca pedis is a very common dermatological condition, especially among children. A contagious viral infection, it is frequently contracted through exposure to the virus at public baths and sports centres. The lesions can be single or multiple, and very painful. They may be inflamed, dry or moist and may also bleed easily, as the blood supply to the virus is contained within a capsule that is covered by callosity.

Most traditional treatments for verrucae and warts are destructive and invasive, causing discomfort or pain – a disadvantage when treating children. The topical applications used contain salicyclic acid, podophyllum or formaldehyde. Alternative treatments are cryo- and electrosurgery.

In a clinical evaluation of marigold therapy preparations in the treatment of verruca pedis, 40 patients were randomly divided into four groups. The first two groups were used to compare the effect of the two samples, with one group receiving the active sample and the other receiving placebo. The third and fourth groups were used to evaluate the effect of wearing a protective pad over the lesion, one group receiving the active sample with a pad, the other using a pad only. Treatment was twice weekly for two weeks, followed by a further four weeks of home treatment. The results were analysed by recording lesion surface area using a wound mapping chart. There was a significant difference between results for the group using the active treatment and the group using placebo ($p<0.005$). Using the pad without the active sample was seen to be effective to a limited extent in removing pressure and friction from the lesion but, in itself, had no curative effect.[3]

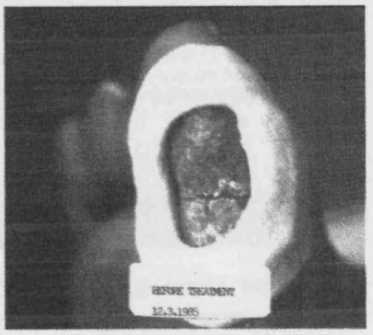

Figure 2a. *Verruca pedis on the toe of a 45-year-old patient*

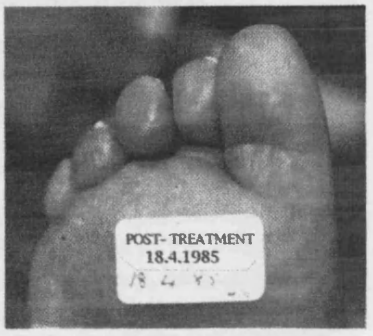

Figure 2b. *Resolution of lesion after four weeks' treatment with marigold therapy. The patient remained symptom-free five years after treament*

During the period 1998–2000, *T occidentalis* (which is part of the formulation used in marigold therapy) was evaluated as a treatment for verruca pedis. The first study was designed to evaluate its efficacy in children and adults.[4] Thirty patients with verruca pedis were randomly selected. All were referrals from GPs or hospital consultants. Patients were aged between seven and 40 years (mean 14 years) and comprised 22 males and eight females. The median duration of lesions was 18 months. The *T occidentalis* extract was applied weekly for three weeks.

Patients were assessed after one month, again after three months, and finally after six months. In 27 out of 30 cases (90%), resolution had occurred by the time of the first assessment, with no recurrence by the time of the final assessment. The study confirmed an antiviral effect of *T occidentalis* and demonstrated that this had a beneficial therapeutic effect on verruca pedis.[4]

Following this open study, a double-blind study of 30 patients with verruca pedis was performed.[5] Patients were aged between seven and 40 years (mean 16 years) and comprised 18 males and 12 females. The median duration of lesions was 17 months. As before, the extract was applied weekly for three weeks and patients were assessed after one month, three months and six months. Patients were assigned to treatment regimens

according to a randomisation table, 15 receiving active *T occidentalis*, and 15 receiving placebo. Samples were prepared at the Centre for Pharmacognosy, University of London.

In 12 out of 15 active cases (80%) and five out of 15 placebo cases (33%), resolution occurred by the time of the first assessment, with no recurrence by the time of the final assessment ($p<0.001$). The study confirmed an antiviral effect of *T occidentalis* (Figures 2a and 2b).

Conclusion

As well as plantar hyperkeratosis, HAV and bunions, and verruca pedis, the authors have also used marigold therapy in the treatment of fungal infections and diabetic ulcers. This non-invasive, homeopathic treatment is safe and effective for the management of such chronic conditions of the feet ■

Conflict of interest statement
Taufiq Khan is Managing Director of Marigold Footcare Ltd

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Key points

- **Marigold therapy is a painless, non-invasive form of topical treatment that combines homeopathic medicinal plants with different species of *Tagetes* for the relief of podiatric disorders.**
- **The effectiveness of marigold therapy preparations, which include pastes, tinctures, oils, ointments and creams, has been demonstrated clinically and in double-blind, placebo-controlled trials.**
- **Marigold therapy preparations are used with a felt protective pad in three once-weekly clinic sessions, followed by further applications at home, the number of which will depend upon the nature of the lesion.**

PCDS profile

North of England meeting

Primary Care Dermatology Society
PO Box 6
Princes Risborough
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Our inaugural North of England meeting, in Prestbury, Cheshire, got under way at 9:30 with Chris Griffiths from Hope Hospital taking a fascinating look at new developments coming our way in psoriasis treatment. Vicky Yates was next, with an insight into what can be achieved in a paediatric eczema clinic – particularly when nurses are given extended time with parents to explain and demonstrate treatments.

