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Für

meine Mama

die mir alles im Leben ermöglicht und so leicht gemacht hat

meinen Papa

der mich stets unterstützt und auf die Pharmazie aufmerksam gemacht hat

meine Großeltern

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und

meine Schwester

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Abstract

During the last few years the importance of plants as source of medicine has increased, mostly depending on the idea that herbal remedies are safer and less damaging to the body than synthetic drugs. Australia's flora has always been used as medicine: by the Aborigines as well as by European settlers (or in some rare cases by other immigrant groups). There are a lot of plants, whose essential oils are used for healing purposes [1]. One of them is *Callitris glaucophylla*, a small to medium-sized tree which usually grows to about 18 m tall and 0.45 m in diameter, but occasionally can reach a height of 30m.

The aim of this study was to explore whether there are differences in the *Callitris glaucophylla* wood oil from different accessions or not. The oils obtained from three different accessions have been profiled by GC/MS and LC/MS to obtain MS and UV data and they showed similarities but also differences in their composition. Guaiol, a sesquiterpene, was the principal component in two of these three oils, as well as bulnesol and α- and β-eudesmol. 10-epi-γ-Eudesmol and the two sesquiterpene lactones columellarin and dihydrocolumellarin were found in all three wood oils. The third oil from *Callitris glaucophylla* showed dihydrocolumellarin as principal component, then a variety of other compounds such as limonene, 1,8-cineole, methyl myrtenate, citronellic acid, α- and β-selinene, γ- and β - costol and sandaracopimarinal.

Pharmacological assays were performed on the crude oil and on fractions as well. Antioxidant activity in the plant material was measured using the ORAC assay. ORAC values were situated between 300 and 1400 μmolTE/g of crude oils, some fractions of different compositions showed even more antioxidant activity than their oil of origin. The cytotoxicity assay was based on adenosine triphosphate (ATP), however the results should be seen as preliminary. Anti-inflammatory properties were tested with a competitive ELISA assay, but none of the tested fractions showed any anti-inflammatory activity.

Kurzzusammenfassung

In den letzten Jahren gewannen Pflanzen als Quelle neuer Wirkstoffe mehr und mehr an Bedeutung, vor allem durch den weit verbreiteten Glauben, herbale Medikamente wären sicherer und würden dem Körper weniger Schaden zufügen als synthetisch erzeugte Produkte.

Australiens Flora und Fauna wurde sowohl von den Ureinwohnern, den Aboriginies, als auch von europäischen Zuwanderern und anderen Immigranten immer schon als Medizin benutzt. Unzählige Pflanzen liefern ätherische Öle, die zur Heilung und Linderung von Beschwerden und Erkrankungen genutzt werden [1]. Eine dieser Pflanzen ist *Callitris glaucophylla*, ein klein bis mittelgroßer Baum, der für gewöhnlich 18 Meter Höhe und 0,45 Meter Durchmesser erreicht, in seltenen Fällen aber auch 30 Meter hoch werden kann.

In dieser Diplomarbeit wurden ätherische Öle aus dem Stamm und der Borke des *Callitris glaucophylla* von drei verschiedenen Sammelplätzen auf ihre chemische Zusammensetzung hin geprüft. Die drei Öle wurden mittels GC/MS und LC/MS untersucht. Die massenspektroskopischen und UV-Daten wurden verglichen. Dabei fanden sich Gemeinsamkeiten aber auch Unterschiede in Zusammensetzung und Quantität der Inhaltsstoffe. Guaiol, ein Sesquiterpen war Hauptkomponente in zwei der drei Öle. Bulnesol, α- und β-Eudesmol fanden sich in allen drei Ölen. 10-Epi-γ-Eudesmol und die zwei Sesquiterpenlactone Columellarin und Dihydrocolumellarin fanden sich in allen drei Ölen. Das dritte Öle zeigt Dihydrocolumellarin als Hauptinhaltsstoff, gefolgt von einer Vielfalt an Komponenten wie Limonen, 1,8-Cineol, Methylmyrtenat, citronellic acid, α- und β-Selinen, γ- und β Costol und Sandaracopimarinal.

Pharmakologische Tests wurden sowohl an den durch Wasserdampfdestillation gewonnenen Ölen als auch an den Fraktionen vorgenommen. Auf antioxidative Aktivität wurde durch den ORAC assay getestet. Die ORAC Werte der drei Öle lagen zwischen 300 und 1400 µmolTE/g. Einige Fraktionen mit verschiedensten Inhaltsstoffen zeigten sogar höhere Aktivität als das Öl selbst. Die Zytotoxizität der *Callitris* Öle wurde an Assays durchgeführt, die auf der Messung des Adenosintriphosphates (ATP) beruhte. Leider müssen die Ergebnisse der Zytotoxizitätsassays als vorläufig angesehen werden, da die positive Kontrolle und der Referenzwert nicht konstante Werte lieferten. Antiinflammatorische

Eigenschaften wurden mit einem kompetitiven ELISA bestimmt. Leider zeigte keine Fraktion Aktivität.

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1. INTRODUCTION

1.1. Background

During the last few years the importance of plants as source of medicine has increased, mostly depending on the thought that herbal remedies are safer and less damaging to the body than synthetic drugs. Australia's flora and fauna has always been used as medicine: by the Aborigines as well as by European settlers (or in some rare cases by other migrant group). There are lots of plants, whose essential oils are used for healing purpose [1]. Nearly every form of pain or disease can be cured or improved with natural medicines. The oil of *Melaleuca cajuputi* and *Melaleuca quinquenervia* are natural painkillers and are used internally against coughs and colds, stomach cramps, colic and asthma and externally for the relief of neuralgia and rheumatism and against toothache and earache. The Aborigines use the fragrant leaves of *Chenopodium rhadinostachyum* soaked in water to bathe the head for the relief of colds and headaches. It is possibly the lemon-scented oil from *Cymbopogon procerus* and the essential oil rich in safrole and methyleugenol of *Eremophila longifolia* which are responsible for their healing activities of colds. The oil of *Eristemon brucei* is also used against colds. The crushed leaves of *Melaleuca hypericifolia* are sniffed for the relief of headache, containing an essential oil with about 80 percent 1,8-cineole. The fragrant sandalwood *Santalum spicatum* is rich in α- and β-santalols and used as cough medicine [1]. The leaves of *Cinnamomum laubatii* contain an oil rich in eugenol and sesquiterpenoid compounds and are used as a carminative, diuretic, stimulant, diaphoretic, lactagogue and deobstruent. *Mentha satureioides* contains an oil rich in pulegone, *l*-menthone, *l*-menthol and menthyl acetate and its tea is used as a tonic, blood purifier and an invigorator of the whole system in general. The resin of *Canarium muelleri* contains α-pinene and α-terpineol and is a very good healing agent for cuts, sores and chronic ulcers. The oil of *Melaleuca alternifolia* is applied externally and used in the treatment of boils, abscesses, sores, cuts and abrasions. The bark of *Cinnamomum oliveri* containing an essential oil rich in camphor, safrole and methyleugenol is used as a tincture for diarrhoea. *L*-piperitone in the volatile oil of *Eucalyptus piperita* has been used in stomach upsets. The volatile oil of *Prostanthera rotundifolia* is carminative. The

composition of the essential oil of *Mentha diemenica* is yet not known. It is used in the same way as ordinary peppermint to treat stomach cramps, as a diuretic and diaphoretic, and additional to treat menstrual disorders in women. The essential oil of *Eucalyptus citriodora* contained in the leaves has bacteriostatic activity towards *Staphylococcus aureus*, due to the synergism between citronellol and citronellal present in the oil. All these plants are native to Australia and their essential oils are responsible for the listed activity. The essential oil of *Callitris glaucophylla* has been used as medicinal remedy by a few aboriginal tribes for a long time. The aim of this thesis was to figure out the chemical composition and pharmacological activities of its components.

1.2. *Callitris glaucophylla*

1.2.1. Botany

Callitris glaucophylla is a small to medium-sized tree, which usually grows to about 18m tall and 0.45m in diameter, but occasionally can reach a height of 30m by 0.9m.

The trunk is usually straight, the branch development varies from appearing over the greater part of the trunk or as a dense conical crown for woodland trees, to short branching in the upper trunk only and a relatively flat top for trees in dense stands. The bark appears deeply furrowed and dark grey, sometimes a bit lighter grey on large trees [2], [3].

Figure 1. *Callitris glaucophylla* tree [4].

The foliage colour is variable but usually glaucous, the leaves are reduced to tiny scales, 1-3 mm long, arranged in whorls of three, sheathing the needle-like green branchlets, and the fruits are spherical cones, which are dark-brown coloured. When aged they get woody, with three large and three smaller alternating scales separating to the base and wrinkled outside with a small point near the tip. The cones open to release their seeds [5]. The wood is coloured light yellow or straw to dark brown and is – like the leaves – rich in oils and resins. The *Callitris glaucophylla* tree hybridizes with the subspecies of *Callitris preissii* [6].

1.2.2. Distribution

Callitris glaucophylla is widespread across Australia, south of the Tropic of Capricorn. It appears from central Queensland to Victoria, over most of Western New South Wales, with outliers in South Australia and in southern parts of the Northern Territory and Western Australia. The most extensive stands are located in the Tambo-Dalby-Inglewood region of southern Queensland and the Baradine-Narrabi and Cobar districts of northern NSW. Its preferred regions are undulated, but also lower slopes and rocky hills. In common the *Callitris* builds monocultures, but it is also found mixed with eucalypts species. *Callitris* itself is very fire sensitive, and in stands with eucalypts the danger of being eliminated by fire is increasing because of the high amount of easily inflammable oil in eucalypt trees [2].

Figure 2. Distribution of *Callitris glaucophylla* in Australia [7].

1.2.3. Use

The timber of *Callitris glaucophylla* is fragrant and durable and has high resistance to decay and termite attack and does not shrink much in maturity. It is also resistant against marine borers Teredo and Limmoria [2]. These useful features made the *Callitris* timber of high commercial importance. The wood is largely used for balks in building construction, and for house blocks, flooring, ceilings and weatherboards. The tree itself is planted as shelter belts and for ornamental purpose [8]. The Aborigines use the *Callitris* tree in many different ways: as an adhesive, as firewood, as implements, as medicine, and it has an importance in their mythological world. The Walpiri people use the resins mixed with kangaroo dung as an adhesive and as a substitute for sandarac resin and make implements out of the wood [9]. Its burnt wood is gladly used as firewood because of its good smell and is also used to produce a pleasant odour for babies. However, it is not appropriate to be used for cooking because it taints the food with its odour [10]. Most of the tribes use it for medicinal treatments, except the Pintupi and the Pitjantjatjara people. Every tribe has his own word for the *Callitris glaucophylla* tree, as shown in Table 1 below.

Table 1. Each tribe got its own aboriginal name for *Callitris glaucophylla* [10].

Tribe	Aboriginal name
Alyawarr	apmikw
Anmatyerr	anngart, rlwek
Eastern Arrernte	irlweke
Western Arrernte	alkngarte
Pintupi	mulku
Pitjantjatjara	kuli, kulpuru, kulilypuru
Walpiri	wanngardi

A book by an Aboriginal woman belonging to the Arrernte people, describes all the traditional medicines and healing methods her aboriginal tribe uses. They use different parts of the tree to heal their patients. The leaves of *Callitris glaucophylla*, called *Irlweke*, were hackled and put into boiling water, then they are used to wash an itchy body or mixed with fat to be rubbed on the chest of a patient suffering from flu. The inner bark of the *Irlweke* was peeled off the tree and the patient got enwrapped with it to heal stomach-ache. An important part of Aboriginal medicine

treatments are sweating treatments – *Antyeye itnyetyeke mpwareme*. This is to cure bad influenza, including fever, pain all over the body and hot or cold flushes. The fresh sticky bark builds a "bed" where the patient is laid on, then he gets enwrapped with the bark, so that he can sweat out the illness [11].

1.3. Aims and objective of research

The work described in this master thesis details the phytochemical and pharmacological investigation of essential oils from three different accessions of *Callitris glaucophylla*. Preliminary work had been done by the Austrian master student Angela Oprava in 2007. The major interest was the chemical characterization of the three oils, with focus on the isolation of major components out of two oils. Therefore the oils have been fractionated using normal phase column chromatography. For further fractionation the fractions with the highest yield have been chosen to be fractionated with preparative HPLC. The collected fractions were investigated using GC/MS and LC/MS systems to attain spectral and physical data (MS, UV) and to check the purity. Selected fractions were run on NMR to get their structural information. Pharmacological activity was improved by different assays, which were done on all fractions and the original oils. For showing if they have antioxidant activity, ORAC assays have been done, followed by testing their cytotoxicity in cytotoxicity assays and their anti-inflammatory attributes in a PG E_2-inhibition assay.

2. REVIEW OF LITERATURE

2.1. Taxonomic description

Callitris is member of the family of Cupressaceae, which is the largest of seven extant families in the order Coniferales. The family has been divided into two subfamilies. The Cupressoideae are located on the northern and the Callitroideae on the southern hemisphere. The Callitroideae itself is divided in Libocedreae and Actinostrobeae, to which the genus *Callitris* belongs. *Callitris* is confined to Australia (including Tasmania) and New Caledonia, with 16 species known: 14 are native to Australia and the other 2 to New Caledonia [9].

The botanical nomenclature of this tree has a long, but entertaining history. Its first name was *Callitris robusta* R. Br., published in 1825. A few decades later, in 1910, the name *Callitris glauca* R. Br. Ex R. T. Bak et H. G. Sm. was published. There was silence until 1956, when the name *Callitris hugelii* (Carr.) Franco appeared in an Australian botanical publication. In 1959 Blake found out that the name *Callitris hugellii* was incorrectly given, and after a review of several species of the genus, he included *Callitris glauca* and *Callitris intratropica* in the description of *Callitris columellaris* F. Muell.. This caused confusion, because this term included 3 until now separate known species, so it was decided to retain the names given before 1956 with the knowledge that there has to be a revision of the nomenclature and taxonomy in the future. In 1986, Thompson & Johnson provided clarity: in a taxonomic study, they defined *Callitris glaucophylla* as an independent species, which belongs to the white cypress-pine complex, consisting of *Callitris glaucophylla*, *Callitris intratropica* and *Callitris columellaris* [12]. The most widely common name is white cypress-pine, beside Western sand cypress and Western cypress [8]. The origin of the term *Callitris* is the greek word for beauty: kalos. The second element –tris is often associated to the greek word threis (three), which means that the leaves usually appear in whorls of three [13]. Glaucus, whose origin is Latin means bluish grey or bluish green, phyllon is Greek meaning leaf – according to the glaucous colour of the foliage [2].

2.2. Previous phytochemical studies on *Callitris glaucophylla*

The initial beginning of chemical research on *Callitris* species has been done by Brecknell and Carman in 1979 [14]. They isolated five new sesquiterpene lactones from the *Callitris columellaris* heartwood (at this stage, the *Callitris glaucophylla* was handled as a subspecies of the *Callitris columellaris*, as reported before in section 2.1.). These lactones were callitrin **(1)** (an elemanolide), callitrisin **(2)** and dihydrocallitrisin **(3)** (both eudesmanolides), columellarin **(4)** and dihydrocolumellarin **(5)** (guaianolides) and a germacranolide, which was reported before only as a result of synthesis and not as a natural product. Those compounds were the first described sesquiterpene lactones isolated from the Cupressaceae. It was also found that callitrin, callitrisin and dihydrocallitrisin show a novel stereochemical arrangement of the lactone ring [14].

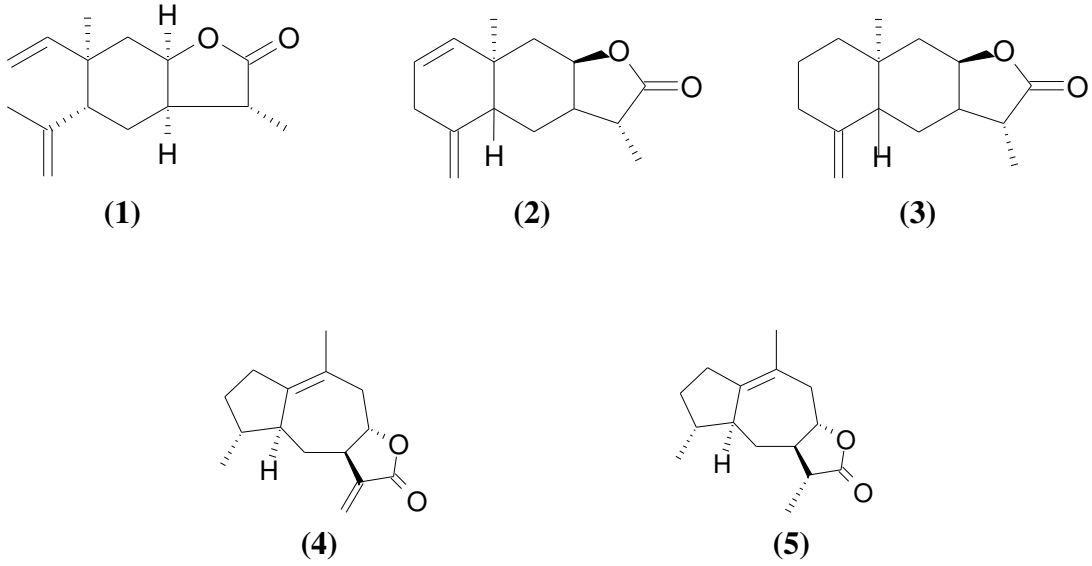
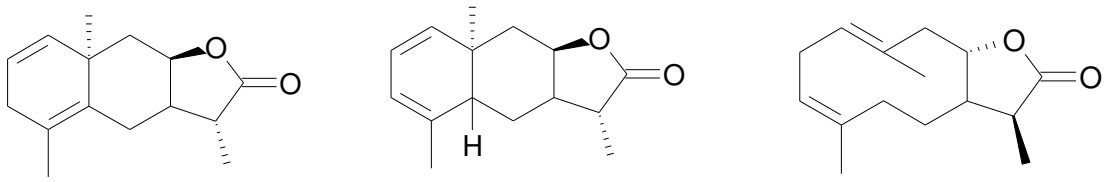


Figure 3. Structures of callitrin **(1)**, callitrisin **(2)**, dihydrocallitrisin **(3)**, columellarin **(4)** and dihydrocolumellarin **(5)** [14].

Further work has been done by Adams and Simmons in 1987 [15], who attempted to elucidate the chaotic taxonomy of the *Callitris* genus (see section 2.1.). They used the volatile oils as the taxonomic marker to define species and subspecies. In 1986,

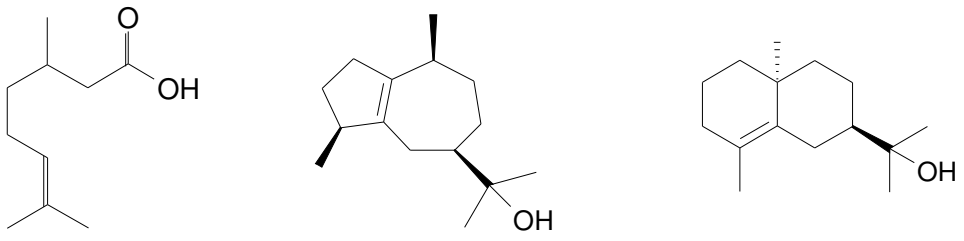
the mystery was resolved, when Thompson & Johnson redefined the *Callitris glaucophylla* as an independent species [12]. Doimo, Fletcher and D'Arcy [12] continued working on *Callitris glaucophylla* and compared the different composition of the oil, obtained either with distillation or as solvent extract. They found out that the amount of γ-lactones is poor in distilled oils but rich in solvent extracts. Further they discovered three previously unidentified lactones: two as isomers of callitrisin **(6)**, **(7)** and one as an isomer of germacranolide **(8)**.



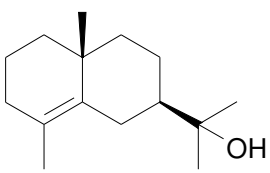
(6) **(7)** **(8)**

Figure 4. Structures of callitrisin isomer 1 **(6)**, callitrisin isomer 2 **(7)** and isomer of germacranolide **(8)** [12].

The fraction containing the highest amount of γ-lactones showed the hightest potential as a termite repellent. They also detected potential against insects, tumor and as an insect anti-feedant [12]. As a follow-up study, Doimo [16] compared the three species of the white cypress-pine complex with a fourth sample – an unusual sample of *Callitris glaucophylla* outside the normal range (Chinchilla, Queensland), with surprising results: the typical sample of *Callitris glaucophylla* (collected in NSW) shows differences to the unusual one. These results suggest a few variation in *Callitris* volatiles. In total, the obtained oils had high amounts of citronellic acid **(9)**, guaiol **(10)** and eudesmols (10-epi-γ-eudesmol **(11)**, γ-eudesmol **(12)**, β-eudesmol **(13)** and α-eudesmol **(14)**), whereas the methanol extracts were rich in γ-lactones (dihydrocolumellarin **(5)**, callitrisin **(2)** and columellarin **(4)**).



(9) **(10)** **(11)**



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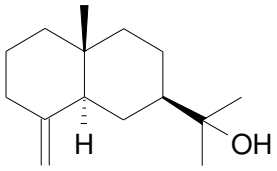
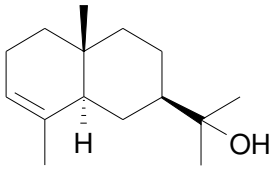


(13)



(14)

Figure 5. Structures of citronellic acid **(9)**, guaiol **(10)**, 10-epi-γ-eudesmol **(11)**, γ-eudesmol **(12)**, β-eudesmol **(13)** and α-eudesmol **(14)** [16].

Doimo also described, that azulenes are responsible for the blue colour of the oil, which have not previously been reported as components in a coniferous oil [16]. In 2005, Watanabe, Mitsunaga and Yoshimura took a closer look at the antitermitic potential of *Callitris* and found, that columellarin is the most responsible for the activity, followed by a sesquiterpene lactone fraction [17].

3. MATERIALS AND METHODS

3.1. Materials and sample preparation

The materials (stems and wood log) were collected a year ago by Paul I. Forster (PIF), Queensland Herbarium, Environmental Protection Agency, Brisbane Botanic Gardens, Mt. Coottha Road, Toowong Qld 4066 Australia. They were attributed code numbers, as shown in Table 2.

Table 2. *Callitris glaucophylla* oils: origin, used parts, code numbers.

Code No.	used parts	Locality	Latitude degrees	Latitude minutes	Longitude degrees	Longitude minutes
PIF 31973	stem & bark	State Forest 341 Bringalily, 22 km NNE of Inglewood	28	14	151	10
PIF 32352	stem & bark	State Forest 50 Glenhaughton to Mapala road	25	21	149	20

The stems of the *Callitris* species (see Figure 6) were ground into coarse powder using a grinder Retsch SM 2000 (diameter of sieve: 4 mm) to maximise the particle surface area and to facilitate efficient solvent extraction. The oils were obtained through water steam distillation. A set – up of the water steam distillation apparatus can be seen in Figure 7. The distilled oils were then transferred quantitatively into vials and water was removed by using a molecular sieve. The yield was attained using an analytical balance, Sartorius BP 210 S.

Figure 6. Plant material of *Callitris glaucophylla* 31973 [18].

