Chapter 8

Antibacterial, antioxidant and cytotoxic activity of taraxerol, a pentacyclic triterpenoid, isolated from *Pteleopsis myrtifolia* leaves

Abstract

A known pure pentacyclic triterpenoid, taraxerol (14-taraxeren-3-ol), that was isolated from *Pteleopsis myrtifolia* leaves by bioassay-guided fractionation in a previous investigation (Chapter 7), was investigated for its bioactivity. It had antibacterial activity and MIC values of 0.04, 0.016, 0.63 and 0.31 mg/ml for the bacteria *Staphylococcus aureus*, *Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli,* respectively. It did not have significant antioxidant activity. It had cytotoxic activity and showed significant growth inhibition of the human cancer cell line $WHCO_3$ (oesophagus).

8.1 Introduction

8.1.1 Triterpenoids

Probably no other group of metabolites throughout the Plant and Animal kingdom has such diversity, so many functions and is produced by so many organisms than terpenoids (Harrewijn *et al*., 2001). Plant hormones are often derivatives of terpenoids, such as cytokines, gibberellins and abscissic acid. The steroid hormones of mammals are terpenoids with an advanced but not very complex structure (Harrewijn *et al*., 2001).

Terpenes have a unique structure: they consist of an integral number of five-carbon (5C or isoprene) units. Two such units can form a monoterpene (C-1O), and sesquiterpenes (C-15), diterpenes (C-20), triterpenes (C-30), tetraterpenes (C-40) and polyterpenoids (>40C) are also

possible. Many terpenoids are produced via the mevalonic acid pathway (MAD) that probably had its origin during the early development of life on this planet. Other terpenoids are biosynthesised via a recently discovered pathway, a mevalonate independent route (MAI) ((Harrewijn *et al*., 2001). The mechanisms that regulate the biosynthesis of mevalonate are finely tuned. In many organisms, end products in which isoprenoids are incorporated can reduce the activity of β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase(s) via a feedback and regulatory system, in this way achieving for example; cholesterol homeostasis. Figure 8.1 shows the several places in steroid biosynthesis where feedback for regulation takes place. Terpenoids can have a simple aliphatic or a cyclic structure. The cyclic structure(s) can exist in mono-, bi-, tri- and polycyclic formations and many of them can be polymerised. Polymerisation can be artificially induced by strong acids, such as nitric acid, UV light, temperature, oxygen and co-polymers that result in complicated structures.