Nick Evans, Programme Director for Action on Dermatology, stimulated much debate with his ideas on 'GPs with special interests' and the move to bring a lot more dermatology out of hospitals and into the community.

The final shift of the morning fell to Neil Cox (*DIP* Editor) who had agreed (with not too much coercion) to take on the almost impossible task of distilling vasculitis and the connective tissue disorders into 40 minutes. His presentation certainly knocked the rust off my knowledge of this area.

After a delicious lunch each delegate attended two of three workshops; cryosurgery with Arthur Jackson: nurse management with Linda Rowan; and urticaria with Rhona Knight and Tom Poyner. People appeared to enjoy leaving the lecture theatre behind and having a bit more input into the teaching. Hopefully, this is an idea we can repeat, though suggestions on subjects for new workshops would be gratefully received.

Having had to work through the afternoon there was only one way to wind down – the inimitable Tony Burns entertained and educated us with his 'Dermatological Mish Mash'. The educational part of the meeting was then closed by the chairman (despite a distinct loss of voice).

Sadly, many of our delegates more local to the venue then departed for home. However, there were plenty of stalwarts for an evening of food and dancing – even though the removal of the chairman's jacket caused such an electric reaction in the disco that a short power cut ensued!

Thanks must go to all the speakers and workshop organisers, our sponsors, and especially to Carol Gifford and Elaine Abrey ■

John Young MB ChB MRCGP GP, Barrow-in-Furness

Phytotherapy with subfractions of topical Thuja Occ on verruca pedis

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Verruca Pedis is a common condition seen in podiatric and dermatology practice and sometimes can be resistant to treatment. Many different types of treatments are available which include liquid nitrogen, electro-cautery, salicylic acid and podophyllin. Thujas are a group of trees belonging to the cedar family. Thuja contains a volatile oil, flavonoids, mucilage and an astringent. It has been documented that Thuja, a common conifer, possesses antiviral properties, which have been successful on herpes simplex (I) virus.[1]

The aim of this study was to evaluate the efficacy of compounds present in Thuja on verruca pedis. Partitioning Thuja with three different solvents hexane, chloroform and ethyl acetate carried this out. In previous studies presented at the EADV (1998, 1999), Thuja Occ showed antiviral action on verruca pedis (HPV); *in vivo*, 90% of patients treated, had complete resolution within a period of 3 months. A double-blind study on 30 patients followed and showed 80% resolution in active group, compared 33% in placebo group, giving a significance $P > 0{\cdot}001$.[2] Verrucae, which were large, moist, and yellow cheese-like growths, were most responsive to treatment.

In this study 30 patients with verruca pedis were randomly selected referrals from general practitioners and dermatologists. The age range of patients, were 7–40 years, with a mean of 16 years. Males to females ratio 18:12. The median duration of lesions present was 17 months.

Patients were assigned treatment regimes according to a randomization table.[3] 10 receiving hexane fraction, 10 chloroform fraction and 10 ethyl acetate fraction. Samples were prepared at the Centre for Pharmacognosy, University of London.

The results of wart clearance for each subfraction showed 0 out 10 (0%) hexane group, 10 out 10 (100%) chloroform and 4 out 10 (40%) in the ethyl acetate group. Resolution occurred by the time of the first assessment with no recurrence up to 3 months (mean of 14) of the final assessment. In all cases cured there were no signs of relapse, this was monitored over one year. Our study demonstrated that chloroform fraction of Thuja was superior to ethyl acetate and hexane, and that the active HPV compounds are present

Phytotherapy with subfractions of topical Thuja Occ on verruca pedis

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Clinical application of homeopathic podiatry as used at The Royal London Homoeopathic Hospital (RLHH)

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Objectives

It is acknowledged that a number of podiatric disorders seen in foot clinics are relieved only in the short term by traditional treatments, necessitating regular attendance every few weeks. Some chronic conditions do not respond to traditional treatments. The chemical and surgical treatments commonly used in podiatric practice are invasive and can cause pain or discomfort, especially from post-operative complications of surgery. Some treatments are contraindicated for patients who are at risk, eg diabetics and the elderly.