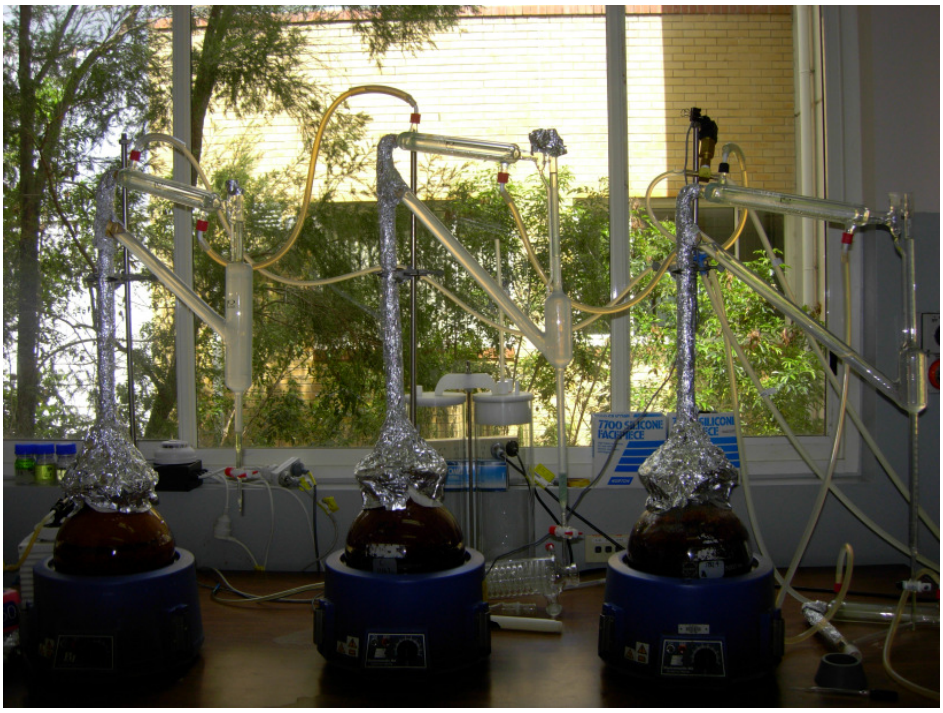
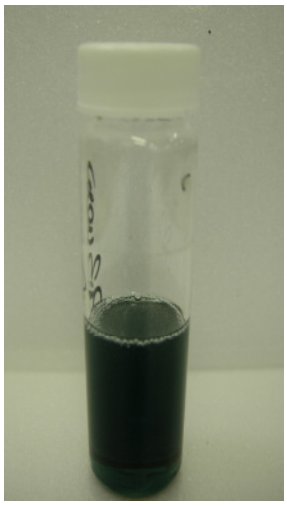


Figure 7. Set-up of water steam distillation apparatus.

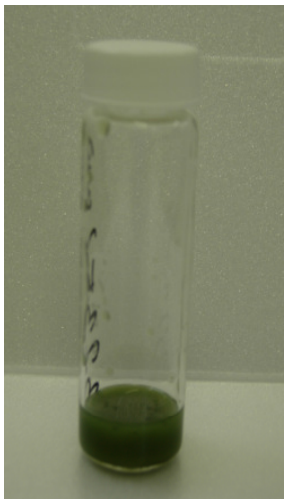
Table 3. Water steam distillation of *Callitris glaucophylla* stem.

Species	starting amount (kg)	hours of distillation	yield (g)	colour of the oil
31973	2.387	144 h	10.867	bluish-green
32352	0.5	72 h	2.95	dark-green
32209	0.64	72 h	7.48	yellow

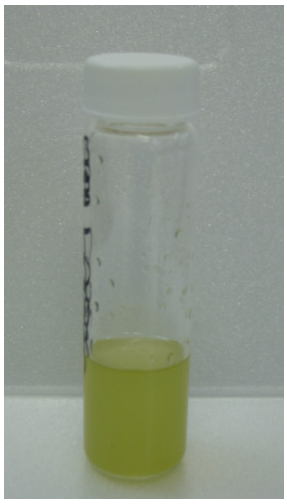
As the oils were obtained, it was observed, that the colour of the oils is different, compared to oils from the same plant origin, which were stored for a year at room temperature. The change in colour can be seen in Figure 8 below. The oil with the code PIF 31973 appears in bluish green colour when it is new obtained, unlike the "old" oil which is honey yellow. PIF 32352 got a dark green colour, compared to the stored one, which turned into a dirty yellow to brown tone. And PIF 32209, at least, turned from yellow to reddish-brown.



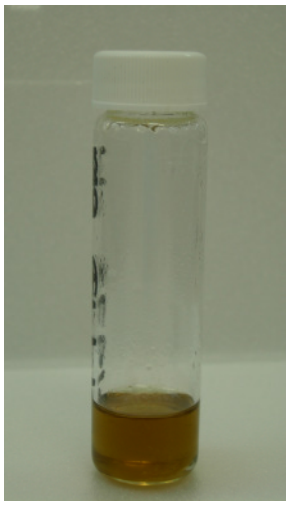
a. PIF 31973 new oil
bluish green



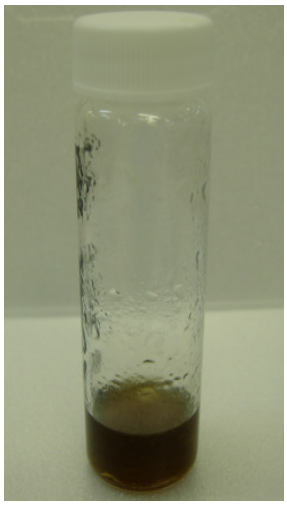
b. PIF 32352 new oil
dark green



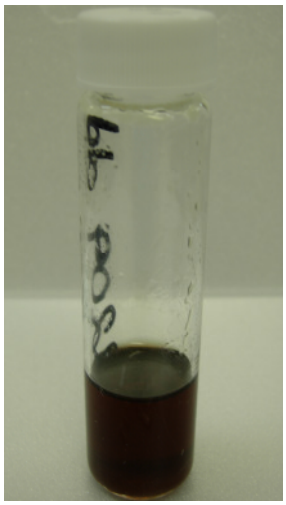
c. PIF 32209 new oil
yellow



d. PIF 31973 old oil
honey yellow



e. PIF 32352 old oil
ochre yellow to brown



f. PIF 32209 old oil
reddish-brown

Figure 8. Appearance of freshly distilled oils: a. PIF 31973, b. PIF 32352, c. PIF 32209; and oils stored for a year: d. PIF 31973, e. PIF 32352, f. PIF 32209.

3.2. Phytochemistry

3.2.1. Isolation and detection of compounds

Different chromatographic techniques were used to fractionate and isolate the compounds of the crude oils.

3.2.1.1. Normal phase column chromatography

Glass columns with sintered glass were used and silica was packed as a slurry in hexane. The top of the silica column was layered with about 1 cm of acid-washed sand. The oils were dissolved in a small amount of n-hexane and were applied using a Pasteur pipette. The column was eluted starting with non-polar solvents and the polarity of the mobile phase was increased stepwise. All solvents used were from LAB-SCAN (HPLC grade), except Milli-Q water, which was purified and filtered by a Millipak® 40 (0.22µm). Column dimensions and details of solvent gradients are described in the individual schemes. Fractions were concentrated to dryness using a rotary vacuum evaporator from Buchi R-114 with a water bath RE 100 B from Bibby, temperature set at 40°C. Fractions were transferred quantitatively into preweighted 20mL vials, dried under nitrogen and weighted on the Sartorius BP 210 S analytical balance to obtain yields.

3.2.1.2. Gas chromatography – Mass spectrometry (GC/MS)

GC/MS was used to attain the chemical profile of the volatile components of the oil, giving detailed information about mass spectra and retention times. The system used was an Agilent 6890 with an Agilent 7683 series autosampler/injector and Agilent 5973 Network Mass Selective Detector (MSD). The column used was a SGE BPX5 Capillary Column, 50.0m x 0.22mm ID x 1µm film thickness.
The method used was the MS-QCIDE method with the following parameters:

Injector parameters:

Injection volume: 1 µL

Injection mode: split

Split ratio: 50:1

Gas type: Helium

Inlet temperature: 280°C

Column parameters:

Carrier gas: Helium

Flow: constant, 2 mL/min

Nominal init pressure: 46.72 psi

Average velocity: 37 cm/sec

Oven parameters:

Initial temperature: 100°C (for 1 minute)

Rate: 8°C per minute

Final temperature: 300°C

MSD parameters:

Transfer temperature: 280°C

Source temperature: 230°C

Quadrupole temperature: 150°C

Ionisation Voltage: 70eV

Scanning mass range: 35–350m/z

3.2.1.3. Liquid chromatography – Mass spectrometry (LC/MS)

LC/MS was also used to obtain additional information about the properties of the components. The system used was an Agilent 1100 Series HPLC with Photo Diode Array Detector and 1100 series Mass Selective Detector. The column was a Phenomenex® Luna 3 u C18 (2) 100A, 100 x 4.6mm 3 micron (P/No. 00D-4251-EO, S/No: 397903-12) with column temperature set at 40°C. The LC/MS parameters were as follows:

Injection volume: 5µL (injection with needle wash)

Solvent A: 90% Milli-Q water with 0.005% TFA

Solvent B: 10% Acetonitrile with 0.005% TFA

Flow rate: 0.5 mL/min

Spray Chamber: Gas Temperature: 350°C

Vaporizer: 350°C

Drying Gas: 5 l/min

Nebulizer Pressure: 60 psig

Diode Array Diode (DAD): Signals (nm): 210, 254, 280, 360

Range: 190 – 600 nm (UV/Vis)

Steps: 2 nm

Mass Spectrometry Detector: Ionization mode: APCI
Active signals: positive 70
positive 150

Method Ruth1 :

Timetable:

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)	Pressure (bar)
0.00	90	10	0.5	400
15.00	5	95	0.5	400
17.50	5	95	0.5	400
20.00	90	10	0.5	400
25.00	90	10	0.5	400

Method Ruth2:

Timetable:

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)	Pressure (bar)
0.00	90	10	0.5	400
15.00	5	95	0.5	400
23.00	5	95	0.5	400
25.50	90	10	0.5	400
30.00	90	10	0.5	400

3.2.1.4. High performance liquid chromatography (HPLC)

3.2.1.4.1. Analytical HPLC

The column used was a Phenomenex® Luna 5u C18 (2), 150 x 4.6mm, 5 micron (P/N° 00F-4252-EO, S/N° 147408-8) with column temperature set at 40.0°C. The mobile phase was a mixture of Acetonitrile (+ 0.05% TFA) and water (+0.05% TFA).

The system used was an Agilent 1100 series (autosampler, degasser, column thermostat, quarternary pump, diode array detector).

Pump:

Column flow: 1mL/min

Max pressure: 400 bar

Injector:

Injection mode: needle wash

Injector volume: 10.00 µL

Diode Array Detector:

Signals: 210, 254, 280, 330, 360 nm

Range: 190 – 400nm

Range step: 2nm

Method: Ruth3

Timetable:

Time (min)	Water (+0.05% TFA)	ACN (+0.05% TFA)	Flow (mL/min)	Pressure (bar)
0.00	50%	50%	1	400
5.00	50%	50%	1	400
10.00	5%	95%	1	400
15.00	5%	95%	1	400
17.00	50%	50%	1	400
22.00	50%	50%	1	400

Method: Ruth6[1]

Timetable:

Time (min)	Water (+0.05% TFA)	MeOH (+0.05% TFA)	Flow (mL/min)	Pressure (bar)
0.00	20%	80%	1	400
3.00	20%	80%	1	400
13.00	2%	98%	1	400
18.00	2%	98%	1	400
20.00	20%	80%	1	400
25.00	20%	80%	1	400

[1] Methods Ruth4 and Ruth5 are not listed; a new method has been developed using methanol instead of acetonitrile and method Ruth6 showed a better separation than Ruth4 and Ruth5.

3.2.1.4.2. Preparative HPLC

The system used was a Gilson® Pump 322 fitted with a Gilson® UV/Vis-156. The column used was a Phenomenex® Luna 5u C18 (2) 100A, 150 x 21.2 mm, 5 micron (P/N° 00F-4252-PO, S/N° 327445 – 1) with column temperature set at 40°C. A guard column was used to protect the preparative column. It was packed with silica (Phenomenex® Sepra C18 – E, 50 μm, 65A). The mobile phases used were Acetonitrile (+0.05% TFA) and Milli-Q water (+0.05%). To wash the column, isopropanol was used (flow 3 mL/min).

A Diode Array Detector was coupled to the preparative HPLC, recording the response at wavelengths 210 and 280 nm. A Gilson® FC 204 fraction collector collected 80 fractions per applicated sample, at a total runtime of 22 min. The flow rate was 20 mL/min.The pressure was observed and usually moved between 600 and 900 psi.

Method: Ruprep1

Timetable:

Time (min)	Water (+0.05% TFA)	Acetonitrile (+0.05% TFA)
0.00	50%	50%
5.00	50%	50%
10.00	5%	95%
18.00	5%	95%
20.00	50%	50%
22.00	50%	50%

Method: Ruprep3[2]

Timetable:

Time (min)	Water (+0.05% TFA)	Methanol (+0.05% TFA)
0.00	20%	80%
3.00	20%	80%
13.00	2%	98%
20.00	2%	98%
22.00	20%	80%

[2] Method Ruprep2 was just used for column wash.

3.2.1.5. Nuclear Magnetic Resonance (NMR) spectroscopy

The system used was a Bruker AVANCE DRX500 (^{1}H at 500.13 MHz, ^{13}C at 125.77 MHz; 5mm QNP probe) spectrometer with Topspin software. NMR was used to elucidate the structure of compounds isolated. The ^{1}H and ^{13}C-NMR spectra were recorded using deuterated solvent ($CDCl_3$) with the solvent peak as reference. The chemical shifts were expressed in parts per million (ppm) as δ values and the coupling constants (*J*) in Hertz (Hz). Multiplicities were abbreviated as s (singlet), d (doublet), dd (doublet of doublets), m (multiplets) and br (broad). For each fraction, which seemed to be pure, 5 experiments have been done.

3.2.1.5.1. One-dimensional NMR Spectroscopy

As a routine, the ^{1}H-NMR spectra were done for all selected fractions. The chemical shifts, coupling constants, peak intensities and splitting patterns of the proton signals characterise the proton and its chemical environment. The carbon resonances were displayed in the *J*-modulated ^{13}C-NMR. CH_3 and CH are pointing down, whilst CH_2 and the quaternary carbons point up.

3.2.1.5.2. Two-Dimensional Homonuclear Correlation Spectroscopy

The technique used was ^{1}H-^{1}H-Correlation Spectroscopy (COSY), correlating the chemical shifts of ^{1}H nuclei that were coupled to each other (cross signal).

3.2.1.5.3. Two-Dimensional Heteronuclear Correlation Spectroscopy

Two of these techniques have been used: HSQC (Heteronuclear Single Quantum Correlation) and HMBC (Heteronuclear Multiple-Bond Correlation) spectroscopy.

3.2.2. Identification of compounds by GCMS

The identification of the components was done both by comparison of fragmentation patterns and with Kovats Indices. The fragmentation patterns of sample peaks were compared to known compounds in the databases Nist98, WILEY 275 and ADAMS [19]. The Kovats Indices of sample peaks were also calculated and compared with either reference compounds or with reported KI in [19].

3.2.2.1. Identification through comparison with reference standards

A stock solution of a variety of terpenes had been prepared. The standards were methyl jasmonate, linalool, aromadendrene, lavandulyl acetate, globulol, α-pinene, limonene, citronellal, guaiazulene and the aromatic compound BHT. Each standard was prepared at 10 mg/mL in methanol. A mixed standard was prepared by combining 1.5 mL and 1 mL of this mixture had been injected into the GC/MS. The retention times of the sample peaks were compared to the retention times of the reference standards.

Table 4. Different terpenes were selected to make a reference standard solution.

Standard	Supplier	Purity	Supplier ID	Amount	RT	KI
methyl jasmonate	Aldrich	95%	13903CI	5 mL	26.80	1649
linalool	Aldrich	97%	03228EQ	100 g	16.72	1096
aromadendrene	Sigma	97%	11067	5 mL	23.93	1441
lavandulyl acetate	Fluka	98%	62684/1	5 mL	20.16	1290
globulol	Fluka	98.93%	49070	tr	26.46	1590
α-pinene	Aldrich	97.85%	05807TW	75 mL	13.28	939
limonene	Aldrich	95.1%	09817MN	45 g	15.46	1029
citronellal	Aldrich	96%	01920AZ	1 g	17.85	1153
BHT	Sigma	≥ 99%	B 1378	100 g	24.42	1515
guaiazulene	Sigma	≥ 99%	50890	10 g	29.31	1770

3.2.2.2. Identification by Kovats Index (KI)

The Kovats Index has been calculated for each sample peak. It is useful because the retention time may vary as columns age. The formula used for calculation of KI is as follows [19]:

$KI(x) = 100\,P_z + [(\log RT(x) - \log RT(P_z)) / (\log RT(P_{z+1}) - \log RT(P_z))]$

3.3. Pharmacology

Pharmacology assays followed the fractionation of the oils, to identify the component which is responsible for the given activity. Initially, the whole oils were tested, to obtain the pharmacological profile, further the fractions were tested too.

3.3.1. ORAC Assay

Oxygen Radical Absorbance Capacity is an assay to measure the total antioxidant power of a substance. The more free radicals a substance can absorb, the higher is its ORAC score. Free radicals are a result of natural body processes, but are exacerbated by unhealthy life styles: tobacco smoke, toxins, pollutants, and of course bad nutrition-habits. This includes inadequate consumption of fruits and vegetable, which are rich sources of antioxidant phytochemicals. Many serious diseases are linked to elevated free radicals such as cancer, cardiovascular disease, rheumatoid arthritis, chronic fatigue and age-related diseases.

3.3.1.1. Sample preparation

All oils and fractions were prepared in methanol at a concentration of 10 mg/mL.

3.3.1.2. Reagents

Phosphate Buffer Solution (75mM): Mono sodium phosphate (NaH_2PO_4; Sigma), 17.10g, and sodium phosphate (Na_2HPO_4; Sigma), 86.24g, were dissolved in 900 mL of Milli-Q water and the pH was adjusted to 7.4. The volume was made up to 1000 mL with Milli-Q water. Milli-Q water, 900mL, were added to 100 mL of this stock solution (750mM). The pH got adjusted again to 7.4 and stored at 2-8°C in a fridge. Fluorescein Solution (6.0×10^{-7} M): Fluorescein sodium salt ($C_{20}H_{12}O_5$. 2Na; Aldrich), 116mg, were dissolved in 63.2 mL of 75 mM phosphate buffer (pH 7.4). This stock solution (4.9×10^{-3} M), 10 µL, was added to 16.5 mL of the 75 mM phosphate buffer (pH 7.4) and stored at 2-8°C with foil to keep it dark in the fridge. Trolox Standard Stock (0.01 M): Trolox [(±)-6-Hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid; $C_{14}H_{18}O_4$; Fluka], 0.25g, were dissolved in 50 mL of 75 mM phosphate buffer (pH 7.4). The volume was made up to 100 mL with 75 mM phosphate buffer. The volumes were aliquoted into 1 mL volumes and stored at -20°C in the freezer. Epicatechin Standard Stock: Epicatechin [cis-2-[3,4-Dihydroxyphenyl]-3,4-dihydro-2H-1-benzopyran-3,5,7-triol; Sigma], 12.5mg, were dissolved in 20 mL of 75 mM phosphate buffer (pH 7.4). The volumes were aliquoted into 1 mL volumes and stored at -20°C in the freezer. AAPH [2,2'-Azobis(2-amidino-propane)dihydrochloride] Solution (20mM): [$HNC(NH_2)C(CH_3)_2N{:}NC(CH_3)_2C(NH_2)NH$. 2 HCl; Wako] has to be prepared immediately before use and is described in section 3.3.1.3.

3.3.1.3. Procedure

A clear 96-well dilution plate (JRH flat bottomed) was used to prepare the dilutions of the samples. For the measurement, the samples were transferred to a Fluorescence 96-well assay plate (black) – a Perkin Elmer Optiplate.

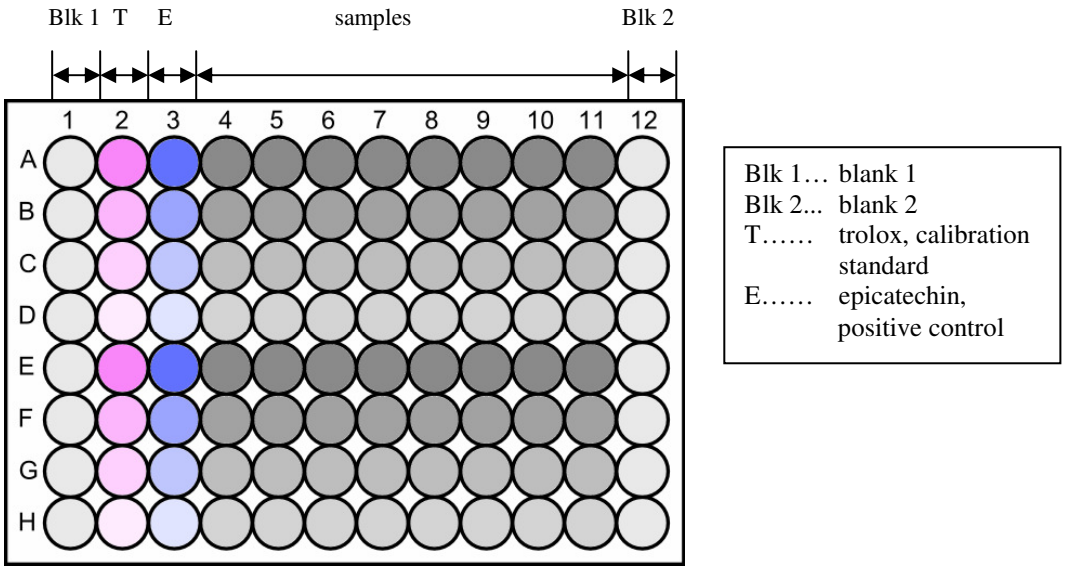


Figure 9. Application of samples, standards and blanks on the 96-well plate: blanks (light-grey), trolox (pink), epicatechin (blue), samples (dark-grey). Colours get lighter as concentration gets less.

Blank 1 just contained 20µL of phosphate buffer with 2% methanol and 170µL phosphate buffer, Blank 2 contained 20µL of phosphate buffer with 2% methanol and AAPH (in both cases no dilutions). Trolox is a water soluble vitamin E analogue and was used as calibration standard to get a standard curve and ORAC values of the test samples were extrapolated from it. Epicatechin was a positive control. Both rows contained each 20 µL trolox or epicatechin in 4 different concentrations (trolox: 0.1mM, 0.05mM, 0.025mM, 0.0125mM and epicatechin: 250µg/mL, 1.25µg/mL, 0.625µg/mL, 0.3125µg/mL) filled up with 170µL AAPH. Samples (dark-grey) were also tested in four different concentrations (200 µg/mL, 66.67 µg/mL, 22.23 µg/mL, 7.41 µg/mL), using phosphate buffer as solvent. They were duplicated at the second half of the plate. In all rows 10µL of fluorescein was added. The azo-compound AAPH produces peroxyl radicals by heating and as a result causes oxidation of fluorescein, which is measured as a loss of fluorescence. If there is antioxidant activity in the oil samples, the fluorescein gets protected. The degree of this protection gets quantified using a fluorometer. AAPH, the azo-compound, is unstable, so the solution was prepared just before adding to the plate and measuring the activity. 200mg were dissolved in 25mL prewarmed phosphate buffer. The measurement was taken using the Wallac Victor 2 reader (Perkin-Elmer). Before the reading begins, the plates were automatically shaken for 10 seconds in a slowly orbital manner. Wallac Victor 2 reads the plate 35 times a minute. The chamber

where the plate was put into, was prewarmed to 37°C to assure good reaction conditions. The decay curves were recorded and the AUC was calculated for each well by Wallac Workout 1.5 (Perkin-Elmer) and was transferred to Excel to determine the micromoles of trolox equivalent value (TE).

3.3.2. Cytotoxicity Assay

The cytotoxicity assay was done using the Perkin Elmer ATPlite kit (Luminescence ATP Detection Assay System). It is an Adenosine Triphosphate (ATP) monitoring system based on firefly (*Photinus pyralis*) luciferase. ATP is present in all metabolically active cells. Its concentration decreases very quickly when the cells undergo necrosis or apoptosis. The ATP reacts with the added luciferase and D-luciferin and produce light, what is shown in the reaction scheme:

$$\text{ATP + D-Luciferin + } O_2 \xrightarrow[Mg^{2+}]{\text{luciferase}} \text{Oxyluciferin + AMP + } PP_i \text{ + } CO_2 \text{ + light}$$

The emitted light is measured and proportional to the ATP concentration [20].

3.3.2.1. Sample preparation

All oils were prepared in ethanol at a concentration of 20 mg/mL.