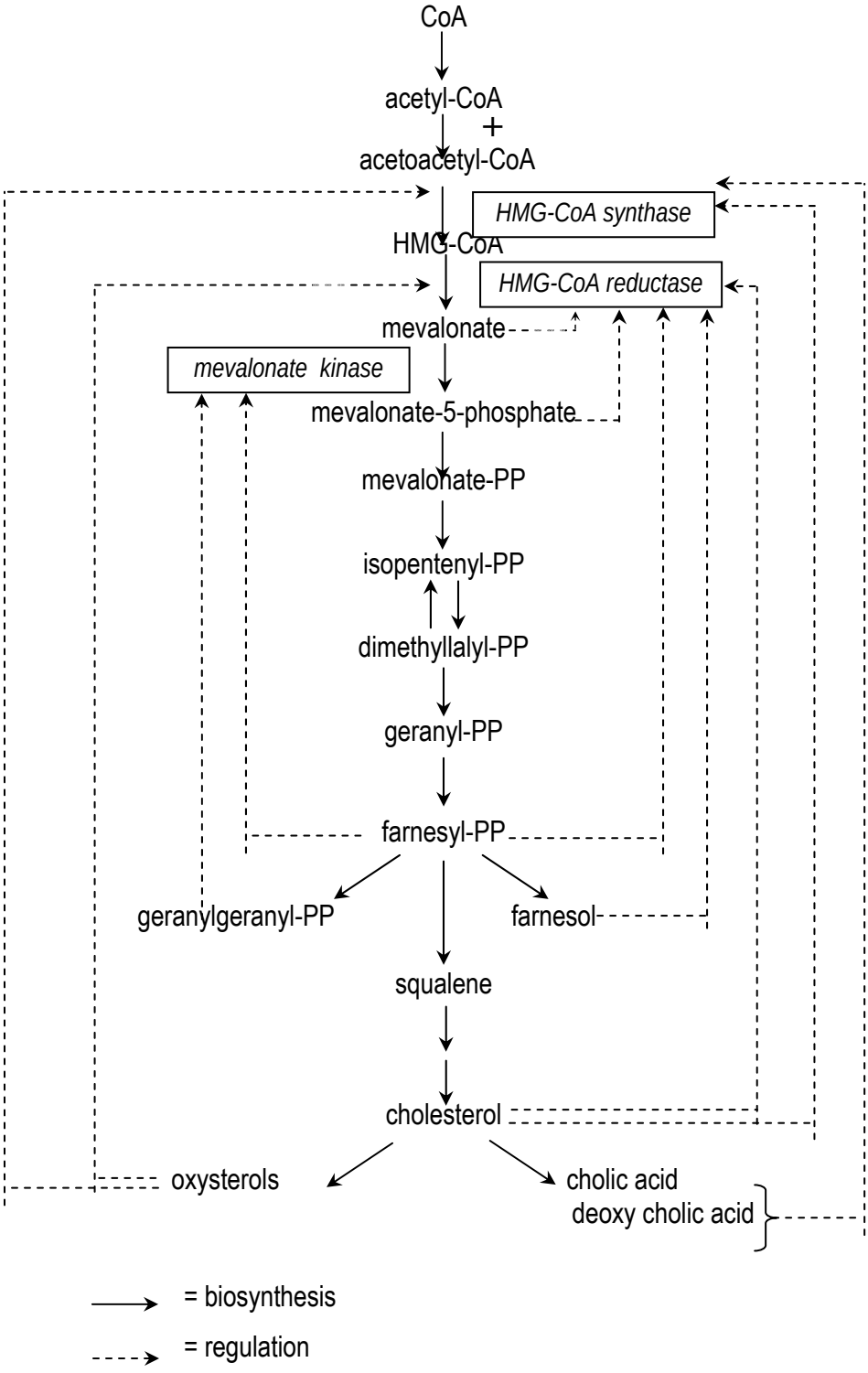

Figure 8.1. Multivalent regulation systems of steroid biosynthesis of the mevalonic acid pathway (MAD) metabolism in vertebrates (Harrewijn *et al*., (2001). (CoA = coenzyme A, HMG = β-hydroxy- β-methylglutaryl, PP = pyrophosphatase).

8.1.2 Specific functions of terpenoids

Terpenoids have a role in the regulation of isoprenoid metabolism and signal transduction and as such can exert a profound effect on cell growth, differentiation, apoptosis and multiplication. According to Penuelas *et al*. (1995), no other biochemical group of secondary metabolites has such a potential to interfere with processes ranging from cell level to ecological interactions. Moreover, the lower terpenes are rather volatile, an essential physical property for air-borne long distance effects. Nature uses terpenes in a "chemical language" between plants, insects, vertebrates and even humans. Terpenes and other isoprenoids have also important functions as messengers: they can act as defensive substances in plants (allomones) and animals; they can be used by plants to deter herbivores or to inform conspecifics, or to attract natural enemies of herbivores; within organs and within the cell body, in particular between the cell surface and the cell nucleus. They can have free radical scavenger capacities as antioxidants, keep homeostasis of cell numbers, have effects on Ras proteins, effect cancer cells in different ways, impair mevalonic acid synthesis in tumours and cause unexpected effects. Terpenoids can be toxic to micro-organisms, insects and other animals.

Often their effects are additive or even synergistic with other mevalonate metabolites, or they are inhibitors of parts of the mevalonate pathway. Thus, their mode of action should be viewed with respect to the role of other mevalonate metabolites in growth, development and behaviour of the organism studied.

8.1.3 Importance of knowledge about terpenoids

From ancient times, humans have utilized the messenger functions (volatile properties) of natural terpenoids for several purposes without knowledge of their structure. Terpenoids' structure elucidation had to wait until the second half of the 20th century for their eventual revelation. Since its identification in 1956, mevalonic acid has been recognized as a key

substance in the biosynthesis of a wide range of isoprenoids, including terpenoids. Mevalonic acid is produced in many organisms from acetate via a generally occurring enzyme, acetyl co-enzyme A. End products of the mevalonic pathway include sterols such as cholesterol, involved in membrane structure; haem A and ubiquinone, active in electron transport; dolichol, required for glycoprotein synthesis; carotenoids with many functions; steroid hormones in animals; hormones in insects and isopentenyl adenine and isoprenoid proteins, both involved in DNA synthesis. A study of a basic function of a messenger molecule in a particular organism increases our understanding of regulatory systems in distant taxa. The more these systems are involved in basic processes (e.g. gene expression), the greater scientific disciplines will benefit from such a study (Harrewijn *et al*., 2001).