Whilst homeopathy has numerous medicines indicated for conditions affecting the skin, bones and nails, the prognosis is influenced in weight-bearing areas by the effects of friction and pressure on the lesion and by biomechanical factors which may predispose or contribute to the problem. Subsequently, there is a need for a non-invasive, painless method of treatment, which would be therapeutic rather than palliative.

Method

Homeopathic podiatry is a method of treatment for conditions of the skin, bones and nails which was developed by combining homeopathy with podiatric practice. An integral part of the speciality is Marigold Therapy, a painless, non-invasive form of topical treatment which has been researched and developed by combining homeopathic medicinal plants, such as *Symphytum officinalis*, *Thuja occidentalis*, *Ruta graveolens*, *Rosemarinus officinalis*, *Bellis perennis*, *Hypericum perforatum* and *Calendula officinalis* with different species of *Tagetes*. Homeopathic and biochemical medicines are prescribed orally when indicated.

The topical preparations are in the form of paste, tincture, oil, ointment, and cream. Treatment consists of weekly applications of the paste for 3 to 4 weeks, followed by the use of tincture, oil, ointment or cream applied by the patient as a preventative measure. The effectiveness and safety in the treatment of foot disorders has been demonstrated clinically and in double-blind placebo-controlled trials.

Results

Double-blind placebo-controlled studies to investigate hyperkeratotic plantar lesions, hallux valgus and verruca pedis have shown a level of significance of $P < 0.001$. Fungal skin and nail infections and diabetic ulcers have responded well to treatment and are currently under investigation.

Conclusions

Homeopathic podiatry treatment is a non-invasive method requiring less frequent follow-up patient consultations when compared with 'conventional treatment'. This also encourages patient satisfaction and a low non-attendance rate, especially in the case of juveniles. The success rate shown by the products and painless, infrequent treatment allows for good patient concordance. The lack of demand for regular ongoing treatments allows for economic savings, both with fewer staff and saving of NHS resources.

Today's patients have greater expectations of the outcome of treatment, an attitude supported by the importance given to evidence-based medicine in the NHS. Homeopathic podiatry meets these demands of the patients and is coherent with Government policy on evidence-based medicine. Most importantly for NHS purchasing authorities, the high rate of resolution makes homeopathic podiatry cost-effective.

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A DOUBLE BLIND PLACEBO STUDY OF TOPICAL THUJA OCCIDENTALIS ON VERRUCA PEDIS IN CHILDREN AND ADULTS

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The aim of this study is to evaluate the efficacy of Thuja Occ using a placebo versus treatment, double-blind trial.

In the previous study presented at The European Academy of Dermatology in 1998 , we showed that Thuja Occ had anti-viral action on verruca pedis (HPV) in vivo. 90% of the patients treated with Thuja Occ had complete resolution in a period of three months. Results found that verruca, which were horny, hard, single were most responsive to treatment.

Thirty patients with verruca pedis were randomly selected referrals from general practitioners and hospital consultants. The age range of patients were between 7 to 40 years, with a mean of 16 years and the mean of male to female were 18 to 12 and the median duration of lesions present were 17 months. The extract was applied weekly for three weeks. Patients were assessed after one month and again after three months and finally after a further six months.
Patients were assigned treatment regimes according to a randomisation table, 15 receiving active Thuja Occ and 15 placebo. Samples were prepared at the Centre for Pharmacognosy, University of London.

12 out of 15 (80%), (active) cases and 5 out of 15 (33%), (placebo) cases resolution occurred by the time of the first assessment with no recurrence by the time of the final assessment. In all cases of resolution there was no signs of relapse, this was monitored over one year. The results show a level of significance $p < 0.001$.The study confirmed anti-viral effect in Thuja and demonstrated that these have a beneficial therapeutic effect on verruca pedis.

This study demonstrated the efficacy of Thuja for a variety of HPV types, and importance of extract to be superior to both placebo and/or spontaneous resolution.