3.3.2.2. Reagents

Culture media:

Colour free medium 1	Colour free DMEM (low glucose)	86 mL
for P388D1 (100mL)	Horse sera	10 mL
	L-Glutamine (200 mM)	2 mL

	Pen/strep (5000 U/mL and 5000 µg/mL)	2 mL
	D-Glucose	350 mg
Colour free medium 2	Colour free DMEM (low glucose)	88 mL
for HEP G-2 and Caco-2	FBS	10 mL
(100 mL)	L-Glutamine (f.c.= 2 mM)	1 mL
	Pen/strep	2 mL
	D-Glucose	350 mg

[All chemicals were purchased from Gibco, except D-Glucose (Sigma)]

Perkin Elmer ATPlite assay kit:
[Mammalian cell lysis solution,
Substrate buffer solution,
Luciferase/Luciferin solution (lyophilized),
ATP standard (lyophilized)]
Cell lines (P388D1, HEP G-2, Caco-2)
Chlorambucil (60 mg/mL in sterilized DMSO)
Curcumin (10 mg/mL in sterilized DMSO)
Ethanol (sterilised)

3.3.2.3. Procedure

Initial, the cell culture media had been prepared, appropriate to the cell line which was used. The first screen for cytotoxic activity has been done on P388D1 cells (mouse lymphoblast), further the samples were tested on HEP G-2 cells (human caucasian hepatocyte carcinoma) and on Caco-2 cells (human colonic adenocarcinoma). Cells were removed from the flask wall using 0.5 mL trypsin, then it got inactivated by adding 0.5 mL of culture medium. A small aliquot of cell suspension had been taken to count the number of cells using the ActDiff cell counter. According to the number of cells, the requested amount of medium has been taken to get a final concentration of 0.1×10^6 cells/mL. To prepare the dilutions of the samples a clear 96-well dilution plate (JRH flat bottomed) was used.

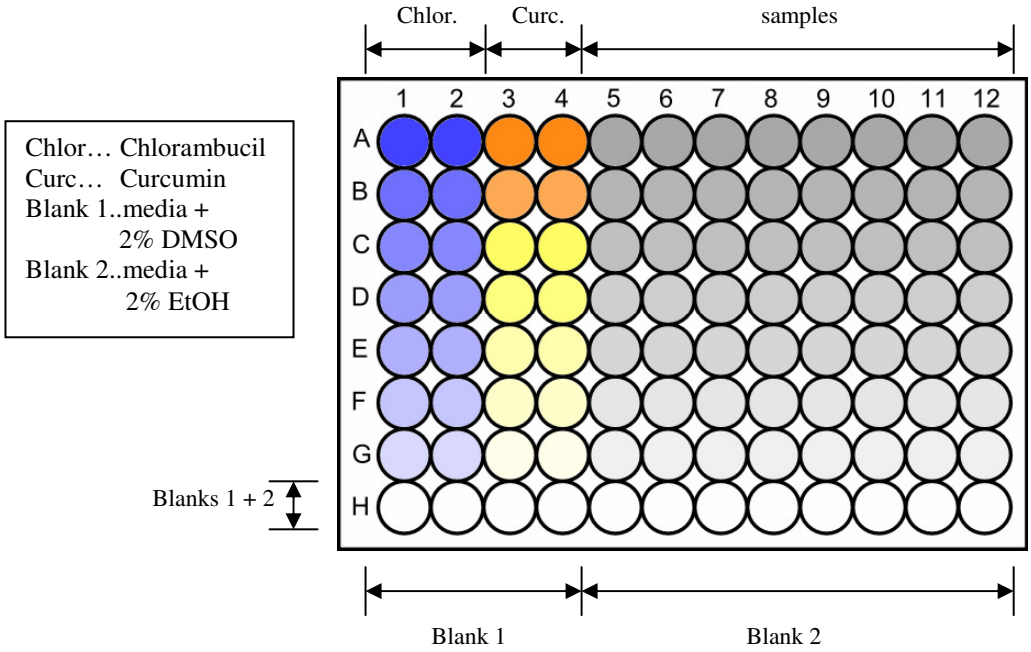


Figure 10. Application of samples, standards and blanks on the 96-well plate: chlorambucil (blue), curcumin (orange), samples (grey) and blanks (white). Colours get lighter as concentration gets less.

Chlorambucil, a cytostatic drug and positive control, got applicated in different concentrations (60, 30, 15, 7.5, 3.75, 1.875 mg/mL). Curcumin got applicated in 7 different concentrations too (10, 5, 2.5, 1.25, 0.625, 0.3125 mg/mL). Both controls were dissolved in sterile DMSO (Dimethyl Sulfoxide). The samples were applicated in concentrations 20, 10, 5, 2.5, 1.25 and 0.625 mg/mL. The samples were dissolved in ethanol. The blanks 1 and 2 contained the culture medium with 2% either DMSO or ethanol. Then 50 µL of each dilution were transferred on the white cell culture plate (with clear bottom). This plate already contained the cell line and was in a CO_2 incubator at 37°C (5% CO_2) overnight (24 hours). At least, 50 µL of the mammalian cell lysis and 50 µL luciferin dissolved in its buffer solution, were put into the wells. Before measuring, the plates have been shaken for 5 minutes on a Wallac 1296-003 Delfia plateshaker, for lysing the cells and stabilising the ATP. The system used was a Wallac Trilux 1450 Microbeta liquid scintillation & luminescence counter. The start of the luminescence counter was delayed 10 minutes to allow the plates to adapt to the dark.

3.3.3. Anti-inflammatory Assay

Most of the chemical classes appearing in *Callitris glaucophylla* oil have been reported as an anti-inflammatory agent before. So are, for example, sesquiterpenes with several double bonds supposed to be good for reducing inflammation caused by stings and bites (Pénoël & Franchomme, 1990). Esters react pretty similar to aspirin (acetyl salicylate), oxides (1,8-cineole) may exhibit anti-inflammatory effects in bronchial asthma by inhibiting the leukotriene B4 and prostaglandin E2 pathways, sesquiterpenols are very potent anti-inflammatory agents, and lactones, especially sesquiterpenoid lactones, seem to have strong anti-inflammatory properties (inhibition of the expression of the gene for interleukin-8 (Mazor et al., 2000) [21]. Selected pure fractions of the oils were tested for their ability to inhibit prostaglandin E_2. Prostaglandin E_2 is one of the primary cyclooxygenase products of arachidonic acid metabolism. The Kookaburra Prostaglandin E2 Enzyme Immunoassay Kit (Catalog No. 133-16359, 96 well kit) from Sapphire Bioscience has been used. This kit is a competitive ELISA for the quantitative determination of PGE_2. It is based on the competition between PGE_2 and a PGE_2-alkaline phosphatase tracer for a limited amount of PGE_2-specific monoclonal antibody [22].

3.3.3.1. Sample preparation

The anti-inflammatory assay was done on selected fractions. All fractions were diluted in ethanol in a concentration of 20 mg/mL, but then diluted in coloured media 2 in two different dilutions: 20 µg/mL and 2µg/mL. Each sample was repeated 8 times.

3.3.3.2. Reagents

Appropriate culture media:

Coloured Media 2	DMEM	86 mL

for 3T3	FBS	10 mL
(100 mL)	L-Glutamine (f.c.= 2 mM)	1 mL
	Pen/Strep	2 mL
	Na pyruvate (100mM)	1 mL

[chemicals were purchased from Gibco]

Cell lines (3T3 Mouse Swiss Albino fibroblast cells)
0.25% trypsin
Sterile phosphate buffered saline (PBS)
Aspirin (acetylsalicylic acid; Sigma A5376)
Calcium ionophore A23187 (calcimycin; Sigma C7522)
Kookaburra Prostaglandin E_2 Enzyme Immunoassay Kit
[Tris Buffer
Wash Buffer
DEA Buffer
Prostaglandin E_2 Standard
Prostaglandin E_2 Alkaline Phosphatase Tracer
Prostaglandin E_2 Monoclonal Antibody]

3.3.3.3. Procedure

Initial, the cell culture media was prepared. According to the 3T3 cells, the coloured medium 2 has been used. Before using the cells, they have been checked for confluence and lack of contamination under the microscope. The growth medium has been sucked off with vacuum, then the flask got rinsed with prewarmed PBS. After removing the PBS, also by using vacuum, 0.5 mL 0.25% trypsin were added to lift the cells, so that they were not adhered to the flask. A little amount of media (about 2 mL) was added to inactivate the trypsin and by sucking up and down with a pipette it was ensured that the cells did not build any cell clumps. Then the cells have been counted (Beckmann Coulter ActDiff Cell Counter) and according to the number of cells, the volume of cell suspension was calculated to get a final concentration of 0.1 x 10^6 cells/mL. Cell suspension (50µL) was pipetted in each well of the used clear

cell culture plate (Perkin Elmer 96 well isoplate) and put in the incubator (37°C, 5% CO_2) overnight. After 24 hours, the samples and controls have been added (each 50 µL). The samples were first diluted in ethanol in a concentration of 20 mg/mL, but then diluted in coloured media 2 in two different dilutions: 20 µg/mL and 2µg/mL. Each sample was repeated 8 times (use of two 96 well plates). The stock of the control, Aspirin, was 15mM in DMSO, and diluted to final concentrations of 200 µg/mL and 20 µg/mL in media 2. Blanks were media (50 µL) and media with 2% DMSO (50 µL). Then the plates were incubated for three hours at 37°C, 5% CO_2. The stock of calcium ionophore (ci) (25mM DMSO) was diluted in media 2 to a concentration of 400 µg/mL and directly added to half of the wells of each plate, so that there are equal repeated samples with and without calcium ionophore. A layout of this plate can be seen in Figure 11. The plates were shaken on a Wallac 1296-003 Delfia plateshaker for 20 seconds before incubated again for 20 minutes (37°C, 5% CO_2). After another short shake (30 seconds) they were centrifuged (Sigma Laboratory Centrifuge 4K15) using program 11150/13220 (1000 RCF, 3 min). The supernatants were transferred into Eppendorf micro tubes and frozen overnight. The following day, all reagents from the kit were prepared: The Tris Buffer Concentrate was diluted with 90 mL MilliQ water. The Wash Buffer (5 mL) was diluted to a final volume of 750mL with MilliQ water. The DEA Buffer (2.5 mL) was diluted to a final volume of 25 mL with MilliQ water. The Prostaglandin E_2 Standard was diluted in MilliQ water to a concentration of 40 ng/mL. For the assay it was further diluted within eight steps (4 ng/mL, 2 ng/mL, 1 ng/mL, 0.5 ng/mL, 0.25 ng/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL). The Prostaglandin E_2 Alkaline Phosphatase Tracer and the Prostaglandin E_2 Monoclonal Antibody were reconstituted with each 6 mL of Tris buffer.

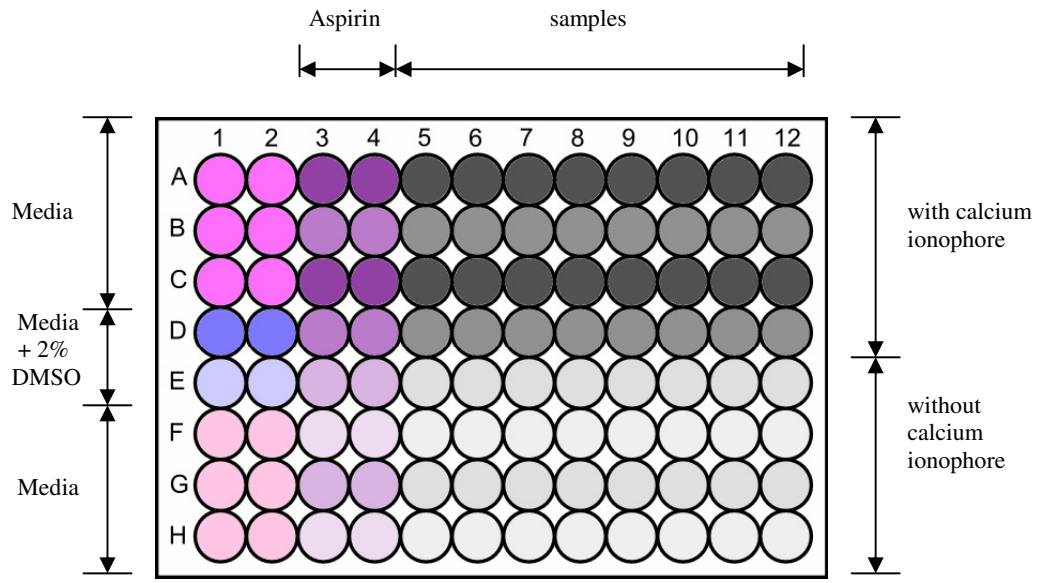


Figure 11. Application of samples, standards and blanks on the 96-well plate: media with calcium ionophore (ci) (pink), media without ci (light pink), media + 2% DMSO with ci (blue), media + 2% DMSO without ci (light blue), Aspirin with ci (in two conc., dark purple and purple), Aspirin without ci (in two conc., light purple and lighter purple), sample with ci (in two conc., dark grey and grey), sample without ci (light grey and lighter grey).

All dilutions were vortexed to mix. For the final assay a goat anti-mouse IgG coated plate (96 well plate) from Sapphire Bioscience was used, as shown in Figure 12.

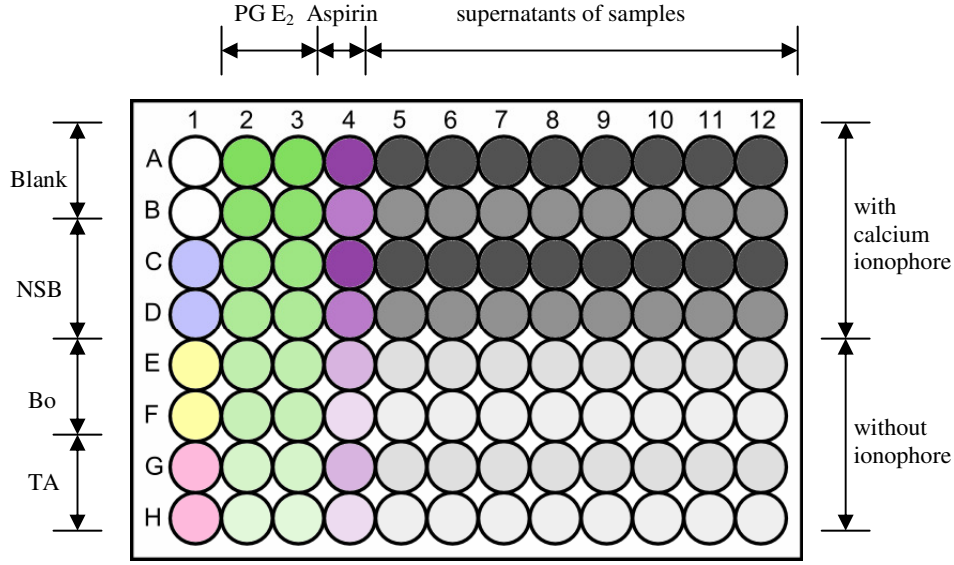


Figure 12. Application of samples, standards and blanks on the 96-well goat anti-mouse IgG coated plate: blanks (white), NSB (blue), B_o (yellow), TA (pink), PG E_2

(green, colour gets lighter as conc gets less), Aspirin with ci (in two conc., dark purple and purple), Aspirin without ci (in two conc., light purple and lighter purple), sample with ci (in two conc., dark grey and grey), sample without ci (light grey and lighter grey).

Blanks contained nothing, NSB (non-specific binding)-wells contained just 150 µL Tris buffer and 50µL Prostaglandin E_2 Alkaline Phosphatase Tracer, B_0 (zero standard)-wells contained 100 µL Tris Buffer, 50 µL Prostaglandin E_2 Alkaline Phosphatase Tracer and 50 µL Prostaglandin E_2 Monoclonal Antibody and TA-wells contained nothing, finally 5 µL of Prostaglandin E_2 Alkaline Phosphatase Tracer. The final sample dilutions were prepared in a dilution plate. For each of the two original dilutions (20 µg/mL and 2 µg/mL), 10 µL supernatant were diluted in 190 µL Tris buffer, and further 10 µL of this dilution in 240 µL Tris buffer. This last concentration was put on the assay plate for both dilutions and duplicated. The whole procedure was done on the eight samples with and those without calcium ionophore. Aspirin was treated the same way as the samples. In all wells containing sample, PG E_2 or Aspirin, 50 µL Prostaglandin E_2 Alkaline Phosphatase Tracer and 50 µL Prostaglandin E_2 Monoclonal Antibody were added. The assay plate got covered with foil and incubated for three hours at room temperature on the Wallac 1296-003 Delfia plateshaker. The wells got emptied and rinsed five times with the wash buffer and afterwards repeatedly blotted on paper towel until there was no drop of buffer left on the plate. 200 µL of prepared *p*NPP (para-nitrophenyl-phosphate) solution (5 tablets dissolved in 25 mL DEA buffer) were added to each well, including all blanks. 5 µL of Prostaglandin E_2 Alkaline Phosphatase Tracer were added to the TA-wells. The plate got covered with an adhesive cover and foil and allowed to develop in the dark on the Wallac 1296-003 Delfia plateshaker for 60 minutes. Finally, the bottom of the plate was wiped to avoid finger prints, smudges or dirt to disturb the reading of absorbance. The plate was read at wavelength 405 nm on the Wallac Victor 2 reader (Perkin-Elmer). The data got exported from Workout to Excel and the percentage inhibition of PGE_2 production was calculated for each well.

4. RESULTS AND DISCUSSION

4.1. Comparison of the freshly distilled oils and oils stored for one year

The *Callitris glaucophylla* oils from three different accessions were dissolved in acetone (at a concentration of approximately 150mg/mL) and profiled by GC/MS. The overlaid chromatograms are shown in Figures 15, 18 and 21. The process of identification of the components is described in section 3.2.2.

4.1.1. *Callitris glaucophylla* 32209

Table 5. Composition of the freshly distilled oil of *Callitris glaucophylla* 32209.

Nº	Compound	Retention Time	CAS #	% distribution	Area	KI	calculated KI
10	guaiol	25.89	489-86-1	47.59	197443744	1600	1579
11	10-epi-γ-eudesmol	26.58	15951-81-7	3.68	15253374	1623	1615
15	bulnesol	27.05	22451-73-6	14.68	60910902	1671	1640
14,13	α- and β-eudesmol	27.10	473-16-5, -15-4	7.11	29500401	1653, 1650	1642
5	dihydrocolumellarin	30.72	66873-38-9	11.76	48794853	1900	1831
4	columellarin	31.49	66873-37-8	13.20	54771967	1952	1871

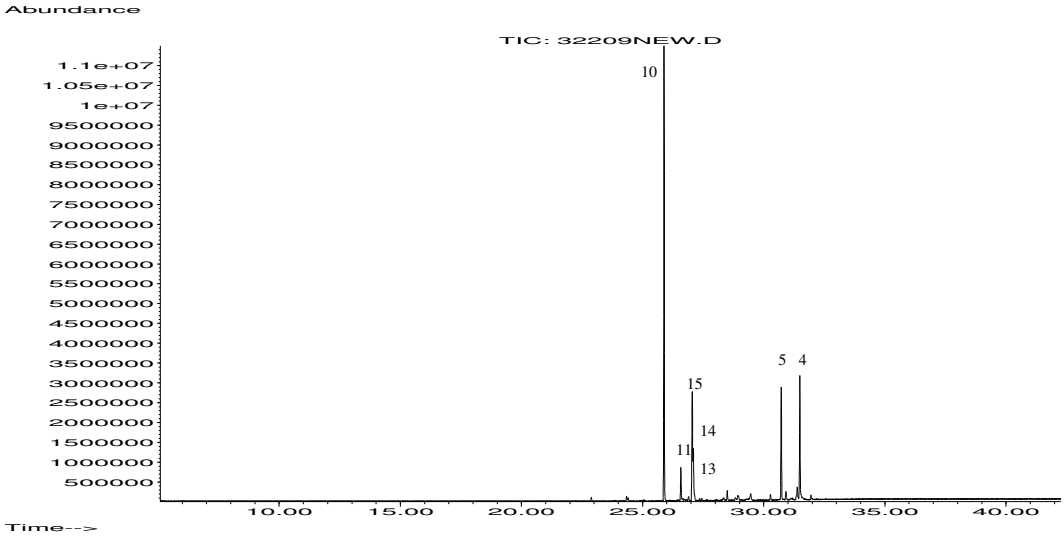


Figure 13. Total Ion Concentration of the freshly distilled oil of *Callitris glaucophylla* 32209.

Table 6. Composition of the oil of *Callitris glaucophylla* 32209 stored for one year.

N°	Compound	Retention Time	CAS #	% distribution	Area	KI	calculated KI
10	guaiol	25.90	489-86-1	79.92	2955254802	1600	1579
11	10-epi-γ-eudesmol	26.58	15951-81-7	1.54	57004580	1623	1615
15	bulnesol	27.05	22451-73-6	10.93	404276505	1671	1640
14,13	α- and β-eudesmol	27.10	473-16-5, -15-4	2.79	103340315	1653, 1650	1642
5	dihydrocolumellarin	30.73	66873-38-9	2.59	95836465	1900	1831
4	columellarin	31.49	6673-37-8	1.01	37290032	1952	1871
27	sandaracopimarinal	34.73	3855-14-9	1.21	44617130	2184	2040

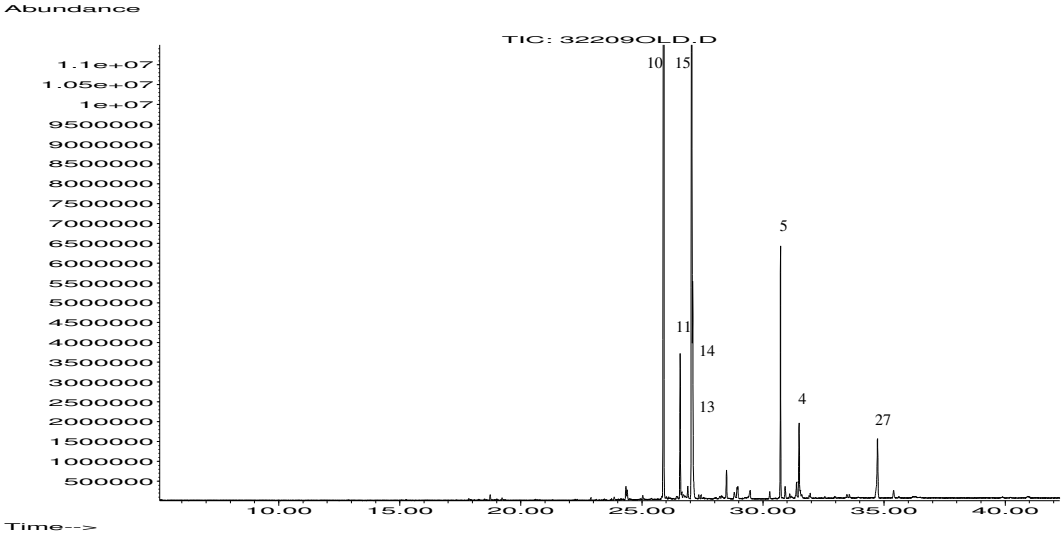


Figure 14. Total Ion Concentration of the oil stored for one year of *Callitris glaucophylla* 32209.

The composition of the freshly distilled oil and the oil stored for one year was very similar, but the amounts of several components changed a bit. Guaiol was the principal component in both oils, showing an increased percent distribution in the old oil (values increased from 47.59 percent to 79.92 percent distribution). Bulnesol showed the second highest percentage, but the value decreased as the oil aged (from 14.68 percent to 10.93 percent). The two sesquiterpene lactones columellarin and dihydrocolumellarin were also present in both oils, even if the amount of both decreased mentionable from 11.76 percent and 13.2 percent to 2.59 percent and 1.01 percent. The level of eudesmols (10-epi-γ-eudesmol, α- and β-eudesmol) was the lowest of all major peaks and decreased also a bit as oil was stored (from 3.68 percent and 7.11 percent to 1.54 percent and 2.79 percent). The appearance of the diterpenealdehyde sandaracopimarinal is limited to the old oil. Differences between the oils are shown in the overlaid chromatogram (Figure 15).

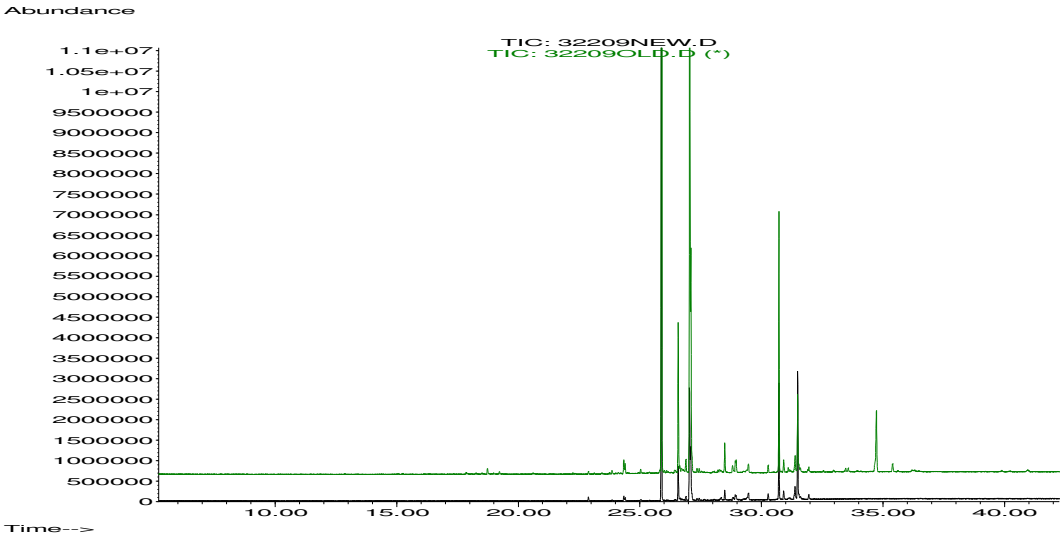


Figure 15. Overlaid chromatograms of the freshly distilled oil (black) and oil stored for one year (green).