Specific targets of messenger molecules in fully developed organisms are usually studied by specialists. It is highly likely that they are unaware of the same compound having profound effects in organisms belonging to other groups, although this knowledge is somewhere amidst a wealth of accessible information. Terpenoids are such compounds. Insects are needed to pollinate our crops, but they can also destroy them and defoliate our forests, or cause epidemic diseases both for livestock and humans. Terpenoids have become of widespread importance in perfumery, detergents, foods and beverages, chemical manufacturing industries, pharmacy and biotechnology, yet knowledge of their potential utilization is only just beginning to be revealed (Harrewijn *et al*., 2001).

8.1.4 Natural terpenoids to the benefit of human health

Examples of benefits of terpenoids to human health form literature are: antimicrobials, analgesics, cholesterolemia and vascular problems, tracheal and bronchial disorders, arthritis, rheumatism, inflammatory disorders, intestinal disorders, stress-related problems and sedatives, cancer therapies, cosmetics, sex attractants, dermatological preparations, terpenoid

analogues and derivatives applied in agriculture and in medicine (Harrewijn *et al*., 2001). Antimicrobial properties and anticancer action of taraxerol are listed in Tables 8.1 and 8.2 respectively.

8.1.4.1 Antimicrobial activity

Only a few examples of terpenoids acting as antimicrobials are given in Table 8.1.

Table 8.1. Effect of volatile terpenoids on bacteria (Harrewijn *et al*., 2001).

Species	Gram-	Gram+	Inhibiting terpenoids
Bacillus subtilis			acorenone, rimulene
Citrobacter freundii (sym-bionts in gut of termites)			thujone
Enterobacter spp. (sym-bionts in gut of termites)			linalool
Escherichia coli			carvacrol (ph.t.), citronellal, citronellyl acetate, geraniol, linalool, neral, pulegone, terpinen-4-ol, α-pinene, α-terpineol, δ-3-carene
Flavobacterium suaveolens			germacrene, sabinene
Klebsiella oxytoca			cineole, thujone
Klebsiella pneumoniae			thujone, 1,8-cineole
Mycobacterium smegmatis			alantolactone, isoalantolactone
Proteus vulgaris			limonene (toxic to cat flea), 1,8-cineole
Pseudomonas aeruginosa			linalool, pulegone
Salmonella spp. *Shigella shiga*			β-caryophyllene, β-caryophyllene oxide β-caryophyllene, β-caryophyllene oxide
Staphylococcus aureus			carvacrol (ph.t.), citronellal, citronellol, manool, pulegone, β-caryophyllene
Streptococcus faecalis			carvacrol (ph.t.), citronellal, citronellol, thymol
Vibrio cholerae			carvacrol, thymol (ph.t.), β-caryohyl-lene, β-caryophyllene oxide

((ph.t) stands for phenolic terpenoids)

8.1.4.2 Cancer therapies

The triterpenoids taraxasterol and taraxerol exhibited potent antitumour-promoting activity in

cacinogenesis tests of mouse skin (induced by a chemical initiator and promoter). In addition, they had an inhibitory effect on mouse spontaneous mammary tumours (Takasaki *et al.,* 1999). The anticancer activity of some terpenoids are listed in Table 8.2.

Table 8.2. Terpenoids' modes of action against tumour cells AG = angiogenesis; cytos. = cytoskeleton; CA = carcinogenes; HMGR = HMG-CoA reductase; diff. = (re)differentiation; ? = unknown (Harrewijn *et al*., 2001).

Terpenoid	AG	DNA	cytos.	G1 arrest	CA	HMGR	diff.	?
aphidicolin		✓					✓	
asprellic acids								✓
betulinic acid derivatives								✓
carotenes							✓	
corosolic acid	✓							✓
curcumins							✓	
dehydrothyrsiferol								✓
farnesol		✓	✓	✓		✓		
geranylgeraniol		✓	✓	✓		✓		
geranylstilbenes								✓
ginsenoides	✓							
gossypol		✓						
β-ionone				✓				
kansuiphorin								✓
limonene				✓	✓	✓		
limonoids								✓
menthol						✓		
perillyl alcohol				✓	✓	✓		
protolichesterinic acid		✓						
oleanolic acid	✓							
pinene						✓		
remangilones								✓
retinoids							✓	
taraxasterol					✓			
taraxerane					✓			
taraxerol					✓			
taxamairins			✓					
taxol			✓					
tingenone								✓
tocotrienols							✓	
ursolic acid	✓							
vitamin K2					✓		✓	

Parallel studies on the different aspects of terpene and steroid chemistry gradually revealed that squalene, a rare C_{30} hydrocarbon, was a conceivable progenitor of the higher terpenoids. Squalene was first isolated from shark liver, *Squalus* spp., which was later found to be

ubiquitously distributed. By folding this compound, one can construct the basic triterpenoid skeleton with the angular methyls and side chain in correct positions, to incorporate into, for example cholesterol.