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Abstract published in the Journal of the European Academy of Dermatology and Venereology. September 1999, Vol 1, pS251

FC13-6 The efficacy of Thuja occidentalis on verruca pedis in children and adults

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Verruca pedis is a common condition seen in the community and hospital clinics, and results in significant morbidity. Massing and Epstein (1963) found that 53% of all warts resolve in one year and 67% in 2 years. Radical therapies are still used including intralesional bleomycin and interferon, but true evaluation is unclear. It has been demonstrated that Thuja Occidentalis an extract from a common conifer possesses anti-viral properties which have been successful on herpes simplex (1) virus (Beuscher, 1986). The aim of this study was to evaluate the efficacy of Thuja on verruca pedis in children and adults. Thirty patients with verruca pedis were randomly selected referrals from general practitioners and hospital consultants. The age range of patients were between 7 to 40 years, with a mean of 14 years and the mean of male to female were 22 to 8 and the median duration of lesions present were 18 months. The extract was applied weekly for three weeks. Patients were assessed after one month and again after three months and finally after a further six months. In 27 out of 30 (90%) cases, resolution occurred by the time of the first assessment with no recurrence by the time of the final assessment. In all cases of resolution there was no signs of relapse, this was monitored over one year. The study confirmed anti-viral effect in Thuja and demonstrated that these have a beneficial therapeutic effect on verruca pedis.

This preliminary study demonstrated the efficacy of Thuja in treating painlessly a variety of HPV types, and the importance of this extract to be superior to both placebo and/or spontaneous resolution. Studies to isolate and purify compounds in Thuja extract and the development of a tissue culture system for HPV propagation to facilitate in vitro investigation are in progress.

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55 THE CLINICAL EVALUATION OF *THUJA OCCIDENTALIS* IN THE TREATMENT OF VERRUCA PEDIS

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The Human Papilloma Virus (HPV), the cause of wart/verruca, represents a group of highly specialised DNA viruses displaying a remarkable host and target-cell specificity. They induce papillomatous proliferation in skin and mucosa via specific differentiation of the genome-harbouring cell. No reproducible method has yet been developed for HPV propagation in *vitro*, which consequently has numerous studies concerning the infectivity in man. This has impeded research into the biological properties of the virus and immunological responses to wart infection (Zur Hausen, 1980).

Verruca pedis is a common condition seen in podiatric and dermatological clinics. Radical therapies are used but can rarely be evaluated successfully, hence not giving positive indications of therapeutic results.

It has been demonstrated that *Thuja occidentalis* possesses anti-viral properties which have been successful on herpes simplex (I) virus (Beuscher, 1986).The aim of this presentation is to evaluate *Thuja* occ. on verruca pedis.

Methanolic extract was obtained by Soxhlet extraction. The crude extract was evaporated to dryness under reduced pressure at 40°C and the dried crude extract was dissolved in 70% methanol and used with chiropody felt cavity pad for the clinical study.

Thirty patients with verruca pedis were randomly selected, according to inclusion and exclusion criteria. Ethical approval was not required as a pharmaceutical preparation in current use was being evaluated. The extract was applied weekly for three weeks. Patients were assessed after one month and again after three months and finally after a further six months.

In the majority of cases, resolution occurred by the time of the first assessment with no recurrence by the time of the final assessment. The findings of the study confirm the presence of anti-viral compounds in *Thuja* occ. and demonstrate that these have an effect on verruca pedis.

Research is now being concentrated on the isolation and purification of compounds present in the *Thuja* extract and the development of a tissue culture system for HPV propagation to facilitate *in vitro* investigation.

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Identification criteria for *Thuja* (species) used in medicine

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This study focuses on the identification of medicinally important properties found in *Thuja* species.

There are four main species of *Thuja* (Fam. Cupressaceae), *occidentalis* (Eastern white cedar), *orientalis* (Chinese arborvitae), *plicata* (Western red cedar) and *standishii* (*Thuja japonica*) (Banthorpe 1971).

Thuja consists of dried young twigs with coniferous pyramidal, flattened branches, the twigs in one plane bearing small scales, each species containing a volatile oil, flavonoids, mucilage and an astringent. The composition of volatile oil shows a great variation within the species and there is seasonal variation of constituent compounds.

Thuja species are traditionally used in medicine and reported to have anti-cancer, anti-haemorraghic, haemostatic, antimicrobialim and antiviral activity (Wren 1988).

Thuja occidentalis has been used in the treatment of verruca pedis (Khan et al 1984) and clinically evaluated in a controlled study of *Thuja* species in the treatment of verruca pedis (Khan 1997).

The plant material from each species was dried, ground and soxhlet extracted with methanol. The crude extracts were evaporated to dryness under reduced pressure at 40°C using a rota-evaporator and the dried extracts dissolved in 70% methanol.

The Rf value of each extract was compared on TLC silica gel $60F_{254}$ aluminium sheet using toluene and ethyl acetate as the solvent system (Wagner et al 1984).

The results of this study showed a significant difference in the Rf value of each species indicating variation in the chemical constituents of each *Thuja* species (Banthorpe 1971).

It was also found in clinical studies that *Thuja occidentalis* was found to be more effective in the treatment of verruca pedis than *Thuja orientalis*, *Thuja plicata* and *Thuja standishii* (Khan 1997).

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