4.1.2. *Callitris glaucophylla* 32352

Table 7. Composition of the freshly distilled oil of *Callitris glaucophylla* 32352.

N°	Compound	Retention Time	CAS #	% distribution	Area	KI	calculated KI
16	α-pinene	12.86	80-56-8	2.34	4350248	939	899
17	limonene	17.85	138-86-3	traces	1421024	1029	1160
18	1,8-cineole	17.85	470-82-6	traces	1421024	1031	1160
19	methyl myrtenate	20.60	30-649-97-9	1.26	2341156	1294	1303
9	citronellic acid	20.60	502-47-6	1.26	2341156	1313	1303
20	β-selinene	24.34	17066-67-0	1.97	3649525	1490	1498
21	α-selinene	24.34	473-13-2	1.97	3649525	1498	1498
11	10-epi-γ-eudesmol	26.58	15051-81-7	traces	-	1623	1615
13	β-eudesmol	27.13	473-15-4	3.35	6217943	1650	1644
22	γ-costol	28.25	65018-14-6	4.83	8976245	1746	1702
23	methyl ester of γ-lactone	28.49	-	6.34	11774470	-	1714
24	β-costol	28.80	515-30-8	5.35	9946597	1767	1731
5	dihydrocolumellarin	30.72	66873-38-9	20.92	38848677	1900	1831
25	unknown, mw 256	30.92	-	8.03	14913801	-	1841
4	columellarin	31.49	66873-37-8	4.04	7502013	1952	1871
26	unknown, mw 207	33.47	-	3.77	7169369	-	1974
27	sandaracopimarinal	34.73	3855-14-9	8.21	15252991	2184	2040
28	unknown, mw 258	36.17	-	8.45	15743603	-	2115

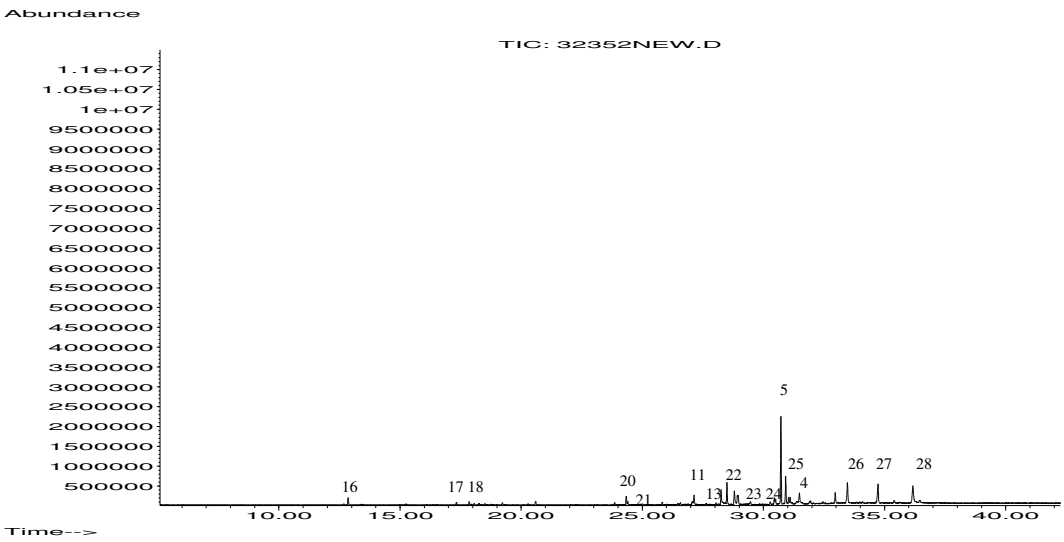


Figure 16. Total Ion Concentration of the freshly distilled oil of *Callitris glaucophylla* 32352.

Table 8. Composition of the oil of *Callitris glaucophylla* 32352 stored for one year.

N°	Compound	Retention Time	CAS #	% distribution	Area	KI	calculated KI
16	α-pinene	12.86	80-56-8	2.02	7849836	939	899
19	methyl myrtenate	20.60	30-649-97-9	0.97	3760552	1294	1303
20	β-selinene	24.34	17066-67-0	2.67	10387321	1490	1498
21	α-selinene	24.40	473-13-2	1.84	7142842	1498	1501
13	β-eudesmol	27.13	473-15-4	1.77	6868984	1650	1644
22	γ-costol	28.25	65018-14-6	1.94	7530462	1746	1702
23	methyl ester of γ-lactone	28.49	-	2.08	8084152	-	1715
24	β-costol	28.80	515-30-8	1.30	5073302	1767	1731
29	α-costol	28.93	65018-15-7	0.25	969599	1774	1738
5	dihydrocolumellarin	30.72	66873-38-9	12.22	47538861	1900	1831
27	sandaracopimarinal	34.74	3855-14-9	60.11	233777189	2184	2040
30	unknown, mw 281	35.39	-	2.96	11507045	-	2074
28	unknown, mw 273	36.17	-	4.67	18168482	-	2115

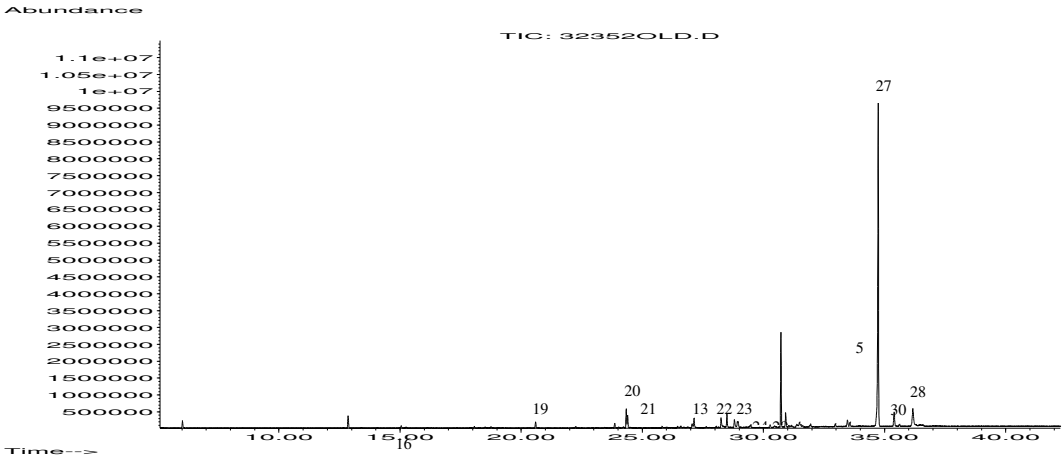


Figure 17. Total Ion Concentration of the oil stored for one year of *Callitris glaucophylla* 32352.

Compared to the oil collected at the accession mentioned above (32209), this oil shows more components in its GC/MS profiles. Instead of guaiol, which was the major component in the oil of 32209 but did not even appear in the profile of the 32352 oil, dihydrocolumellarin was the principal compound in the freshly distilled oil. The amount of dihydrocolumellarin decreased little when oil has been stored (20.92 percent to 12.22 percent). Characteristic for the stored oil is the high increase of sandaracopimarinal, which appeared as a small peak in the freshly distilled oil, from 8.21 percent to 60.11 percent. Small amounts of limonene, 1,8-cineole, citronellic acid, 10-epi-γ-eudesmol, unknown (mw 256), columellarin, unknown (mw 207) and unknown (mw 258) were present in the freshly distilled oils, but could not be observed in the GC/MS profile as oil aged. The minor components α-pinene, methyl myrtenate and α-selinene stayed nearly the same in appearance and amount in the last year. Very little de- or increases happened in the amounts of β-selinene, β-eudesmol, γ-costol, a methyl ester of a γ-lactone and β-costol. The stored oil showed two new peaks at retention times 35.39 and 36.17 which could not get identified and are probably derivatives and α-costol, all three in small amounts (0.25, 2.96 and 4.67 percent distribution). The differences between the freshly distilled oil and the one year stored oil are shown in the overlaid chromatogram below (Figure 18).

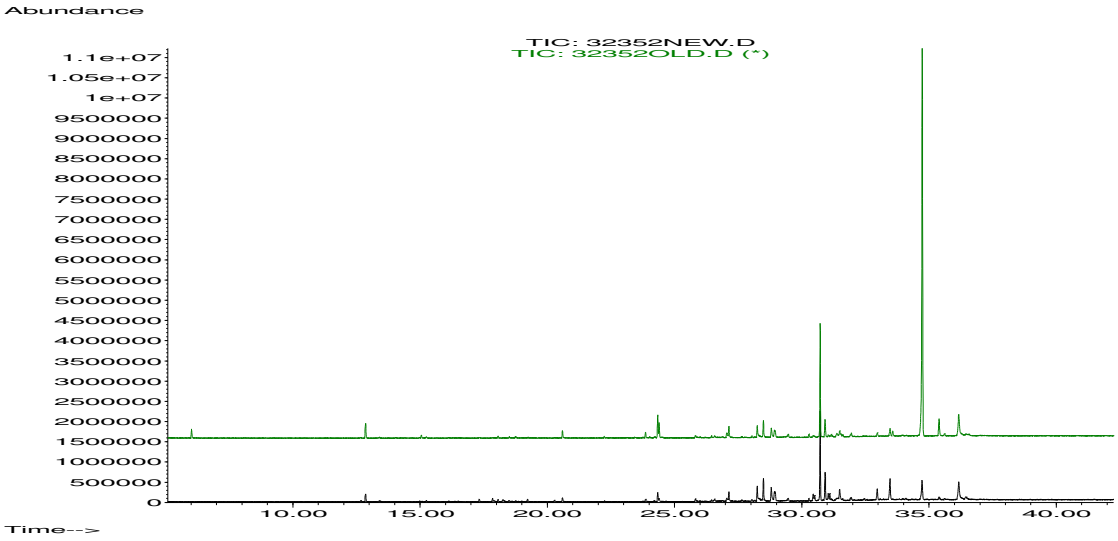


Figure 18. Overlaid chromatograms of the freshly distilled oil (black) and oil stored for one year (green).

4.1.3. *Callitris glaucophylla* 31973

Table 9. Composition of the freshly distilled oil of *Callitris glaucophylla* 31973.

N°	Compound	Retention Time	CAS #	% distribution	Area	KI	calculated KI
16	α-pinene	12.86	80-56-8	traces	-	939	803
10	guaiol	25.89	489-86-1	75.6	1965623080	1600	1599
11	10-epi-γ-eudesmol	26.58	15951-81-7	1.69	43813969	1623	1641
12	γ-eudesmol	26.58	1209-71-8	1.69	43813969	1632	1641
15	bulnesol	27.05	22451-73-6	12.14	315693197	1671	1670
14	α-eudesmol	27.05	473-16-5	12.14	315693197	1653	1670
13	β-eudesmol	27.10	473-15-4	3.59	93274148	1650	1673
5	dihydrocolumellarin	30.72	66873-38-9	2.39	62176509	1900	1894
4	columellarin	31.50	66873-37-8	4.59	119408933	1952	1935

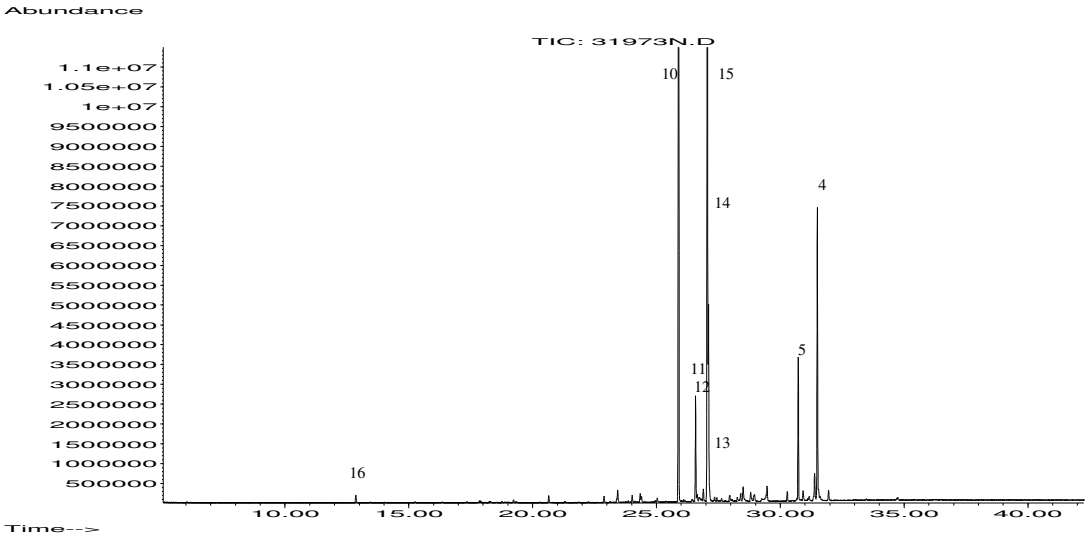


Figure 19. Total Ion Concentration of the freshly distilled oil of *Callitris glaucophylla 31973.*

Table 10. Composition of the oil of *Callitris glaucophylla* 31973 stored for one year.

N°	Compound	Retention Time	CAS #	% distribution	Area	KI	calculated KI
10	guaiol	25.89	489-86-1	67.60	695530040	1600	1599
11	10-epi-γ-eudesmol	26.58	15951-81-7	2.83	29163862	1623	1641
12	γ-eudesmol	26.58	1209-71-8	2.83	29163862	1632	1641
15	bulnesol	27.05	22451-73-6	15.96	164178002	1671	1670
14	α-eudesmol	27.05	473-16-5	15.96	164178002	1653	1670
13	β-eudesmol	27.10	473-15-4	5.29	54413493	1650	1673
5	dihydrocolumellarin	30.72	66873-38-9	3.56	36670023	1900	1894
4	columellarin	31.49	66873-37-8	4.76	48976516	1952	1935

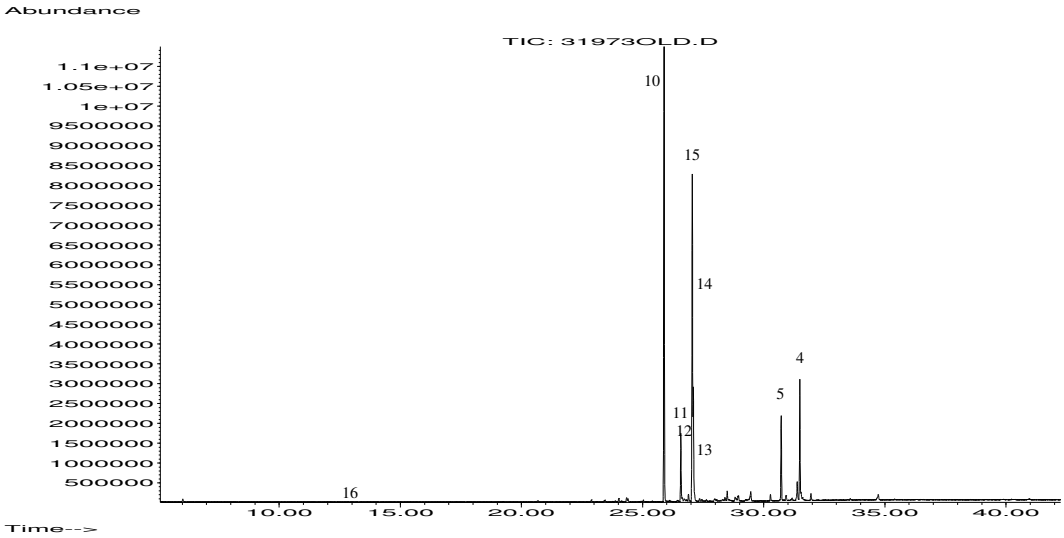


Figure 20. Total Ion Concentration of the oil stored for one year of *Callitris glaucophylla* 31973.

The principal compound in the freshly distilled 31973 oil was guaiol with a distribution of 75.6 percent as well as in the stored oil with a slight decrease of 8 percent. In general, this oil did not show many differences in composition and proportion between the freshly distilled oil and the oil stored for one year. All other compounds (as shown in Tables 9 and 10) slightly increased within the last year of storage, except α-pinene which could not be detected in the stored oil. Differences between the freshly distilled oil and the oil stored for a year are shown in the overlaid chromatogram in Figure 21 below.

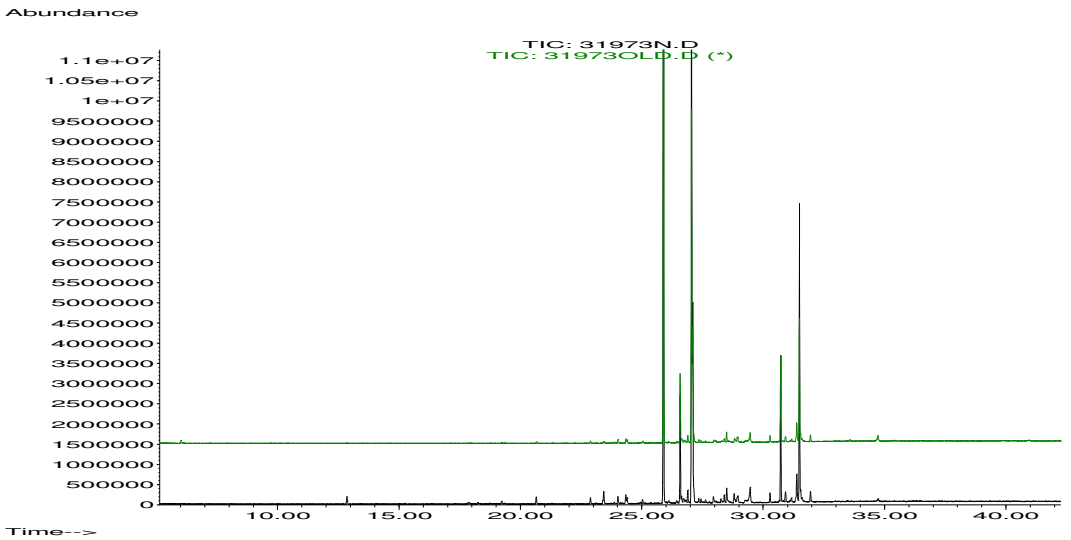
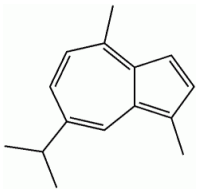


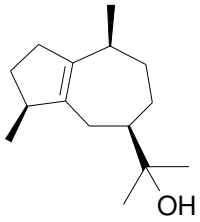
Figure 21. Overlaid chromatograms of the freshly distilled oil (black) and oil stored for one year (green).

It was surprising, that the freshly distilled oils did not seem to have many differences to the one year stored ones, which changed its colour from green and blue tones to brown and ochre yellow tones. However, the amount of some components had changed. Although the GC/MS analysis was not quantitative, the concentrations were approximately 10mg/mL and the percentage distribution of the components had changed as shown in Tables 5 – 10. A closer look at the component, which seemed to have influenced the change of colour was made: The bluish colour of the *Callitris* oils is guaiazulene **(31)**, a dark blue crystalline hydrocarbon, which is a bicyclic sesquiterpene. Doimo also reported the presence of chamazulene (another azulene, known from *Chamomilla recutita*) in *Callitris intratropica*, but there were no traces of it in *Callitris glaucophylla* in his paper. He identified both azulenes being responsible for the blue colour of the *Callitris intratropica* oil, which is a result of the distillation process [16]. Maybe the absence of the second azulene explains the minor-blue of the *glaucophylla* oils, in addition to the lesser amount of guaiazulene. The conjugated double bonds of guaiazulene build a chromophore, that furnishes absorbance both in the visible region (600nm) and in the UV-A (330 nm) [23].

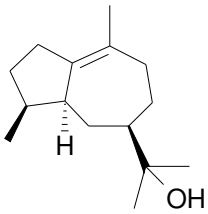


guaiazulene
(31)

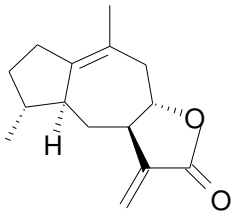
Small changes in the chemical structure of guaiazulene could have ended in the loss of colour. Guaiol **(10)** for example, which was found in high amounts in two oils, has a very similar structure to guaiazulene. The hydroxyl group is missing, beside the loss of the double bonds. It is not coloured. Bulnesol **(15)**, columellarin **(4)** and dihydrocolumellarin **(5)** – components of all three oils – do also have a very similar structure and could be oxidation products. It is possible, that the guaiazulene has transformed into a sesquiterpene lactone (like columellarin **(4)** or dihydrocolumellarin **(5)**) or into a sesquiterpene alcohol (like guaiol **(10)** or bulnesol **(15)**).



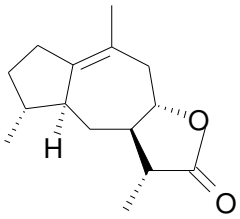
guaiol
(10)



bulnesol
(15)



columellarin
(4)



dihydrocolumellarin
(5)

Guaiazulene could not be detected with GC/MS. This might be caused of its small amount or it is overlapped with other components. A reference standard containing guaiazulene has been run on GC/MS to obtain the expected retention time. In all oils stored for one year, the abundance at this retention time was lower, than in the freshly distilled oils. *Callitris glaucophylla* 31973, which is the bluish-green oil, showed the highest peak at the expected retention time of guaiazulene, followed by the green 32352 and the lowest level was found in the yellow oil 32209.

4.1.4. Results of the pharmacological assays on oils

4.1.4.1. ORAC results of the crude oils

The mean trolox equivalent values of the three *Callitris glaucophylla* oils are shown in Table 11 and illustrated in Figure 22. The oil of 32209 showed the highest activity among the three different accessions, followed by the oil of 31973 and the oil of 32352 showed less antioxidant activity. The antioxidant activity slightly decreased as oils were stored.

Table 11. ORAC results of *Callitris glaucophylla* oils.

sample	ORAC value umolTE/g
31973 freshly distilled	1247.67 ± 116.77
32352 freshly distilled	376.93 ± 92.06
32209 freshly distilled	1426.91 ± 255.88
31973 stored for one year	1114.04 ± 243.01
32352 stored for one year	255.64 ± 58.59
32209 stored for one year	1390.29 ± 194.26

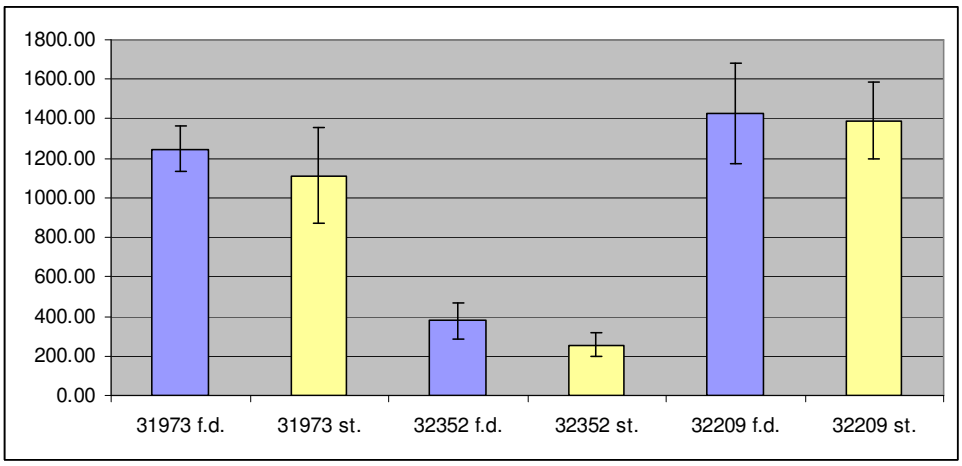


Figure 22. ORAC results (µmolTE/g) of *Callitris glaucophylla* oils. Freshly distilled oils (f.d.) are blue coloured, oils stored for one year (st.) are yellow coloured.