One previous report of taraxerol's isolation in the Combretaceae was from leaves of *Terminalia glabrescens* in Brazil (Garcez *et al*., 2003). No reports of taraxerol's antibacterial activity or effect on human cell lines could be found. In a previous investigation in Angola and the Cape Basin, taraxerol and *Rhizophora* (a mangrove tree, dominant in equatorial and subequatorial west Africa) pollen, found in mid-Pleistocene sediments, was indicative of past mangrove ecosystems (Versteegh *et al*., 2004). *Rhizophora mangle* and *Rhizophora racemosa* leaves are extraordinary rich in taraxerol.

Pteleopsis myrtifolia leaf extracts have antibacterial activity (Chapters 3 and 4). Taraxerol, a pentacyclic triterpenoid, was isolated from *Pteleopsis*' leaves by bioassay-guided fractionation. The aim of this research was to determine taraxerol's bioactivity: - biological activity against various bacteria, against various human cancer cell lines, as well as its free radical scavenging or antioxidant capacity.

8.2 Materials and Methods

8.2.1 Plant material

Plant material were collected and prepared as described in 2.2.1 of Chapter 2.

8.2.2 The isolation of taraxerol

Taraxerol was isolated as described in Chapter 5.

8.2.3 Antibacterial activity of taraxerol

8.2.3.1 Minimum inhibitory concentration

MIC values were determined as described in 3.2.4.1 of Chapter 3.

8.2.3.2 Bioautography

For bioautography on the thin layer chromatograms, 20 µl of a10 mg/ml solution of taraxerol in acetone was applied to Merck Silica gel F_{254} plates) and developed with a solution of *n*-hexane and chloroform, (3:7). The bioautography method is described in 3.2.4.3 of Chapter 3.

8.2.4 Investigation of activity of taraxerol against human cell lines

A dried form of taraxerol was redissolved in dimethylsulfoxide (DMSO) to a final concentration of 1000 mg/ml (which served as a stock from which dilutions were made) and stored in a tightly sealed dark glass container at 5 ºC. The human cell lines used were MCF-12 (non-cancerous mammary gland), MCF-7 (cancerous breast), H157 (cancerous lung), $WHCO_3$ (cancerous oesophagus) and HeLa (cancerous cervix) (detail about cell lines in 6.2.2 of Chapter 6).

8.2.4.1 Human cell line cultures

The human cell lines were seeded in multiwell plates as described in 6.2.5 of Chapter 6.

Initially each cell line was tested at 10 and 100 µg/ml of taraxerol. The experimental layout of the MWP is shown in Figure 8.2. The wells bordering the MWP contained only Triton X-100.

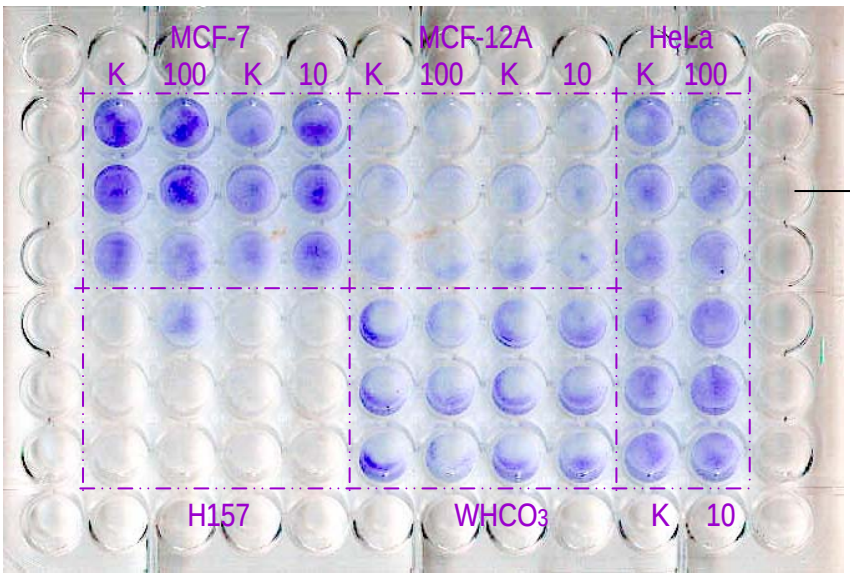

Figure 8.2. Scan of a 96-multiwell plate with the human cell lines MCF-7 (top left), MCF-12A (top right), H157 (bottom left) and $WHCO_3$ (bottom right). Each cell line's reaction to taraxerol was tested at control (K), 10 and 100 µg/ml values in triplicate against taraxerol. • The wells bordering the MWP contained only Triton X-100.

Three days after the plant extracts were added, the medium was discarded from the wells. Fixation, staining and spectrophotometer readings were done as described in 6.2.5 of Chapter 6.

Only the H157 (lung) cell line was tested at more concentrations against taraxerol.

8.2.5 Antioxidant activity

To investigate the free radical scavenger activity of taraxerol, a dried pure form thereof was redissolved in acetone to a 10 mg/ml final concentration.

8.2.5.1 The dot-blot DPPH staining procedure

The method, as described in 7.2.4 of Chapter 7, was followed.

8.3 Results and Discussion

8.3.1 Antibacterial activity of pure compound

8.3.1.1 Minimum inhibitory concentration (MIC)

Taraxerol's MIC values against the bacteria *Staphylococcus aureus*, *Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli* are listed in Table 8.3. The activity for the Gram-positive bacteria, especially *E. faecalis* is very good. In Table 8.4 of section 8.4, its MIC values are compared to that of other compounds from Combretaceae.

Table 8.3. Minimum inhibitory concentration values of taraxerol against the bacteria *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*.

	Minimum inhibitory concentration (MIC) in mg/ml			
	Staphylococcus aureus	*Enterococcus faecalis*	*Pseudomonas aeruginosa*	*Escherichia coli*
Taraxerol	0.04	0.16	0.63	0.31

8.3.1.2 Bioautography

Taraxerol had visible antibacterial activity against the bacteria *S. aureus*, *E. faecalis* and *E. coli* (Figure 8.3).

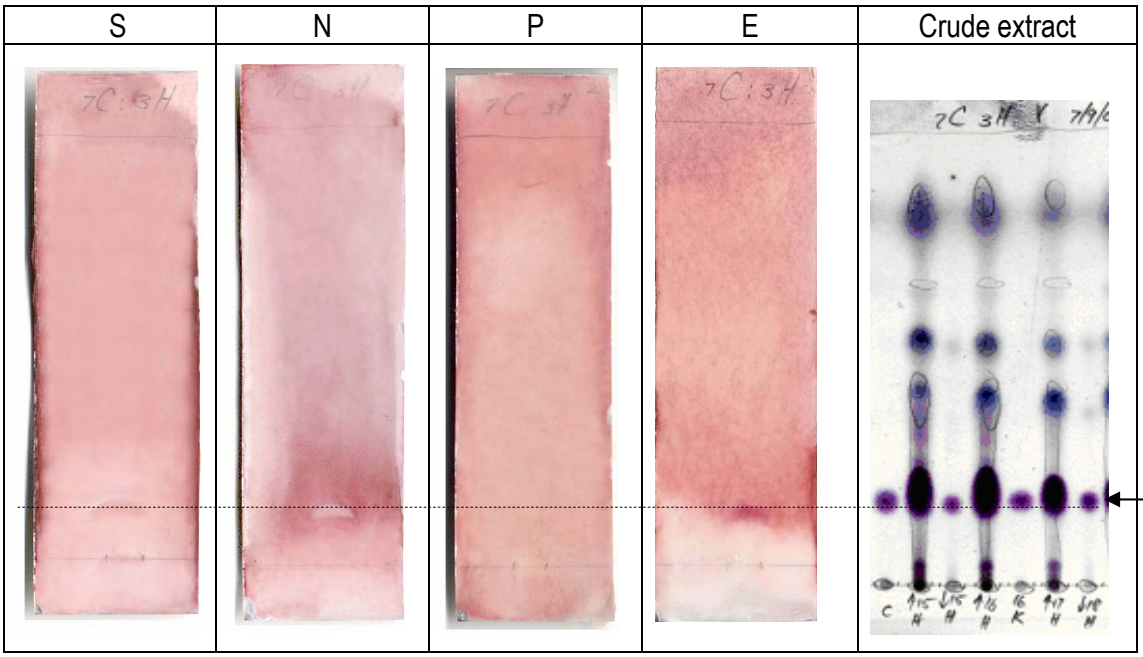

Figure 8.3. Bioautograms showing the effect of taraxerol on *Staphylococcus aureus* (S), *Enterococcus faecalis* (N), *Pseudomonas aeruginosa* (P) and *Escherichia coli* (E), after spraying with an aqueous solution of 2.0 mg/ml p-iodonitrotetrazolium violet solution, and (extreme right) a thin layer chromatogram (which contained taraxerol – position of arrow) developed in same eluent (chloroform : *n*-hexane; 7:3) and sprayed with vanillin.