4.1.4.2. Cytotoxicity results of crude oils

The cytotoxicity of the three *Callitris glaucophylla* oils was tested on P388D1 cells, Caco-2 cells and HEP G-2 cells. The percent inhibition values are shown in Table 12, 13 and 14. The assay was repeated twice for all samples (Rep I and Rep II in Table 12 and 13). All oils showed effects at the illustrated concentrations. The higher the concentration was, the higher the percentage of inhibition. On the P388D1 cells the freshly distilled oil of *Callitris glaucophylla* 31973 showed the highest inhibition, followed by the fresh 32209 oil and the fresh 32352 oil. However, at a concentration of 50 µg/mL all oils showed nearly the same activity. The cytotoxic activity changed little as oils aged. The 32352 oil stored for one year showed higher activity at the highest concentration (208 µg/mL) than the fresh one and was the most active oil from the stored ones. The *Callitris glaucophylla* 32209 oil did not show significant changes in the activity as oil was stored (197 µg/mL: from 99.38% to 99.47%, 99 µg/mL: from 71.83 to 75.24%, 49 µg/mL: from 46.16 to 48.04%). The oil from the third accession (31973) also did not show big changes, but a loss of activity at the second concentration of 106 µg/mL from 81.53 to 65.20%. Chlorambucil did not work on the Caco-2 cells and showed an increase of ATP (which is equivalent to number of cells). All oils showed activity at the highest concentration of approximately 200 µg/mL on the Caco-2 cells. There was a huge loss of activity from the first to the second concentration of approximately 100

µg/mL in the oils from 32352 (from 99.23 to 51.28%) and 32209 (from 99.41 to 66.51%). The 31973 oil did not show any inhibition except at the highest concentration. No significant changes could be reported between the oils stored for a year and the freshly distilled oils. All oils (freshly distilled and stored oils) showed high activity at HEP G-2 cells at the highest concentration. They did not show differences within the different accessions eighter.

Table 12. Cytotoxicity results on P388D1 cells (% inhibition) of *Callitris glaucophylla* oils.

sample	conc. (µg/mL)	% inhibition Rep I	% inhibition Rep II	average
chlorambucil	600	98.04	99.70	98.87 ± 1.17
	300	95.62	93.78	94.70 ± 1.30
	150	80.56	76.40	78.48 ± 2.94
	75	35.40	57.84	46.62 ± 15.87
	38	7.73	30.98	19.36 ± 16.44
	19	10.29	13.48	11.89 ± 2.26
curcumin	100	99.34	99.27	99.31 ± 0.05
	50	55.88	60.02	57.95 ± 2.93
	25	28.60	38.91	33.76 ± 7.29
31973 freshly distilled	211	99.30	99.61	99.45 ± 0.22
	106	79.33	83.74	81.53 ± 3.12
	53	42.05	58.38	50.22 ± 11.55
	26	21.66	41.61	31.63 ± 14.11
	13	25.98	43.78	34.88 ± 12.59
	7	15.03	31.06	23.05 ± 11.34
	3	8.74	32.63	20.68 ± 16.89
32352 freshly distilled	197	44.05	99.63	71.84 ± 39.30
	98	63.98	87.07	75.53 ± 16.33
	49	41.98	53.41	47.70 ± 8.09
	25	22.10	47.77	34.94 ± 18.15
	12	28.88	14.88	21.88 ± 9.90
	6	17.55	12.83	15.19 ± 3.33
32209 freshly distilled	197	99.17	99.59	99.38 ± 0.30
	99	64.23	79.43	71.83 ± 10.75
	49	40.98	51.34	46.16 ± 7.33
	25	27.30	39.35	33.33 ± 8.52
	12	19.47	33.60	26.54 ± 10.00
	6	9.70	16.98	13.34 ± 5.14
31973 stored for one year	213	98.45	98.34	98.40 ± 0.08
	106	57.71	72.69	65.20 ± 10.59
	53	42.96	50.55	46.76 ± 5.37
	27	49.98	43.20	46.59 ± 4.79
	13	27.39	35.94	31.67 ± 6.04
	7	11.54	35.48	23.51 ± 16.93
32352 stored for one year	208	99.47	98.80	99.13 ± 0.47
	104	66.74	80.11	73.42 ± 9.45
	52	27.14	49.47	38.31 ± 15.79
	26	12.83	49.88	31.36 ± 26.20
32209 stored for one year	208	99.27	99.66	99.47 ± 0.27
	104	65.62	84.87	75.24 ± 13.61
	52	39.66	56.43	48.04 ± 11.85
	26	35.87	45.96	40.91 ± 7.14
	13	29.97	45.09	37.53 ± 10.70
	6	9.19	40.41	24.80 ± 22.08

Table 13. Cytotoxicity results on Caco-2 cells (% inhibition) of *Callitris glaucophylla* oils.

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil		no inhibition		
curcumin	100	99.20	97.86	98.53 ± 0.95
	50	94.28	77.56	85.92 ± 11.82
31973 freshly distilled	211	99.34	98.70	99.02 ± 0.46
32352 freshly distilled	197	99.14	99.32	99.23 ± 0.13
	98	29.84	72.72	51.28 ± 30.32
	49	38.71	64.85	51.78 ± 18.49
32209 freshly distilled	197	99.17	99.65	99.41 ± 0.34
	99	44.55	88.47	66.51 ± 31.06
	49	55.29	72.34	63.81 ± 12.06
	25	12.58	73.77	43.17 ± 43.26
	12	21.62	71.21	46.42 ± 35.06
	6	20.41	55.61	38.01 ± 24.89
31973 stored for one year	213	97.19	99.21	98.20 ± 1.43
	106	1.35	69.66	35.50 ± 48.30
	53	48.46	74.21	61.33 ± 18.21
32352 stored for one year	208	99.21	99.67	99.44 ± 0.32
	104	55.01	84.81	69.91 ± 21.07
	52	45.86	70.67	58.26 ± 17.55
	26	3.35	68.49	35.92 ± 46.06
32209 stored for one year	208	99.27	99.54	99.40 ± 0.19
	104	41.32	59.33	50.33 ± 12.73
	52	4.67	55.33	30.00 ± 35.82
	26	7.43	48.56	27.99 ± 29.08

Table 14. Cytotoxicity results on HEP G-2 cells (% inhibition) of Callitris glaucophylla oils.

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil	600	20.30	53.11	36.70 ± 23.20
	300	22.51	64.77	43.64 ± 29.88
	150	6.97	55.11	31.04 ± 34.04
	75	1.05	62.80	31.92 ± 43.67
curcumin	100	99.44	99.70	99.57 ± 0.18
	50	95.64	97.18	96.41 ± 1.08
	25	71.43	77.43	74.43 ± 4.24
	13	38.95	57.07	48.01 ± 12.82
31973 freshly distilled	211	99.63	99.77	99.70 ± 0.10
	106	67.41	78.22	72.81 ± 7.65
	53	53.10	61.00	57.05 ± 5.59
	26	47.54	75.74	61.64 ± 19.94
	13	42.65	59.79	51.22 ± 12.12
	7	0.54	77.45	39.00 ± 54.38
	3	22.98	81.94	52.46 ± 41.69
32352 freshly distilled	197	99.59	99.72	99.65 ± 0.09
	98	69.15	75.64	72.40 ± 4.59
	49	26.43	83.29	54.86 ± 40.20
	25	57.35	65.41	61.38 ± 5.70
	12	29.28	73.82	51.55 ± 31.50
	6	35.84	76.69	56.26 ± 28.88
32209 freshly distilled	197	99.62	99.70	99.66 ± 0.06
	99	80.93	74.74	77.84 ± 4.38
	49	49.46	88.66	69.06 ± 27.72
	25	64.12	64.96	64.54 ± 0.59
	12	48.90	68.19	58.55 ± 13.64
	6	7.80	54.77	31.29 ± 33.21
31973 stored for one year	213	99.57	99.68	99.63 ± 0.08
	106	61.08	66.91	64.00 ± 4.12
	53	46.30	81.57	63.94 ± 24.94
	27	37.62	80.31	58.97 ± 30.19
	13	48.11	80.94	64.53 ± 23.22
	7	3.58	71.75	37.66 ± 48.21
32352 stored for one year	208	99.56	99.73	99.65 ± 0.12
	104	71.73	80.87	76.30 ± 6.46
	52	70.17	67.49	68.83 ± 1.89
	26	58.03	68.71	63.37 ± 7.55
	13	14.38	60.93	37.66 ± 32.91
	6	35.62	75.99	55.81 ± 28.55
32209 stored for one year	208	99.35	99.66	99.51 ± 0.21
	104	50.82	81.00	65.91 ± 21.34
	52	73.77	66.98	70.37 ± 4.81
	26	52.44	57.98	55.21 ± 3.92
	13	66.84	59.38	63.11 ± 5.28
	6	4.17	77.76	40.96 ± 52.04

4.2. *Callitris glaucophylla* 32352

4.2.1. Overview

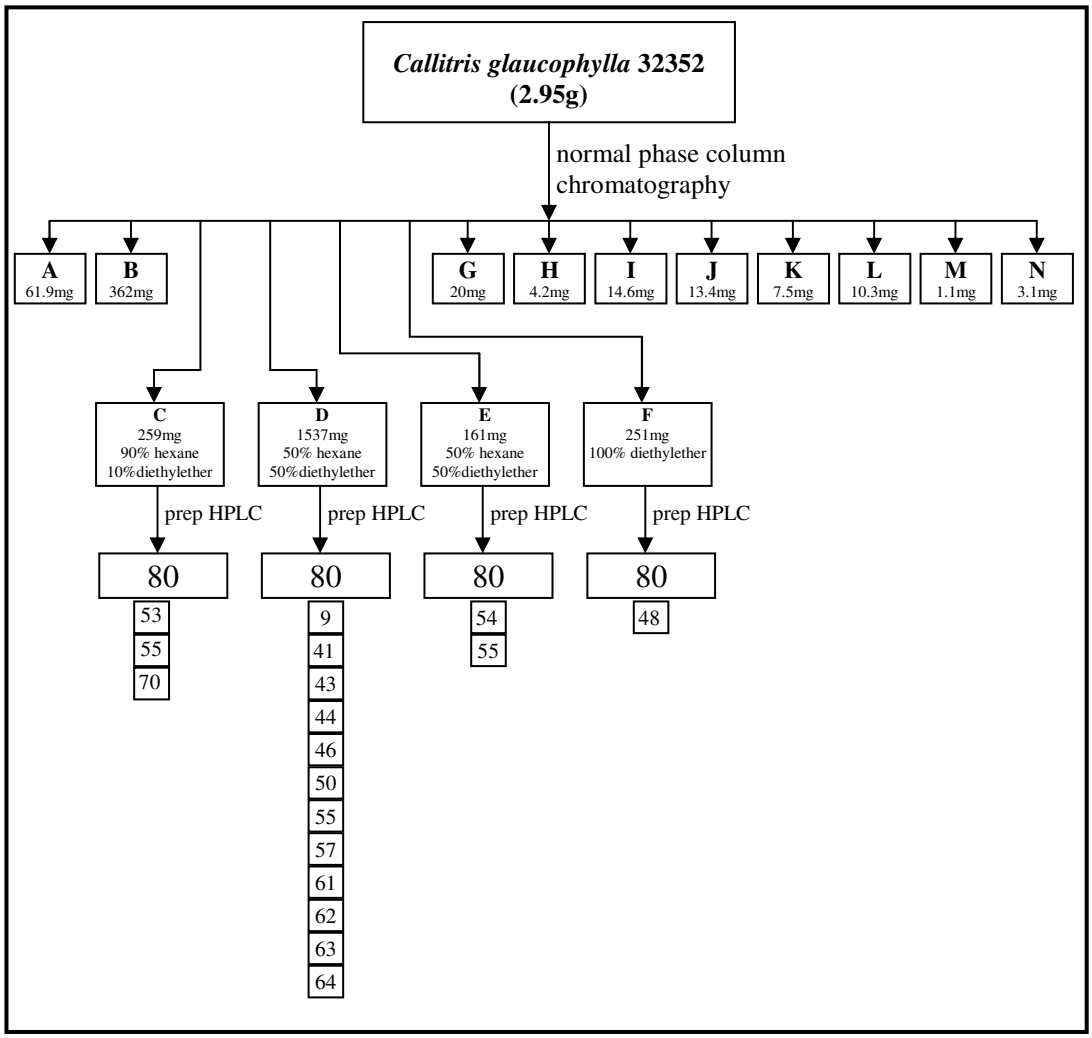


Figure 23. Schematic diagram for the isolation of compounds from *Callitris glaucophylla* 32352.

4.2.2. Column fractionation of 32352

The column had a diameter of 3 cm and a length of 46 cm. Column chromatography has been done using silica as stationary phase. The yield of 32352 was 2.95 g. For getting a 5 – 10% loading, 100 g silica was used. Solvents used were hexane, diethylether, ethyl acetate, methanol and isopropanol, used in ascending order of

polarity. A detailed scheme of the fractionation design is shown in the Table 15 below.

Table 15. Fractionation scheme of *Callitris glaucophylla* 32352.

Fraction	**mobile phase**	**volume (ml)**	**yield (mg)**
A	100% hexane	230	61.9
B	90% hexane / 10% diethylether	230	362
C	90% hexane / 10% diethylether	230	259
D	50% hexane / 50% diethylether	230	1537
E	50% hexane / 50% diethylether	230	161
F	100% diethylether	230	251
G	100% diethylether	230	20
H	50% diethylether / 50% ethyl acetate	230	4.2
I	100% ethyl acetate	230	14.6
J	50% ethyl acetate / 50% methanol	230	13.4
K	100% methanol	230	7.5
L	100% methanol	230	10.3
M	50 % methanol / 50% isopropanol	230	1.1
N	100% isopropanol	460	3.1

4.2.2.1. Chemical analysis

Fractions were profiled on LC/MS and/or GC/MS to obtain the UV and/or MS data.

32352 A

Table 16. Composition of Fraction A.

N°	Compound	Retention Time	CAS #	KI	calculated KI
32	γ-gurjunene	23.85	22567-17-5	1477	1473
20	β-selinene	24.33	17066-67-0	1490	1498
28	unknown, mw 243	30.49	-	-	1819
28	unknown, mw 243	31.09	-	-	1850

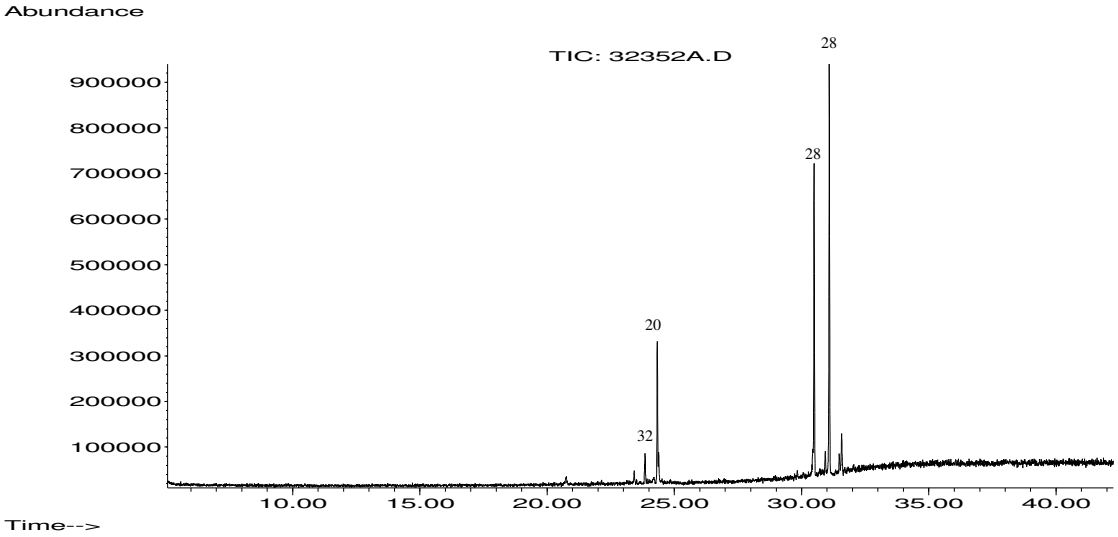


Figure 24. Total Ion Concentration of Fraction A of *Callitris glaucophylla* 32352.

32352 B

Table 17. Composition of Fraction B.

N°	Compound	Retention Time	CAS #	KI	calculated KI
19	methyl myrtenate	20.60	30649-97-9	1294	1302
20	β-selinene	24.36	17066-67-0	1490	1499
23	methyl ester of γ-lactone	28.52	-	-	1716
33	derivative of isopimarol	30.94	-	-	1843
27	sandaracopimarinal	34.76	3855-14-9	2184	2042

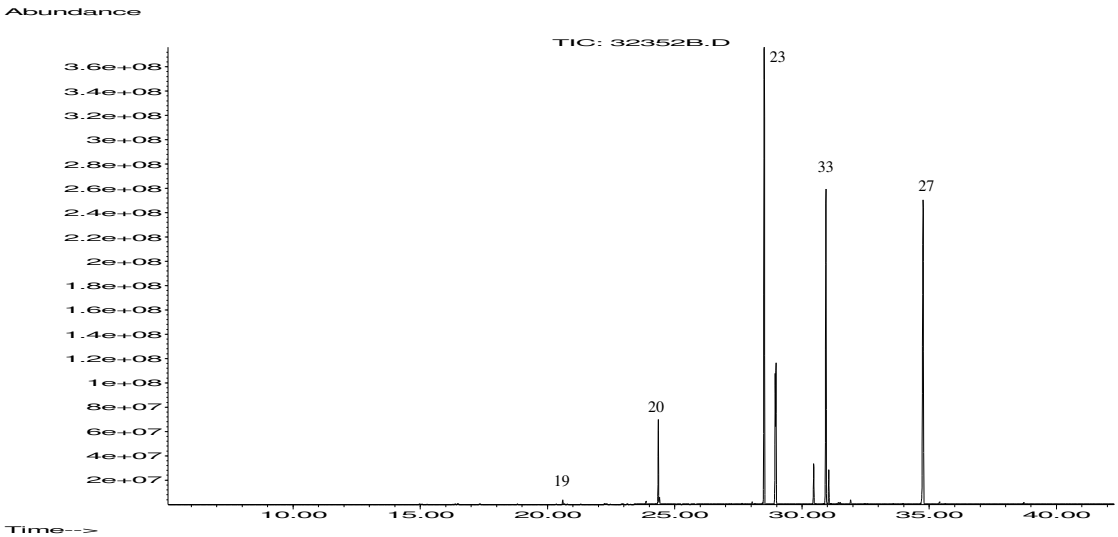


Figure 25. Total Ion Concentration of Fraction B of *Callitris glaucophylla* 32352.

32352 C

Table 18. Composition of Fraction C.

N°	Compound	Retention Time	CAS #	KI	calculated KI
23	methyl ester of γ-lactone	29.37	-	-	1761
27	sandaracopimarinal	35.35	3855-14-9	2184	2073
34	6,7-dehydroferruginol	37.16	34539-85-9	2315	2167

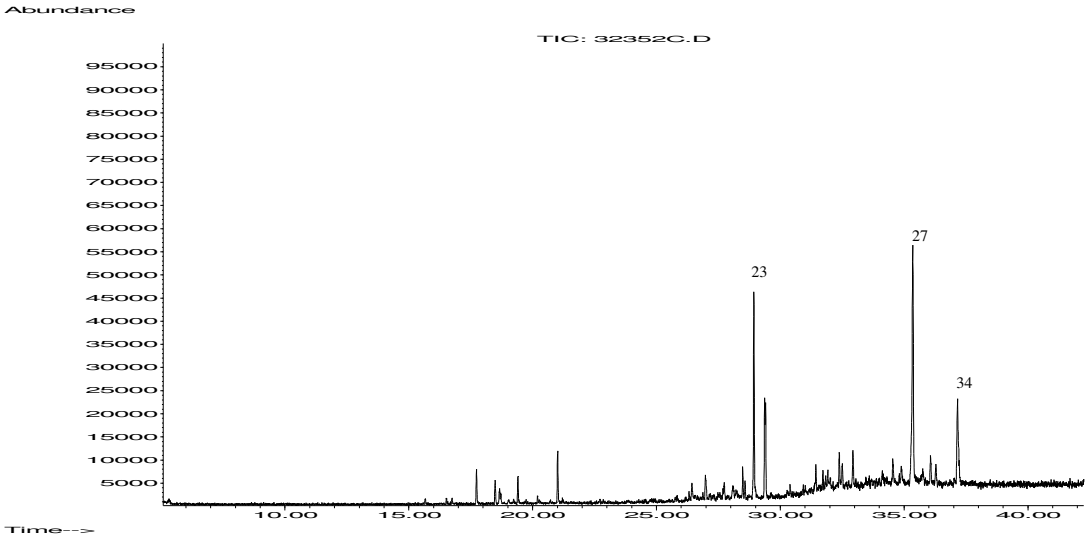


Figure 26. Total Ion Concentration of Fraction C of *Callitris glaucophylla* 32352.

32352 D

Table 19. Composition of Fraction D.

N°	Compound	Retention Time	CAS #	KI	calculated KI
5	dihydrocolumellarin	30.71	66873-38-9	1900	1830

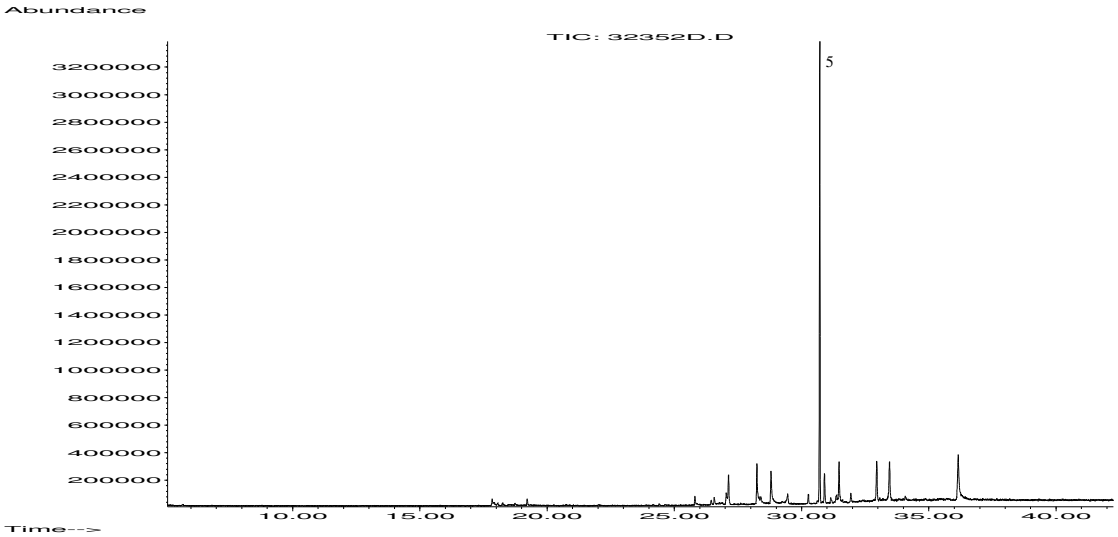


Figure 27. Total Ion Concentration of Fraction D of *Callitris glaucophylla* 32352.

32352 E

Table 20. Composition of Fraction E.

N°	Compound	Retention Time	CAS #	KI	calculated KI
5	dihydrocolumellarin	31.15	66873-38-9	1900	1854
26	unknown, mw 207	34.00	-	-	2002

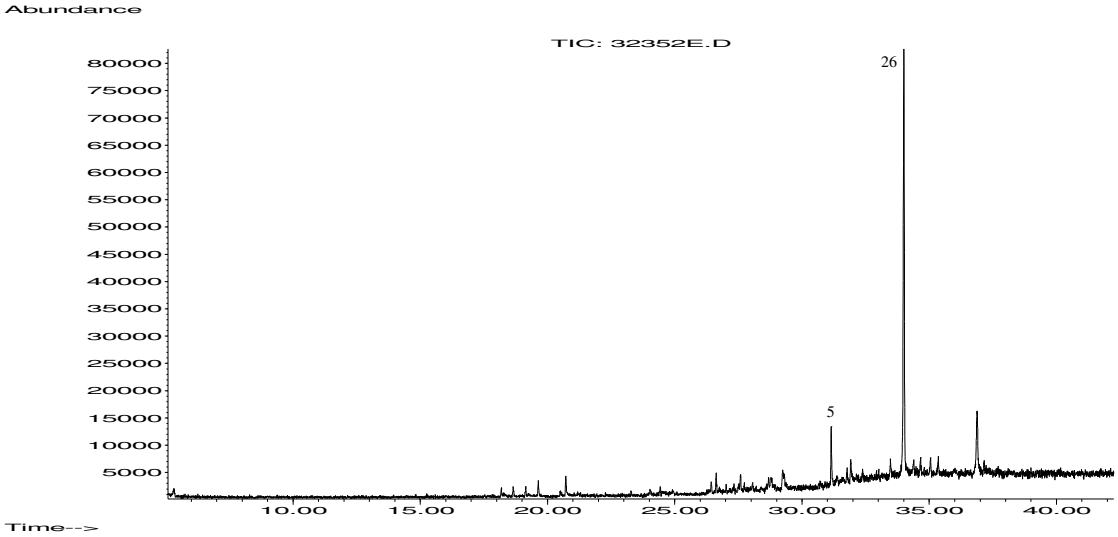


Figure 28. Total Ion Concentration of Fraction E of *Callitris glaucophylla* 32352.