An important observation here is that the R_f value of the inhibition zones were the same as that of the pure compound (taraxerol) isolated (chromatogram at the right).

8.3.2 Cancer cell growth inhibition by taraxerol

The 10 μg/ml concentrations of taraxerol did not inhibit growth of cell lines MCF-7, $WHCO_3$ and HeLa significantly different than the 100 μg/ml concentrations (Figure 8.4).

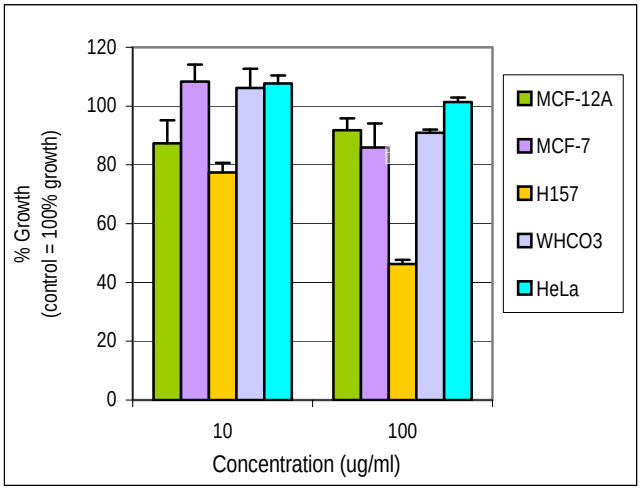

Figure 8.4. Effect of taraxerol, isolated from *Pteleopsis myrtifolia* leaves, on different human cell lines MCF-12A (non-cancerous breast), MCF–7 (breast adenocarcinosma), H157 (cancerous lung), $WHCO_3$ (cancerous oesophagus) and HeLa (cancerous cervix) at 10 μg/ml (left) and 100 μg/ml (right).

Taraxerol was inhibitory to cancer cell line H157 and this significant difference is indicated with a star at the 100 μg/ml in Figure 8.4. Figures 8.5 and 8.6 also show the inhibitory effect of taraxerol to the cancerous cell line H157 (lung).

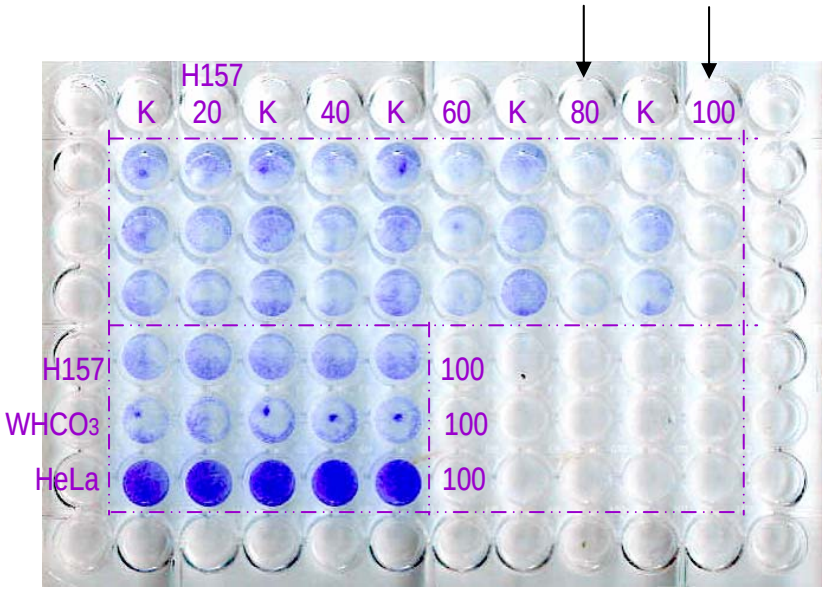

Figure 8.5. Scan of multiwell plates where taraxerol was tested at different concentrations (20, 40, 60, 80 and 100 μg/ml) against the H157 (lung) cancer cell line.

Lighter purple areas in Figure 8.5, (indicated with arrows) indicate less cancer cell growth.

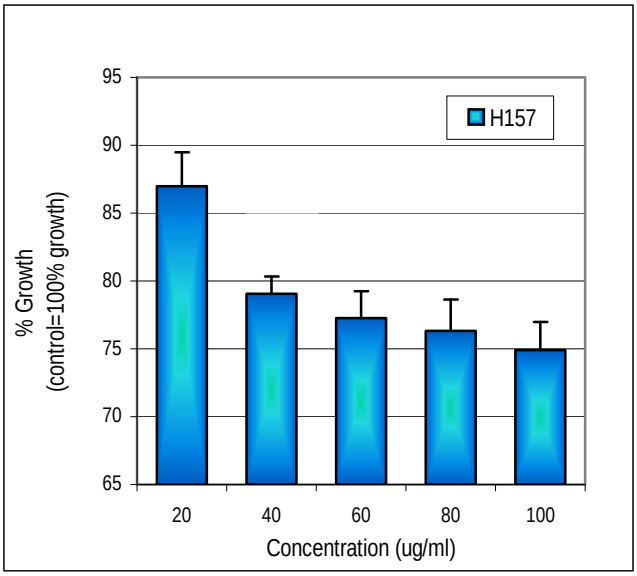

Figure 8.6. Graph of the effect of different concentrations of taraxerol (20, 40, 60, 80 and 100 μg/ml), isolated from *Pteleopsis myrtifolia* leaves, on the human cancer cell line H157 (lung).

Taraxerol's effect on the H157 cell line at 20 μg/ml concentration's were significantly less than the 40, 60, 80 μg/ml concentration's effects and this is indicated by a above the 20 μg/ml. In addition, its effect at 60 μg/ml was significantly less than at 100 μg/ml and this is indicated by a above the 60 μg/ml.

Taraxerol did not reach GI50 or LC values for the concentrations examined. No other reports of taraxerol's activity for the human cell lines tested could be found.

8.3.3 Antioxidant activity of taraxerol

8.3.3.1 Dot-blot DPPH staining procedure

The dot-blot assay indicates coloured (yellow) spots where aliquots of compounds with free radical scavenger activity were placed on the TLC plate. A more intense yellow colour is indicative of increased antioxidant activity. The purple area on the plate indicates no free radical scavenging (antioxidant) activity. Although extracts of *P. myrtifolia* leaves gave

antioxidant (free radical scavenger) activity (the extract 14-taraxen-3-ol was isolated from as well) in a previous study (Chapter 7), taraxerol did not have any antioxidant activity (lane at the right in Figure 8.7, indicated with an arrow).

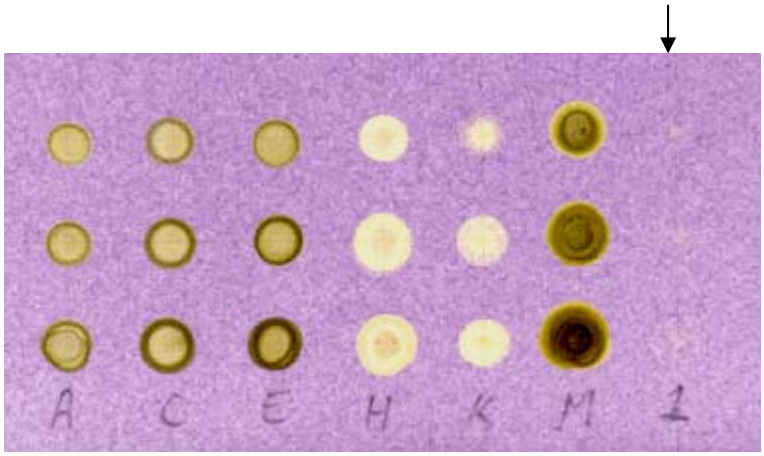

Figure 8.7. Scan of a dot-blot test of a thin layer chromatography plate sprayed with a 0.4 mM solution of 1,2-diphenyl-2-picrylhydrazyl in methanol after extracts A, C, E, H, K, M and 1 were applied (A = acetone extract, C = chloroform extract, E = ethanol extract, H = hot water extract, K = cold water extract, M = methanol extract and 1 = taraxerol). The dot blots applied were 20 μg (bottom row), 10 μg (middle row) and 5 μg (top row).

Cisplatin is a standard anticancer drug that does not have antioxidant activity (like taraxerol). It is extremely toxic and it acts as an alkylating agent (Yasuda *et al.,* 2000).