32352 F

Table 21. Composition of Fraction F.

N°	Compound	Retention Time	CAS #	KI	calculated KI
35	BHT (butylated hydroxy toluene)	23.99	128-370	1515	1480
5	dihydrocolumellarin	31.33	66873-38-9	1900	1863

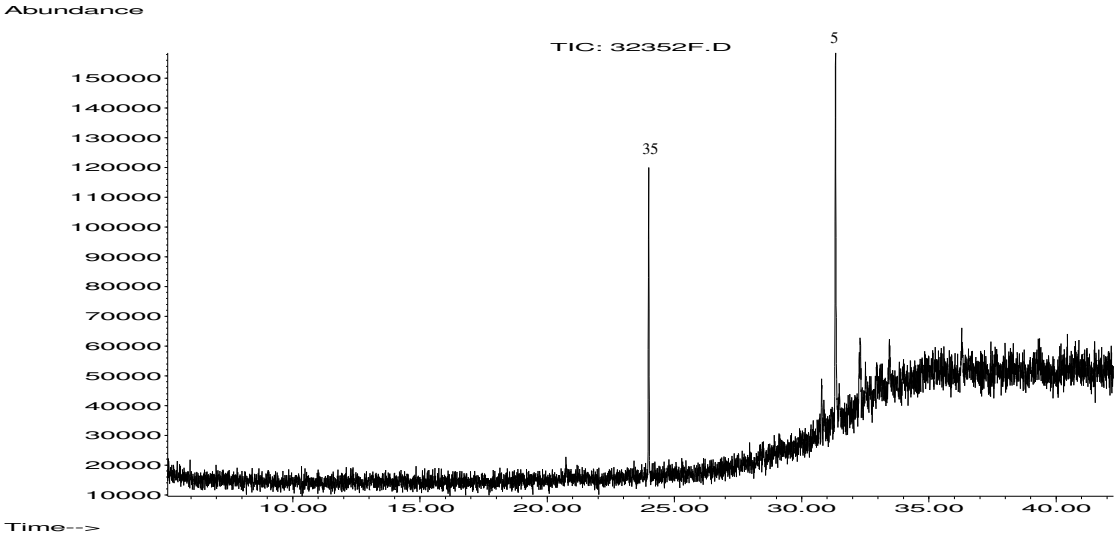


Figure 29. Total Ion Concentration of Fraction F of *Callitris glaucophylla* 32352.

32352 G

Table 22. Composition of Fraction G.

N°	Compound	Retention Time	CAS #	KI	calculated KI
35	BHT (butylated hydroxy toluene)	23.98	128-370	1515	1479
5	dihydrocolumellarin	30.70	66873-38-9	1900	1830

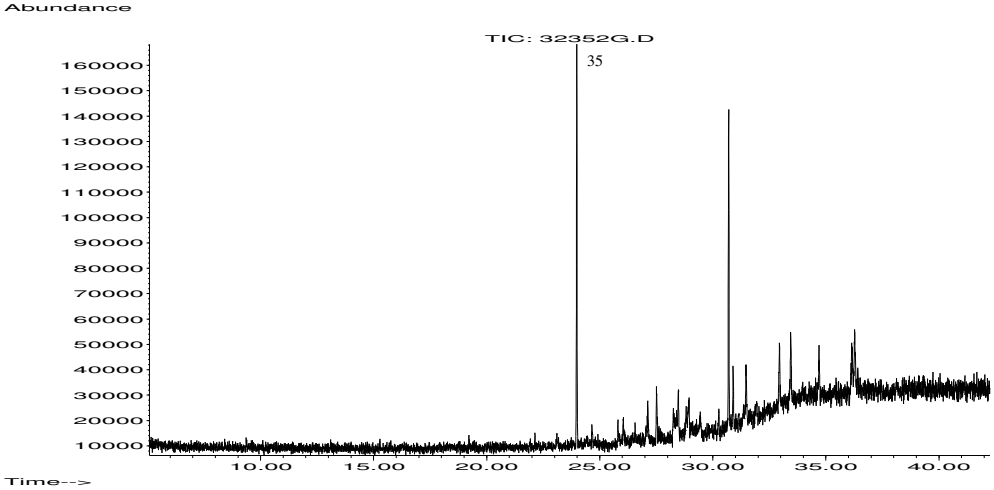


Figure 30. Total Ion Concentration of Fraction G of *Callitris glaucophylla* 32352.

32352 H

Table 23. Composition of Fraction H.

N°	Compound	Retention Time	CAS #	KI	calculated KI
35	BHT (butylated hydroxy toluene)	23.98	128-370	1515	1480

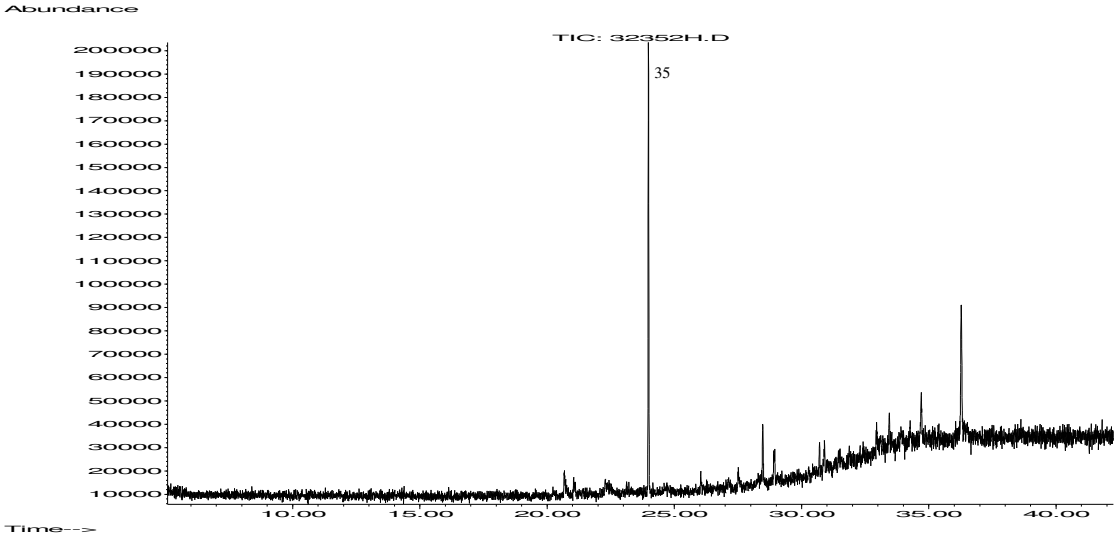


Figure 31. Total Ion Concentration of Fraction H of *Callitris glaucophylla* 32352.

32352 I, 32352 J, 32352 K, 32352 L, 32352 N

Fractions I, J, K, L and N did not show any volatile compounds in the GC/MS profile. The LC/MS profile of fraction J, K and L showed little UV response at a wavelength of 210 nm at retention times of approximately 15 min and 17 min. Fraction N showed no response at all.

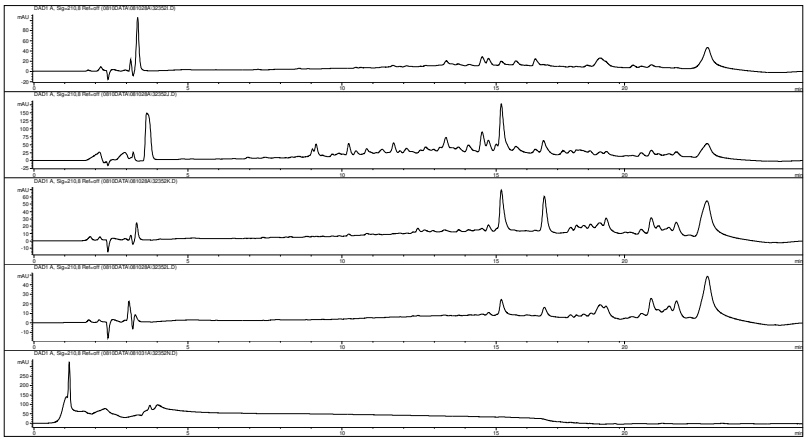


Figure 32. LC/MS profiles of fractions 32352 I, 32352 J, 32352 K, 32352 L, 32352 N at a wavelength of 210 nm.

32352 M

Table 24. Composition of Fraction M.

N°	Compound	Retention Time	CAS #	KI	calculated KI
36	methyl ricinoleate	35.13	141242	-	2061

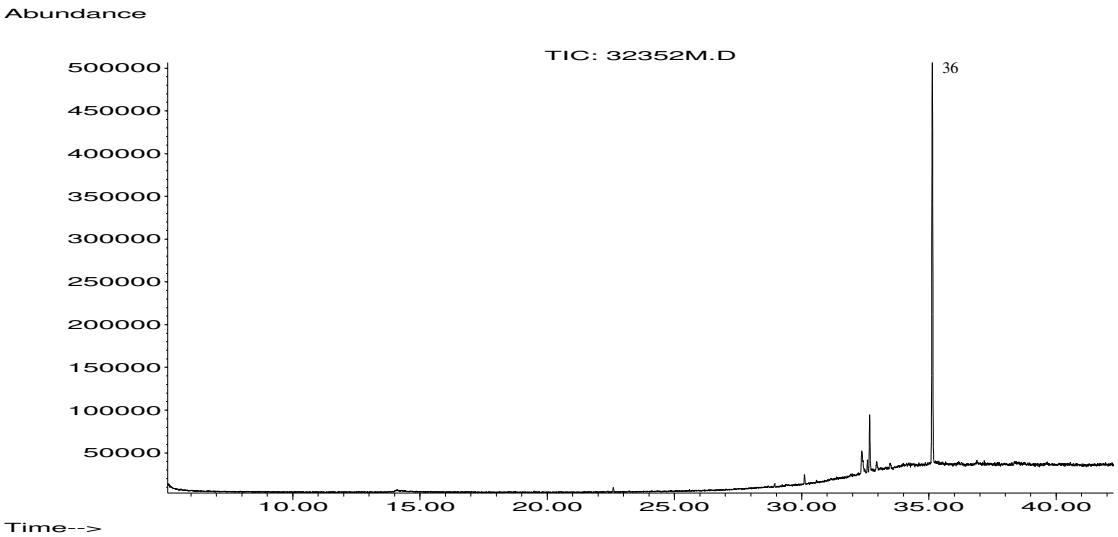


Figure 33. Total Ion Concentration of Fraction M of *Callitris glaucophylla* 32352.

4.2.2.2. Results of the pharmacological assays on 32352 fractions

4.2.2.2.1. ORAC results of 32352 fractions

The results of the antioxidant assay are shown in Table 25 and illustrated in Figure 34. Fractions D and F showed higher antioxidant activity than the crude oil (505.75µmolTE/g and 419.86µmolTE/g compared to 376.93µmolTE/g of the crude oil). The principal component of fraction D was the sesquiterpene lactone dihydrocolumellarin, which could mean that dihydrocolumellarin is responsible for the given activity. Its percentage was also high in fraction F, which also contained BHT (butylated hydroxy toluene). The presence of BHT in fractions of the *Callitris* oil was very doubtful. It is produced by alkylation reaction of *p*-cresol with isobutylene. BHT is a synthetic phenol, which acts as antioxidant in foods and cosmetics by being oxidized instead of the protected substance [24]. Fractions C, E

and G did not show differences in the antioxidant activity compared to the crude oil (318.30µmolTE/g, 350.60µmolTE/g and 381.48µmolTE/g compared to 376.93µmolTE/g). However, fraction G contained BHT and dihydrocolumellarin as well, but did not show as high values as fraction F. The low activity of fraction H was surprising, because of its high level of BHT it was expected to be very high. An explanation would have been, that BHT did not come from a contamination of the samples after being steam distilled, but from the GC/MS column. All other fractions were far under the trolox equivalent values of the crude oil.

Table 25. ORAC results of fractions of *Callitris glaucophylla* 32352.

sample	ORAC value µmolTE/g
32352 freshly distilled	376.93 ± 92.06
32352 A	29.55 ± 15.61
32352 B	62.95 ± 20.56
32352 C	318.30 ± 72.78
32352 D	505.75 ± 87.61
32352 E	350.60 ± 75.88
32352 F	419.86 ± 113.61
32352 G	381.48 ± 104.92
32352 H	70.57 ± 23.42
32352 I	114.45 ± 21.33
32352 J	75.85 ± 22.92
32352 K	28.65 ± 32.55
32352 L	13.85 ± 32.83
32352 M	22.20 ± 35.27
32352 N	21.14 ± 103.24

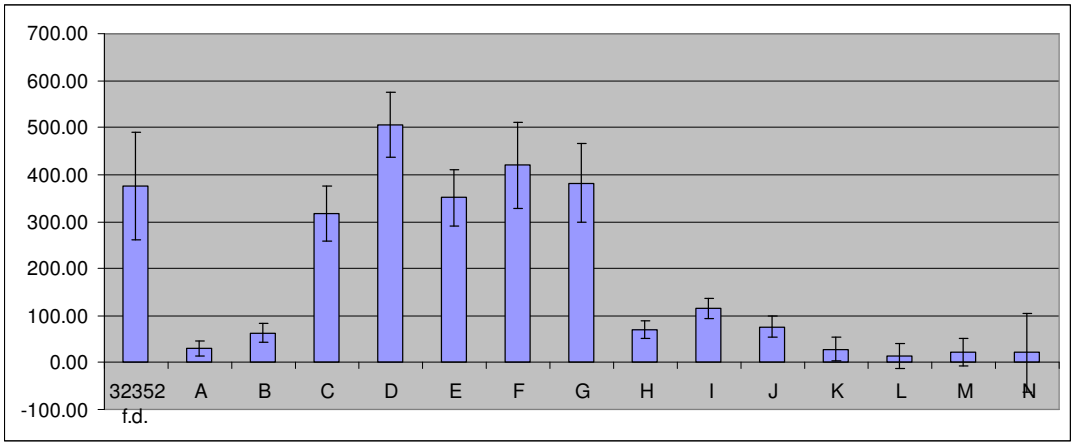


Figure 34. ORAC results (µmolTE/g) of fractions 32352.´

4.2.2.2.2. Cytotoxicity results of 32352 fractions

The cytotoxicity assays were done on two different cell lines. The percent inhibition values on P388D1 cells are shown in Table 26 and the values on HEP G-2 cells are shown in Table 27. The assay was repeated twice for all samples (Rep I and Rep II in Table 26 and 27). Samples got spilt and have been tested on two plates. The figures show the first plate with its control values, followed by the second plate with its curcumin and chlorambucil values below. The positive controls did not yield consistent results, so the data is doubtful and should just be evaluated as preliminary data. With great distance, fraction B was the most cytotoxic fraction, even in low concentrations. On P388D1 cells it showed still 94.21 percent inhibition at a concentration of 6 μg/mL, on HEP G-2 the activity was a little less with 83.75 percent inhibition at a concentration of 23.75 μg/mL. It showed also more activity than the crude oil, which did not show as much activity in low concentrations as fraction B (oil on P388D1 cells at a concentration of 6.15 μg/mL showed only 15.19 percent inhibition, see Table 12 in section 4.1.4.2.). None of the components (methyl myrtenate, β-selinene, methyl ester of γ-lactone, derivative of isopimarol and sandaracopimarinal) were reported as cytotoxic agents before. The reason why it was the most cytotoxic fraction could have also been a synergistic effect of all components. No other fraction contained as much major peaks and components as Fraction B. But this would have not explained why the fraction showed more activity than the crude oil itself. Fraction A also showed high cytotoxic activity on P388D1 cells (98.36 percent at a concentration of 50 μg/mL) but less activity on HEP G-2 cells (just 59.09 percent at a concentration of 100 μg/mL). On both cell lines, most of the other fractions only showed high activity in high concentrations. Fractions K, M and N did not show any effect on both cell lines, Fraction L showed no inhibition on HEP G-2 cells but little activity on P388D1 cells at its highest concentration (24.75 percent at concentration 206 μg/mL).

Table 26. Cytotoxicity results on P388D1 cells (% inhibition) of 32352 fractions.

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
Chlorambucil	600	79.84	65.98	72.91 ± 9.80
Curcumin	100	98.45	95.13	96.79 ± 2.35
	50	31.76	52.77	42.26 ± 14.86
	25	7.85	10.57	9.21 ± 1.92
32352 A	200	93.36	99.74	96.55 ± 4.51
	100	99.61	99.61	99.61 ± 0.00
	50	98.97	97.76	98.36 ± 0.85
	25	80.67	59.45	70.06 ± 15.01
32352 B	190	99.76	99.75	99.76 ± 0.01
	95	99.72	99.67	99.69 ± 0.04
	48	99.56	99.26	99.41 ± 0.22
	24	99.29	98.76	99.02 ± 0.37
	12	98.86	98.25	98.56 ± 0.43
	6	95.90	92.52	94.21 ± 2.39
	3	62.92	55.21	59.06 ± 5.45
32352 C	200	99.66	99.63	99.65 ± 0.02
	100	95.27	96.93	96.10 ± 1.17
	50	63.98	42.53	53.25 ± 15.17
32352 D	200	98.46	98.74	98.60 ± 0.20
	100	75.13	68.02	71.58 ± 5.03
32352 E	254	99.35	99.30	99.32 ± 0.03
	127	51.18	56.46	53.82 ± 3.74
32352 F	222	98.91	98.84	98.87 ± 0.05
	111	20.21	55.25	37.73 ± 24.77
32352 G	164	62.18	56.72	59.45 ± 3.86
	82	7.14	18.68	12.91 ± 8.16
32352 H	168	25.67	17.80	21.73 ± 5.56
	42	17.35	9.36	13.35 ± 5.65
	21	4.19	31.21	17.70 ± 19.10

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil		no inhibition		
curcumin	100	77.6303	87.3198	82.48 ± 6.85
	50	4.76525	3.05545	3.91 ± 1.21
32352 I	200	91.0701	93.757	92.41 ± 1.90
	100	30.3323	29.7116	30.02 ± 0.44
	50	14.0826	11.7102	12.90 ± 1.68
32352 J	200	25.6171	18.8044	22.21 ± 4.82
32352 K		no inhibition		
32352 L	206	20.7652	28.7271	24.75 ± 5.63
	26	28.5427	0.40789	14.48 ± 19.89
32352 M		no inhibition		
32352 N		no inhibition		

Table 27. Cytotoxicity results on HEP G-2 cells (% inhibition) of 32352 fractions.

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil		no inhibition		
curcumin	100	98.87	97.60	98.23 ± 0.90
	50	85.97	86.43	86.20 ± 0.32
	25	30.12	8.64	19.38 ± 15.19
32352 A	100	74.47	43.71	59.09 ± 21.75
	50	18.84	1.11	9.97 ± 12.53
32352 B	95	98.50	96.68	97.59 ± 1.29
	48	98.09	95.00	96.55 ± 2.18
	24	95.08	72.70	83.89 ± 15.82
	12	40.62	26.66	33.64 ± 9.87
32352 C	100	98.52	98.25	98.39 ± 0.19
	50	71.26	65.42	68.34 ± 4.13
	25	25.90	21.06	23.48 ± 3.43
	13	6.63	8.37	7.50 ± 1.23
32352 D	100	98.53	98.59	98.56 ± 0.04
	50	41.65	39.00	40.32 ± 1.87
	25	22.64	9.00	15.82 ± 9.65
	13	17.21	2.43	9.82 ± 10.45
	2	12.10	15.08	13.59 ± 2.11
32352 E	127	98.39	98.25	98.32 ± 0.10
	64	37.20	33.30	35.25 ± 2.76
	32	23.37	16.19	19.78 ± 5.08
	16	3.57	12.23	7.90 ± 6.12
	8	10.19	5.96	8.08 ± 2.99
	4	2.56	11.18	6.87 ± 6.09
32352 F	111	104.56	92.85	98.71 ± 8.28
	56	25.34	47.31	36.32 ± 15.54
	28	26.75	8.23	17.49 ± 13.10
	14	18.77	6.67	12.72 ± 8.56
32352 G	82	68.49	68.73	68.61 ± 0.17
	41	20.96	19.22	20.09 ± 1.23
	21	21.64	16.18	18.91 ± 3.86
	10	13.11	2.92	8.02 ± 7.21
	5	4.82	0.51	2.66 ± 3.05
32352 H	84	16.15	5.43	10.79 ± 7.58
	21	4.70	12.72	8.71 ± 5.67
	11	3.70	4.02	3.86 ± 0.23
32352 I	100	33.74	18.21	25.98 ± 10.98
	50	13.72	14.21	13.97 ± 0.34
32352 J	100	11.52	4.19	7.85 ± 5.18

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil		no inhibition		
curcumin	100	97.71	98.21	97.96 ± 0.36
	50	73.61	75.37	74.49 ± 1.25
32352 K		no inhibition		
32352 L		no inhibition		
32352 M		no inhibition		
32352 N		no inhibition		

High yielding fractions C, D, E and F were further fractionated using preparative HPLC. Eighty fractions have been collected.

4.2.3. Preparative HPLC of subfraction 32352 C

Preparative HPLC was done using method Ruprep1. The chromatographic profile obtained from analytical HPLC (method: Ruth3) is shown in Figure 35 below, followed by the UV data of selected fractions. The selected fractions showed single peaks at wavelengths 210 and 280 nm. They were all tested for pharmacological activity as shown in section 4.2.7.

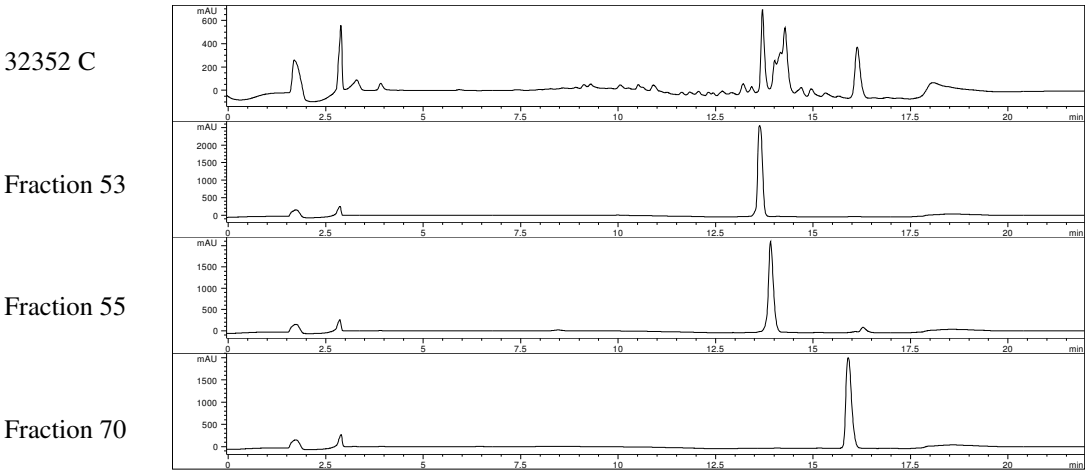


Figure 35. UV response at wavelength 210 nm (analytical HPLC) of 32352 C and selected fractions.

4.2.4. Preparative HPLC of subfraction 32352 D

Preparative HPLC was done using method Ruprep1. The chromatographic profile obtained from analytical HPLC (method: Ruth3) is shown in Figure 36, followed by the UV data of selected fractions. The selected fractions showed single peaks at wavelengths 210 and 280 nm. They were all tested for pharmacological activity as shown in section 4.2.7.