8.4 Summary and comparison of taraxerol's activity

Taraxerol had MIC values of 0.04, 0.016, 0.63 and 0.31 mg/ml for the bacteria *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli* respectively. A *Terminalia sericea* extract that contained terminoic acid and had a MIC value of 0.33 mg/ml for *S. aureus* (lower than taraxerol's MIC for *S. aureus*), was developed into a topical ointment for use (Kruger, 2004). In an experiment with mice, this ointment was found to be more effective than commercial Gentamycin cream. This ointment may find a veterinary application. If Taraxerol could be commercialised as an ointment it may be even more effective than the ointment prepared from *Terminalia* extracts because of

its lower MIC. This might however, not be a viable option due to limited distribution and numbers of *P. myrtifolia* trees, insufficient leaves and considering conservational aspects. The Pharmaceutical developer would have to find ways to synthesize taraxerol. Alternatively, other plants that contain this terpenoid and that occur more abundantly, might be used to isolate it from. In this study 7.7 mg was isolated from 1 kg of dried leaf material, indicating that a big amount of dry leaves will be needed. To prepare 30 ml of a 10 mg/ml cream, provided the yield of taraxerol is similar, 39 kg of dried leaves would have to be extracted. A leaf extract from *Pteleopsis* trees may find an application by rural people who live in the vicinity of trees and who can gather leaves to apply a crude leaf extract to skin infections.

MIC values found from pure compounds isolated from members of Combretaceae in previous investigations, are listed in Table 8.4.

Taraxerol's MIC values indicated that it was very active against *E. faecalis* and *S. aureus*. Its' MIC values to the Gram-negative bacteria were not as low.

Taraxerol did not have significant antioxidant activity (although the fraction it was isolated from, had).

Taraxerol significantly inhibited growth of the human lung cancer cell line H157. It was previously reported to have a less defined mode of action against tumour cells (Harrewijn *et al*., (2001). It, together with 10 other triterpenoids from the roots of *Taraxacum japonicum* (Compositae) were examined for its inhibitory effects on Epstein-Barr virus early antigen (EBV EA) at the University of Kyoto (Takasaki *et al*., 1999). This antigen was induced by the tumour promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), in Raji cells as a primary screening test for anti-tumour promoters (cancer chemopreventive agents). Of these triterpenoids, taraxasterol and taraxerol exhibited significant inhibitory effects on EBV-EA induction. Further-

Table 8.4. Minimum inhibitory concentration values from pure compounds isolated from members of Combretaceae.

Compounds from Combretaceae	MIC values in μg/ml			
	S. aureus	*E. faecalis*	*P. aeruginosa*	*E. coli*
Taraxerol	40	16	630	310
Combretastatin B5[1]	16	>250	125	125
5 hydroxy-7,4'-dimethoxyflavone [2]	>100	50	100	50-100
Rhamnazin [2]	>100	25	100	100
Rhamnocitrin [2]	50-100	25-50	100	100
Genkwanin [2]	50-100	50-100	100	100
Terminoic acid [3]	330	-	-	-
Alpinentin [4]	40	40	130	250
Pinocembrin [4]	80	40	300	130
Flavokwavaine [4]	40	400	300	600
Ampicillin[c]	80	160	125	160
Chloramphenicol[c]	160	40	125	160

[1] = Famakin (2003), [2] = Martini (2002), [3] = Kruger (2004), [4] = Serage (2003), [c] = control

more, these two compounds exhibited potent anti-tumour promoting activity in the two-stage carcinogenesis tests of mouse skin using 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and TPA as a promoter. (Takasaki *et al*., 1999). The inhibition of TPA co-carcinogenesis took place because signal-regulated cyclic AMP-dependant protein kinases were inhibited. Taraxerol can also inhibit proteases by targeting trypsin and being anti-inflammatory to phorbol ester-induced inflammation (Polya, 2003).

8.5 Conclusions

Results found in this study contributed to knowledge of the phytochemistry of Combretaceae – that taraxerol occur in the leaves of *P. myrtifolia.* This is the first time it was isolated from *P. myrtifolia* leaves.

No literature reporting on the MIC values of taraxerol could be found, and this might be the first report of taraxerol's MIC values against the bacteria *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli.* It had good antibacterial activity, a MIC of 0.016 mg/ml against the Gram-positive *E. faecalis.*

Taraxerol significantly inhibited growth of the human lung cancer cell line H157. Growth inhibition of the H157 cell line was significantly less at 20 μg/ml than at 60 μg/ml and 100 μg/ml. No other reports of taraxerol's effect on human cell lines could be found.

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