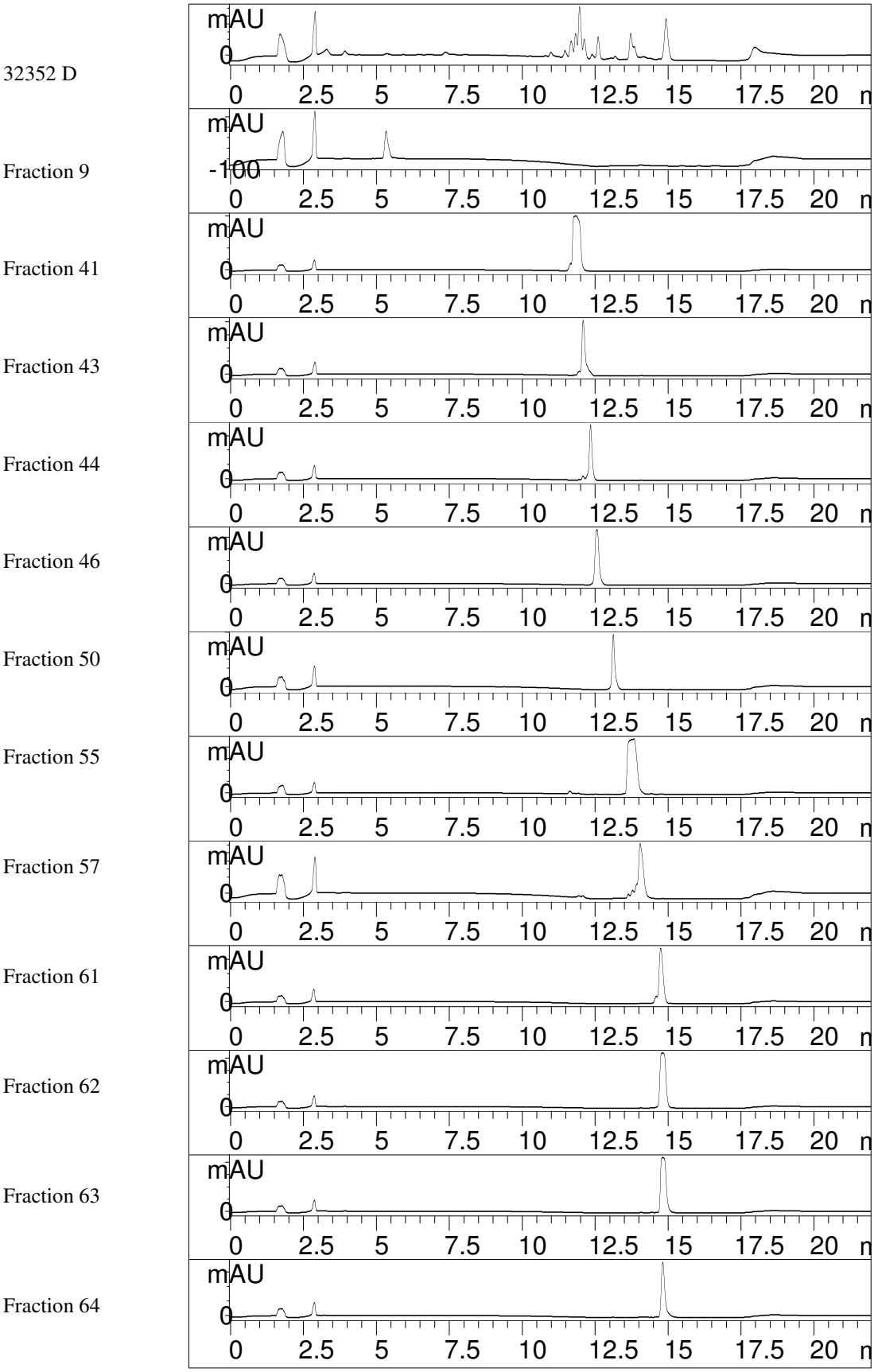


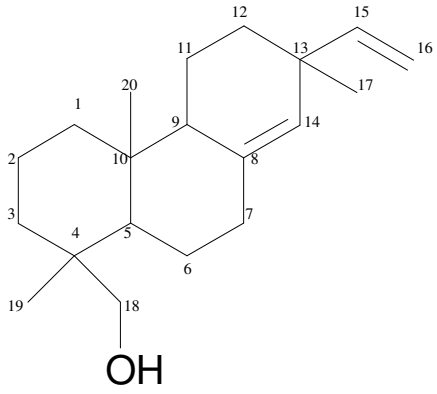
Figure 36. UV response at wavelength 210 nm (analytical HPLC) of 32352 D and selected fractions.

4.2.4.1. Isolation of pure compounds

4.2.4.1.1. Isolation of sandaracopimarinol

Sandaracopimarinol has been isolated from Fraction D 63. Fractions D 62 and D 64 turned out to be sandaracopimarinol too after the investigation on the NMR. The ^{1}H and ^{13}C spectra values of Fraction D 63 have been compared with literature values and did not show big differences.

CAS - Number:	24563-84-6
molecular formula:	$C_{20}H_{32}O$
molecular weight:	288.472
physical description:	crystal
melting point:	63 – 65°



(37)

Figure 37. Chemical structure of sandaracopimarinol **(37)**, Fraction D 63.

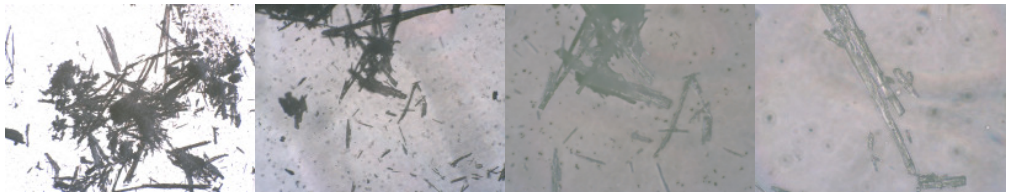


Figure 38. Crystals of sandaracopimarinal under miscroscope (magnifications: 40, 100, 200, 400).

Table 28. ^{1}H and ^{13}C-NMR spectral data of sandaracopimarinol **(37)**.

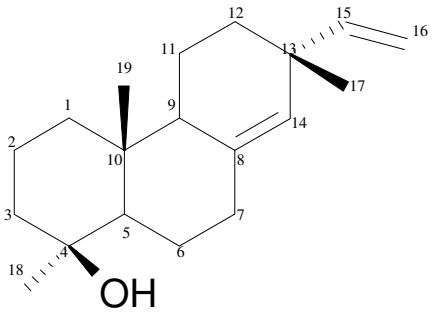
C/H Position	Chemical Shift, ppm ^{1}H	Chemical Shift, ppm ^{13}C	Literature values, ppm [25] ^{1}H	Literature values, ppm [25] ^{13}C
1	1.55 - 1.40[a], m 1.40 - 1.25[b], m	35.70		38.88
2	1.70 - 1.55[c], m 1.55 - 1.40[a], m	19.00		18.32
3	1.73, br 1.04, m	39.10		35.72
4	-	38.90		37.37
5	1.40 - 1.25[b], m	48.10		47.87
6	1.55 - 1.40[a], m 1.40 - 1.25[b], m	22.60		22.38
7	2.24, m 2.08, m	35.90		35.44
8	-	137.20		136.98
9	1.76, m	50.80		50.53
10	-	38.40		38.12
11	1.70 - 1.55[c], m 1.55 - 1.40[a], m	18.60		18.78
12	2.08, m 1.55 - 1.40[a], m	34.80		34.54
13	-	37.60		37.76
14	5.21, s	128.90	5.19, s	128.68
15	5.78, dd (17.45, 10.60)	149.30	5.75, dd (17.50, 10.60)	149.09
16	4.91, d (17.45) 4.87, d (10.60)	110.20	4.88, dd (17.50, 1.3 4.85, dd (12.00, 1.3)	109.66
17	1.04, s (3H)	26.20	1.02, s (3H)	25.94
18	3.39, d (10.90) 3.12, d (10.90)	72.40	3.37, d (10.90) 3.10, d (10.90)	72.23
19	0.84, s (3H)	18.10	0.78, s (3H)	17.92
20	0.81, s (3H)	15.80	0.82, s (3H)	15.58

[a, b, c]…. overlapping peaks

4.2.4.1.2. Isolation of 18-Nor-8(14),15-pimaradien-4-ol

18-Nor-8(14),15-pimaradien-4-ol has been isolated from Fraction D 61. The values of the 13C spectra showed comparable numbers to the literature, except an inconsistency at carbon 18.

CAS:	35930-17-7
molecular formula:	$C_{19}H_{30}O$
molecular weight:	274.445
physical description:	crystal
melting point:	119 – 121°



(38)

Figure 39. Chemical structure of 18-Nor-8(14),15-pimaradien-4-ol (**38**), Fraction D 61.

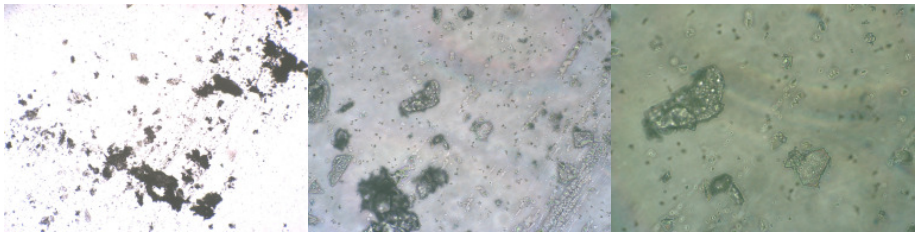


Figure 40. Crystals of 18-Nor-8(14),15-pimaradien-4-ol under microscope (magnifications: 40, 200, 400).

Table 29. ^{1}H and ^{13}C-NMR spectral data of 18-Nor-8(14),15-pimaradien-4-ol (**38**).

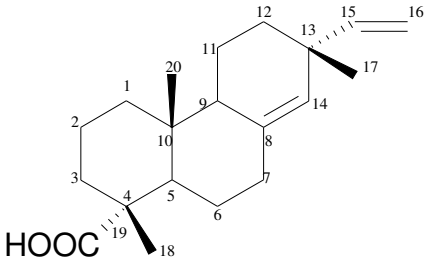
C/H Position	Chemical Shift, ppm ^{1}H	^{13}C	Literature values, ppm [26] ^{13}C
1	1.80 - 1.67[a], m 1.05, m	39.10	38.60
2	1.80 - 1.67[a], m 1.64 - 1.57[b], m 1.57 - 1.52[c], m	18.90	18.90
3	1.80 - 1.67[a], m 1.42 - 1.32[d], m	41.30	42.90
4	1.19, s (3H)	72.30	72.40
5	1.12, dd (12.6, 2.7)	53.40	56.40
6	1.80 - 1.67[a], m 1.5 - 1.42[e], m	21.60	21.60
7	2.31, m (14.5, 3.7, 1.8) 2.09, m	35.70	35.60
8	-	137.00	136.60
9	1.80 - 1.67[a], m	50.00	50.30
10	-	38.30	39.00
11	1.80 - 1.67[a], m 1.64 - 1.57[b], m 1.57 - 1.52[c], m	18.40	20.30
12	1.5 - 1.42[e], m 1.42 - 1.32[d], m	34.80	34.50
13	-	37.60	37.40
14	5.25, br s	129.20	129.10
15	5.79, dd (10.6, 17.4)	149.30	148.90
16	4.92, d (17.4) 4.88, d (10.6)	110.20	110.10
17	1.05, s (3H)	26.10	26.00
18	1.19, s (3H)	31.30	23.50
19	0.97, s (3H)	14.80	14.50

[a, b, c, d, e] … overlapping peaks

4.2.4.1.3. Isolation of sandaracopimaric acid

Sandaracopimaric acid has been isolated from Fraction D 55.

CAS:	471-74-9
molecular formula:	$C_{20}H_{30}O_2$
molecular weight:	302.456
physical description:	crystal
melting point:	171 – 173°



(39)

Figure 41. Chemical structure of sandaracopimaric acid (**39**), Fraction D 55.

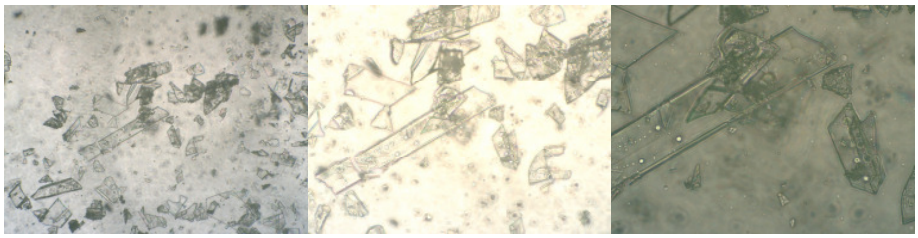


Figure 42. Crystals of sandaracopimaric acid under microscope (magnifications: 100, 200, 400).

Table 30. ^{1}H and ^{13}C-NMR spectral data of sandaracopimaric acid **(39)**.

C/H Position	Chemical Shift, ppm ^{1}H	^{13}C
1	1.83 - 1.75[a], m 1.15, m	38.50
2	1.65 - 1.53[b], m	18.40
3	1.83 - 1.75[a], m 1.66, m	37.30
4	-	47.50
5	1.93, dd (2.55, 12.45)	49.10
6	1.53 - 1.43[c], m 1.28, m	25.20
7	2.23, m (1.90, 4.60, 14.20) 2.14, m	35.70
8	-	136.90
9	1.83 - 1.75[a], m	50.80
10	-	37.60
11	1.65 - 1.53[b], m	18.80
12	1.53 - 1.43[c], m 1.39, dd (3.05, 11.65)	34.70
13	-	38.00
14	5.23, br s	129.40
15	5.78, dd (10.60, 17.40)	149.10
16	4.92, d (17.40) 4.89, d (10.60)	110.40
17	1.05, s (3H)	26.30
18	1.22, s (3H)	17.00
19	-	184.80
20	0.85, s (3H)	15.50

[a, b, c] … overlapping peaks

4.2.5. Preparative HPLC of subfraction 32352 E

Preparative HPLC was done using method Ruprep1. The chromatographic profile obtained from analytical HPLC (method: Ruth3) is shown in Figure 43 below, followed by the UV data of selected fractions. The selected fractions showed single peaks at wavelengths 210 and 280 nm. They were all tested for pharmacological activity as shown in section 4.2.7.

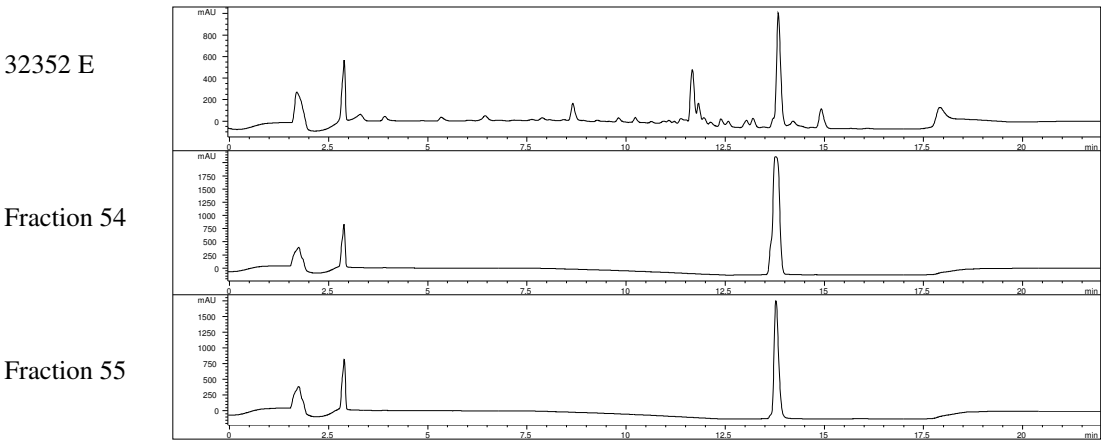
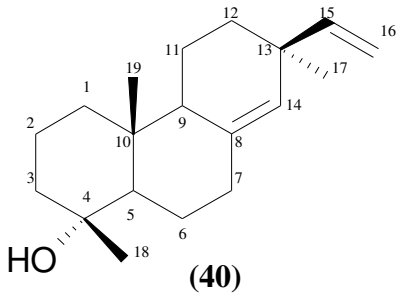


Figure 43. UV response at wavelength 210 nm (analytical HPLC) of 32352 E and selected fractions.

4.2.5.1. Isolation of pure compounds

4.2.5.1.1. Isolation of an isomer of 18-Nor-8(14),15-pimaradien-4-ol

An isomer of 18-Nor-8(14),15-pimaradien-4-ol **(40)** has been isolated from Fraction E 54. Fraction E 55 turned out to be this isomer too. The molecular formula and the molecular weight of Fraction E 55 are the same than 18-Nor-8(14),15-pimaradien-4-ol, there is just a difference in the stereochemistry at carbon 13.



(40)

Figure 44. Chemical structure of an isomer of 18-Nor-8(14),15-pimaradien-4-ol **(40)**, Fraction E 54.

Table 31. ^{1}H and ^{13}C-NMR spectral data of an isomer of 18-Nor-8(14),15-pimaradien-4-ol **(40)**.

C/H Position	Chemical Shift, ppm 1H	13C
1	1.72, br (13.15) 1.1, m	38.90
2	1.66 - 1.61[a], m 1.40 - 1.29[b], m	20.60
3	1.81 - 1.77[c], m 1.40 - 1.29[b], m	43.20
4	-	72.70
5	1.40 - 1.29[b], m	56.70
6	1.81 - 1.77[c], m 1.40 - 1.29[b], m	21.90
7	2.31, br (13.5) 2.09, m	35.80
8	-	136.80
9	1.81 - 1.77[c], m	50.60
10	-	39.30
11	1.57 - 1.52[d], m 1.50 - 1.44[e], m	19.10
12	1.50 - 1.44[e], m 1.40 - 1.29[b], m	34.80
13	-	37.60
14	5.25, s	129.40
15	5.78, dd (10.60, 17.45)	149.10
16	4.91, d (17.45) 4.89, d (10.6)	110.40
17	1.05, s	26.30
18	1.17, m (3H)	23.80
19	0.78, s	14.80

[a, b, c, d, e] …. overlapping peaks

4.2.6. Preparative HPLC of subfraction 32352 F

Preparative HPLC was done using method Ruprep1. The chromatographic profile obtained from analytical HPLC (method: Ruth3) is shown in Figure 45 below, followed by the UV data of selected fractions. The selected fractions showed single peaks at wavelengths 210 and 280 nm. They were all tested for pharmacological activity as shown in section 4.2.7.

32352 F

Fraction 48

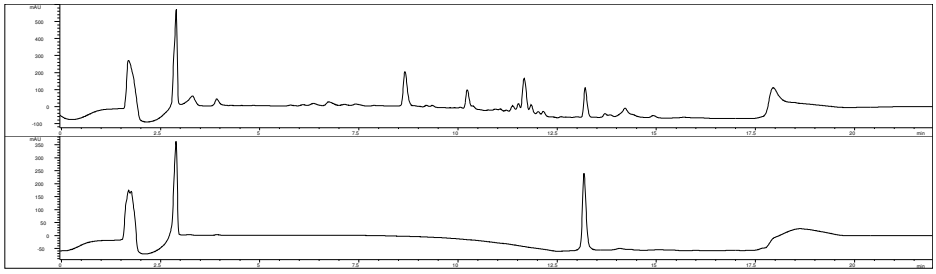


Figure 45. UV response at wavelength 210 nm (analytical HPLC) of 32352 C and selected fractions.

4.2.7. Results of the pharmacological assays on selected fractions of subfractions obtained from *Callitris glaucophylla* 32352

4.2.7.1. ORAC results of selected fractions of subfractions obtained from *Callitris glaucophylla* 32352

The results of the antioxidant assay on the selected fractions are shown in Table 32 and illustrated in Figure 46. Fraction D41 showed the highest antioxidant activity with 5566.94 μmolTE/g, even if the standard deviation was very high. The GC/MS profile showed just one peak in this fraction and was identified as dihydrocolumellarin. This sesquiterpenelactone was also found in accompany of γ-costol in Fraction 43, but its ORAC score was must lower (880.94 μmolTE/g, with a high standard deviation as well). Fraction D50 had a trolox equivalent value of

998.03 µmol/g, but no component could be found out. Several peaks came out between retention times 26.39 – 34.56. An unknown compound with the molecular mass of 284 was responsible for the high activity (981.17 µmolTE/g) in Fraction C 53. The principal component of Fraction D44 was 10-epi-γ-eudesmol and showed an activity of 502.39 µmolTE/g. A methyl ester of a γ-lactone with the m/z 248 in Fraction C55 showed high antioxidant activity as well. The isolated compounds of Fractions D64, D63 and D 61, sandaracopimarinal, 18-Nor-8(14),15-pimaradien-4-ol and sandaracopimaric acid, all showed activity with values 156.39 µmolTE/g, 145.47 µmolTE/g and 137.88 µmolTE/g. Fractions D62 until D64 which turned out to be the same compound, showed comparable values. The isomer of D61, Fractions E54 and E55, also showed similar activity. The lowest values came from Fraction D9, with an unknown principal component at retention time 19.63 with m/z 150 (43.32 µmolTE/g) and Fraction F48, containing six major peaks, from which 3 could be identified as columellarin, BHT and an unknown molecular weight of 206 (52.36 µmolTE/g).

Table 32. ORAC results of selected fractions of all subfractions obtained from *Callitris glaucophylla* 32352.

sample	ORAC value umolTE/g		
C 53	981.17	±	354.59
C 55	330.33	±	85.52
C 70	101.95	±	98.26
D 9	43.32	±	14.50
D 41	5566.94	±	1802.36
D 43	880.94	±	587.05
D 44	502.39	±	151.61
D 46	498.59	±	429.94
D 50	998.03	±	120.24
D 55	99.47	±	5.30
D 57	277.04	±	72.01
D 61	137.88	±	68.17
D 62	194.56	±	110.55
D 63	145.47	±	27.23
D 64	156.39	±	30.39
E 54	80.18	±	39.88
E 55	121.68	±	29.27
F 48	52.36	±	51.92

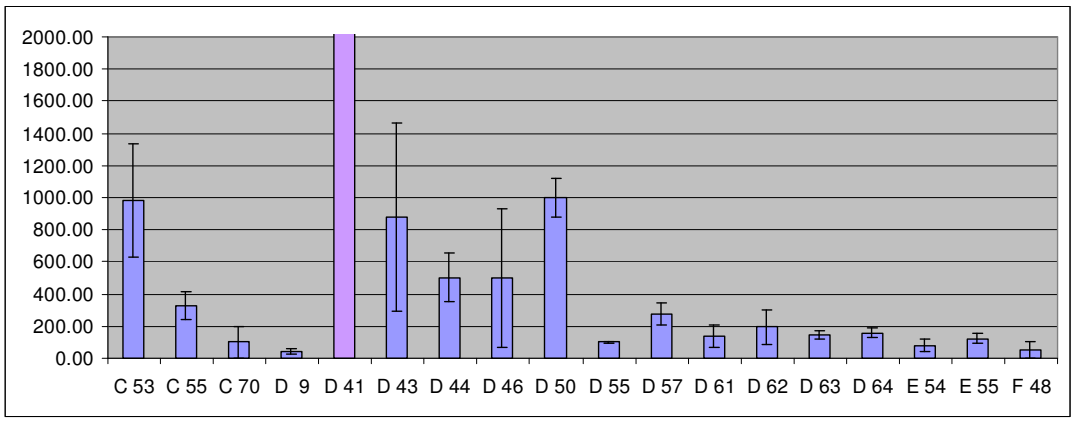


Figure 46. ORAC results (µmolTE/g) of selected fractions of 32352 C, 32352 D, 32352 E, 32352 F.

4.3. *Callitris glaucophylla* 31973

4.3.1. Overview

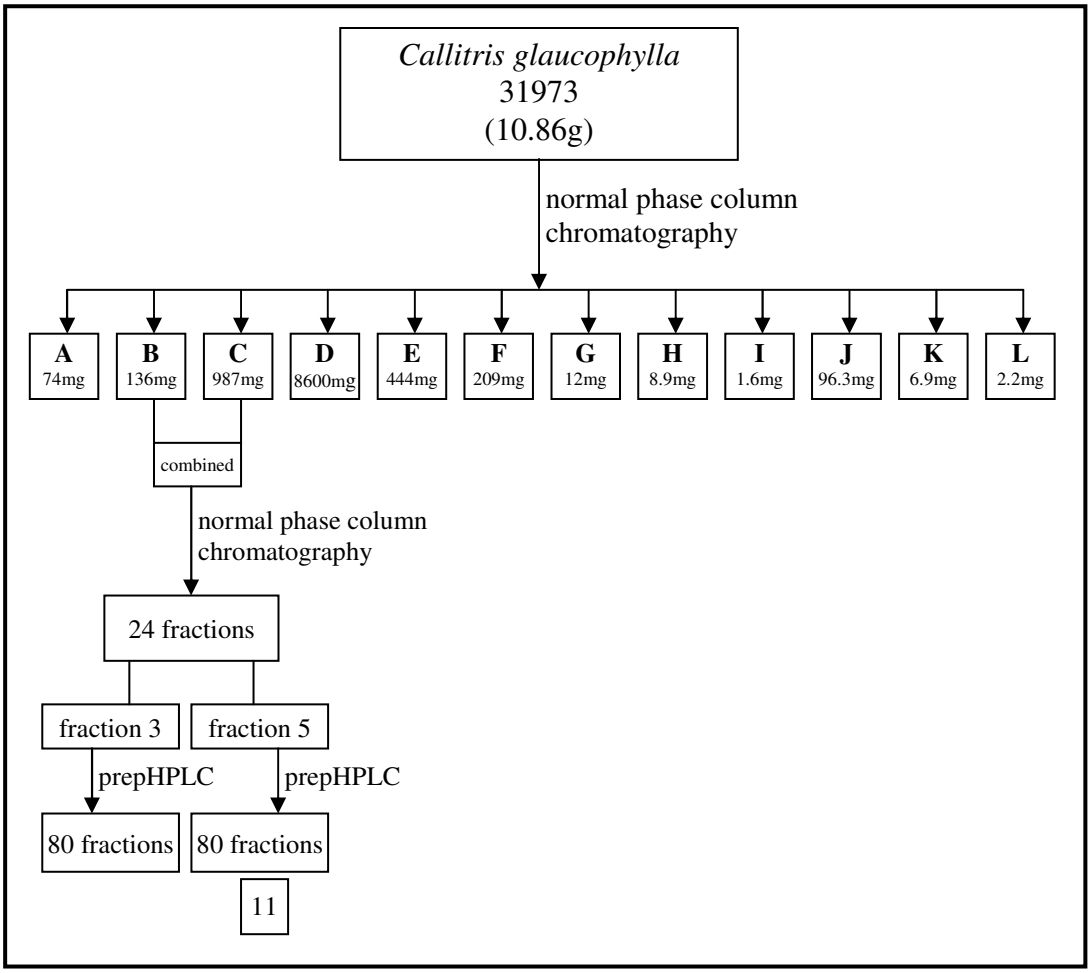


Figure 47. Schematic diagram for the isolation of compounds from *Callitris glaucophylla* 31973.

4.3.2. Column fractionation of 31973

The column had a diameter of 4.2 cm and a length of 55 cm. Column chromatography has been done using silica as stationary phase. The yield of the *Callitris glaucophylla* 31973 oil was 10.86 g. For getting a 5 – 10% loading, 250 g silica was used. Solvents used were hexane, diethylether, ethyl acetate and methanol, used in ascending order of polarity. A detailed description of the fractionation design is shown in Table 33.

Table 33. Fractionation scheme of *Callitris glaucophylla* 31973.

Fraction	**mobile phase**	**volume (ml)**	**yield (mg)**
A	100% hexane	500	74
B	90% hexane / 10% diethylether	500	136
C	90% hexane / 10% diethylether	500	987
D	50% hexane / 50% diethylether	500	8600
E	50% hexane / 50% diethylether	500	444
F	100% diethylether	500	209
G	100% diethylether	500	12
H	50% diethylether / 50% ethylacetate	500	8.9
I	100% ethylacetate	500	1.6
J	50% ethylacetate / 50% methanol	500	96.3
K	100% methanol	500	6.9
L	100% methanol	500	2.2

4.3.2.1. Investigation of 31973 fractions using GC/MS

31973 A

Table 34. Composition of Fraction A.

N°	Compound	Retention Time	CAS #	KI	calculated KI
41	α-guaiene	23.56	3691-12-1	1439	1457
42	6,9-guaiadiene	24.32	37839-64-8	1444	1503
20	β-selinene	24.82	17066-67-0	1490	1534
21	α-selinene	24.87	473-13-2	1498	1537
28	unknown, mw 258	30.98	-	-	1819
28	unknown, mw 258	31.59	-	-	1850
43	sandaracopimara-8(14),15-diene	32.07	1686-56-2	1968	1977

6,9-guaiadiene has not been reported before in a *Callitris* oil.

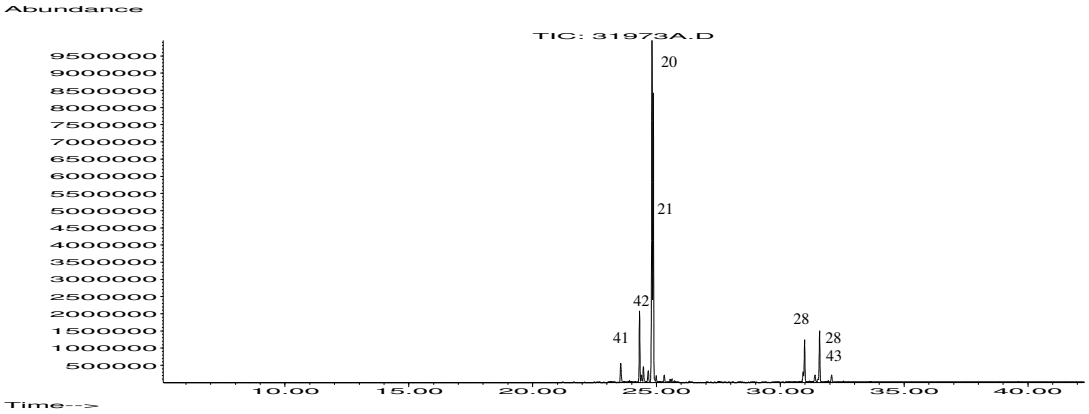


Figure 48. Total Ion Concentration of Fraction A of *Callitris glaucophylla* 31973.

31973 B

Table 35. Composition of Fraction B.

N^{o}	Compound	Retention Time	CAS #	KI	calculated KI
44	methyl geranate	21.05	2349-14-6	1324	1303
17	limonene	22.67	138-86-3	1029	1403
32	γ-gurjunene	24.47	22567-17-5	1477	1512
20	β-selinene	24.80	17066-67-0	1490	1533
21	α-selinene	24.85	473-13-2	1498	1536
23	methyl ester of a γ-lactone	28.93	-	-	1785
28	unknown (mw 258)	30.98	-	-	1910
25	unknown (mw 256)	31.39	-	-	1935
28	unknown (mw 258)	31.58	-	-	1948

The identity of the peaks at retention time 22.67 (limonene) and 24.47 (γ-gurjunene) is doubtful. Whilst the fragmentation pattern would fit to limonene, it was uncommon that it came out after methyl geranate. That would mean that the monoterpene methylester methyl geranate moves faster through the column than the monoterpene limonene. The fragmentation pattern of γ-gurjunene did not fit exactly to the peak at retention time 24.47, but the calculated KI did not allow any other identification. γ-Gurjunene has not been reported before in a *Callitris* oil.

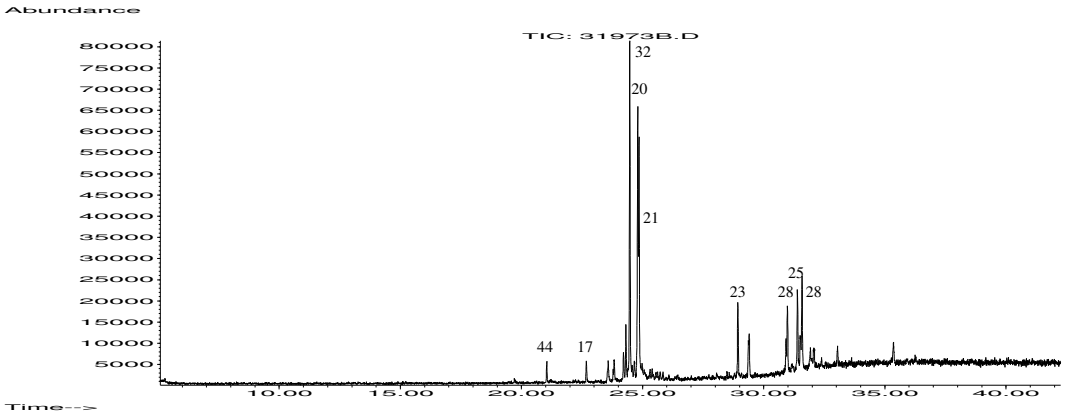


Figure 49. Total Ion Concentration of Fraction B of *Callitris glaucophylla* 31973.

31973 C

Table 36. Composition of Fraction C.

N°	Compound	Retention Time	CAS #	KI	calculated KI
44	methyl geranate	21.04	2349-14-6	1324	1303
19	methyl myrtenate	23.83	30-649-97-9	1294	1473
20, 21	β- and α-selinene	24.81	17066-67-0, 473-13-2	1490, 1498	1533
45	amorpha-4,9-dien-14-al	28.06	394251-65-1	1707	1732
23	methyl ester of a γ-lactone	28.93	-	-	1785
4	columellarin	31.92	66873-37-8	1953	1968
27	sandaracopimarinal	35.35	3855-14-9	2184	1935

Amorpha-4,9-dien-14-al has not been previously reported in a *Callitris* oil.

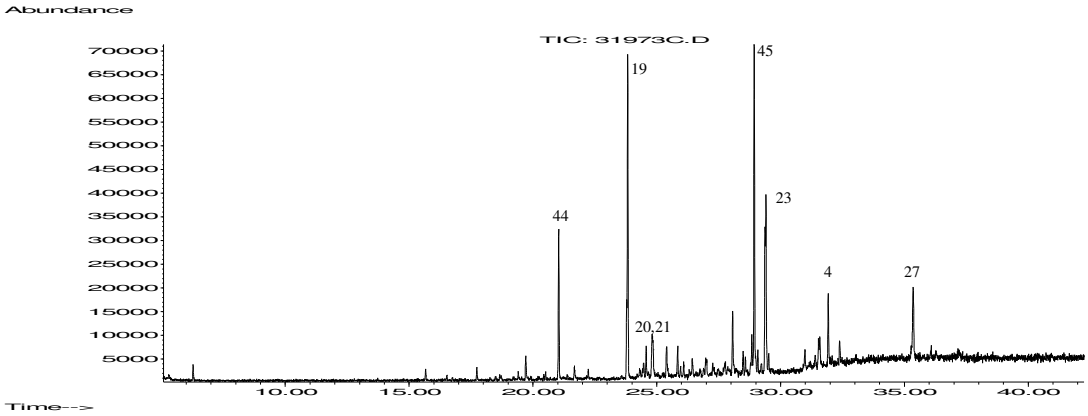


Figure 50. Total Ion Concentration of Fraction C of *Callitris glaucophylla* 31973.

31973 D

Table 37. Composition of Fraction D.

N°	Compound	Retention Time	CAS #	KI	calculated KI
10	guaiol	26.32	489-86-1	1600	1601
11	10-epi-γ-eudesmol	27.02	15951-81-7	1623	1638
13	β-eudesmol	27.49	473-15-4	1650	1662
14	α-eudesmol	27.54	473-16-5	1653	1665
5	dihydrocolumellarin	31.15	66873-38-9	1900	1853
2	callitrisin	31.92	66964-62-3	1942	1893
4	columellarin	31.83	66873-37-8	1953	1889
3	dihydrocallitrisin	32.39	72523-74-1	1969	1918

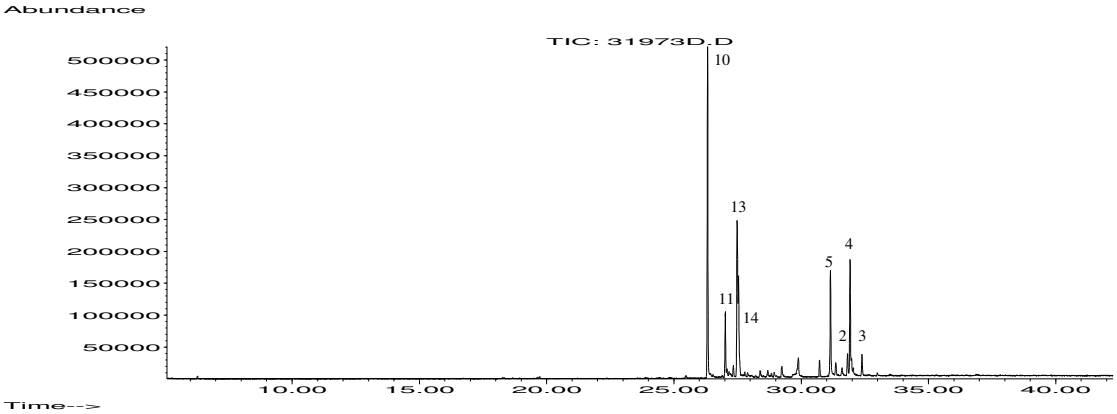


Figure 51. Total Ion Concentration of Fraction D of *Callitris glaucophylla* 31973.

31973 E

Table 38. Composition of Fraction E.

N°	Compound	Retention Time	CAS #	KI	calculated KI
46	uk, mw 168	23.28	-	-	1443
35	BHT (butylated hydroxy toluene)	24.43	128-370	1515	1503
15	bulnesol	27.54	22451-73-6	1671	1665
47	uk, 177	27.90	-	-	1684
28	uk, 258	34.01	-	-	2002

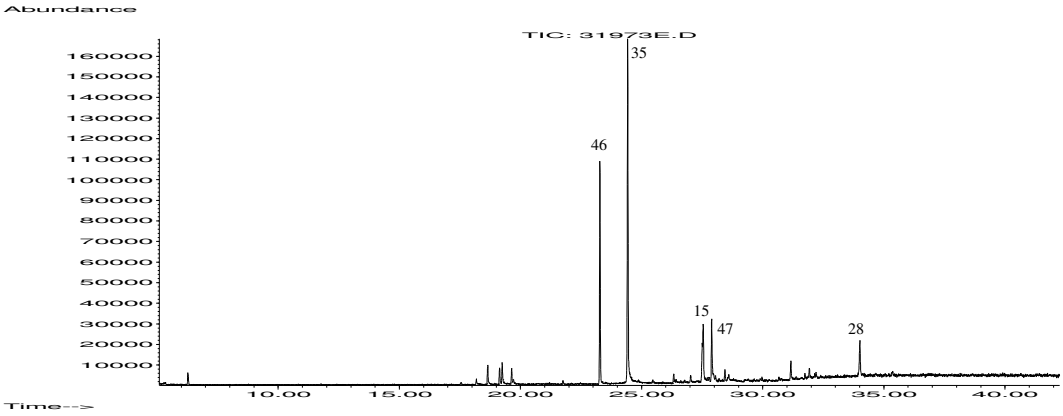


Figure 52. Total Ion Concentration of Fraction E of *Callitris glaucophylla* 31973.

31973 F

Table 39. Composition of Fraction F.

N°	Compound	Retention Time	CAS #	KI	calculated KI
9	citronellic acid	21.15	502-47-6	1313	1332
35	BHT (butylated hydroxy toluene)	24.01	128-370	1515	1481
48	davanone	26.44	20482-11-5	1587	1608
49	uk, mw 274	31.50	-	-	1872

A reference standard verified the identity of citronellic acid and BHT, as well as the calculated KI. Davanone fitted to the fragmentation pattern in [19] and the KI was within the expected range. Davanone was never reported before in a *Callitris* oil.

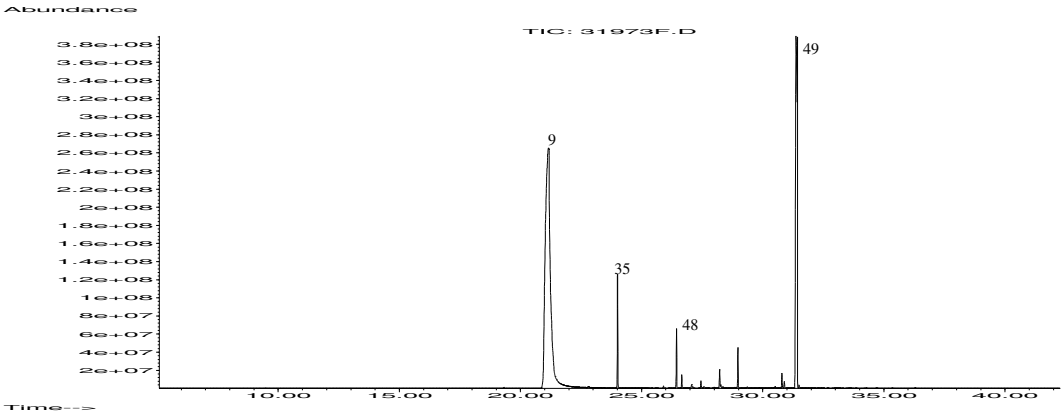


Figure 53. Total Ion Concentration of Fraction F of *Callitris glaucophylla* 31973.

31973 G

Table 40. Composition of Fraction G.

N°	Compound	Retention Time	CAS #	KI	calculated KI
35	BHT (butylated hydroxy toluene)	24.43	128-370	1515	1503
50	uk, mw 164	29.41	-	-	1763
27	sandaracopimarinal	35.35	3855-14-9	2184	2072

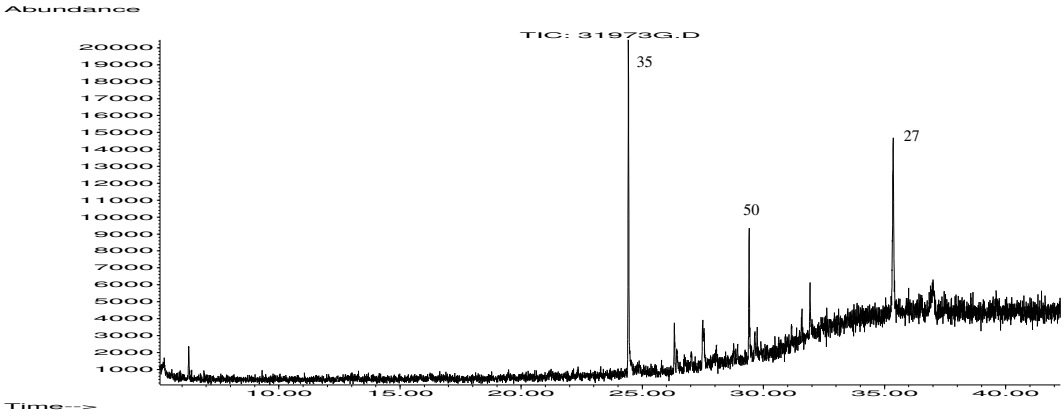


Figure 54. Total Ion Concentration of Fraction G of *Callitris glaucophylla* 31973.

31973 H

Table 41. Composition of Fraction H.

N°	Compound	Retention Time	CAS #	KI	calculated KI
35	BHT (butylated hydroxy toluene)	23.99	128-370	1515	1483
10	guaiol	25.87	489-86-1	1600	1598
11	10-epi-γ-eudesmol	26.57	15051-81-7	1623	1641
13	β-eudesmol	27.04	473-15-4	1650	1669
14	α-eudesmol	27.08	473-16-5	1653	1672
22	γ-costol	28.81	65018-14-6	1746	1777
5	dihydrocolumellarin	31.27	66873-38-9	1900	1928
51	uk, 149	36.29	-	-	2235

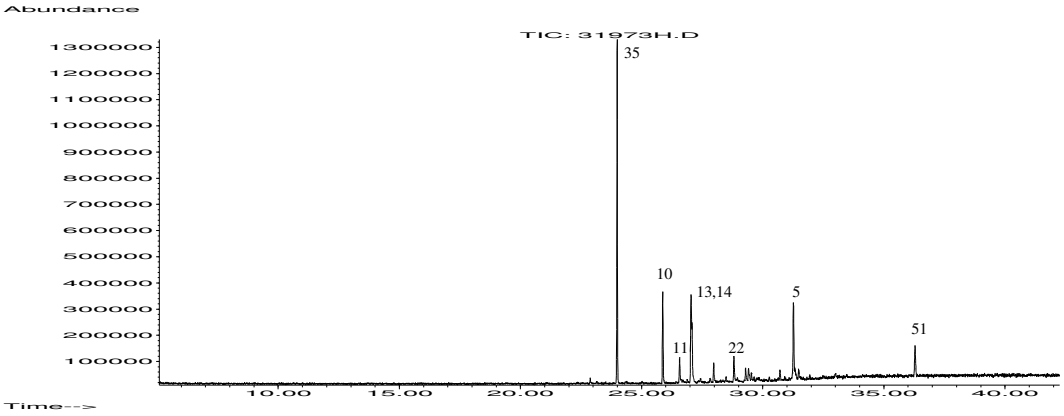


Figure 55. Total Ion Concentration of Fraction H of *Callitris glaucophylla* 31973.

31973 I

Table 42. Composition of Fraction I.

N^{o}	Compound	Retention Time	CAS #	KI	calculated KI
52	δ-selinene	23.90	28624-28-4	1492	1475
35	BHT (butylated hydroxy toluene)	24.42	128-370	1515	1502
12	γ-eudesmol	26.44	1209-71-8	1632	1608
31	guaiazulene	29.31	489-84-9	1700	1757
26	uk, 207	31.93	-	-	1894

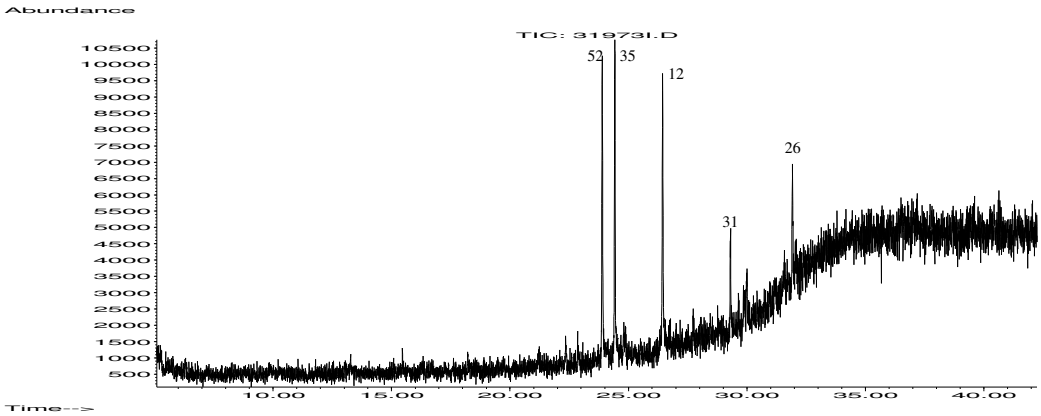


Figure 56. Total Ion Concentration of Fraction I of *Callitris glaucophylla* 31973.

31973 J

Table 43. Composition of Fraction J.

N°	Compound	Retention Time	CAS #	KI	calculated KI
53	cryptomeridiol	29.54	4666-84-6	1813	1813
5	dihydrocolumellarin	30.92	66873-38-9	1900	1902
4	columellarin	31.48	66873-37-8	1953	1941

Cryptomeridiol was never reported before in a *Callitris* oil.

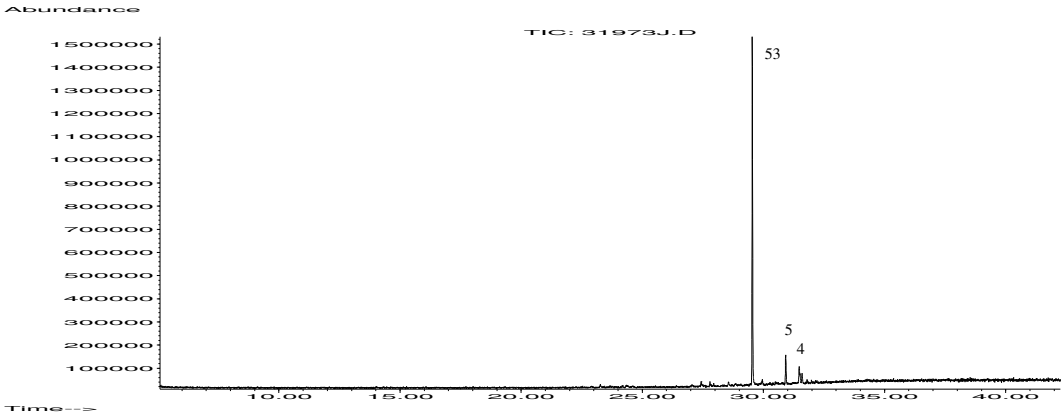


Figure 57. Total Ion Concentration of Fraction J of *Callitris glaucophylla* 31973.

31973 K

Table 44. Composition of Fraction K.

N°	Compound	Retention Time	CAS #	KI	calculated KI
10	guaiol	26.33	489-86-1	1600	1602
13	β-eudesmol	27.49	473-15-4	1650	1662
14	α-eudesmol	27.54	473-16-5	1653	1665
5	dihydrocolumellarin	31.15	66873-38-9	1900	1853
2	callitrisin	31.92	66964-62-3	1942	1894

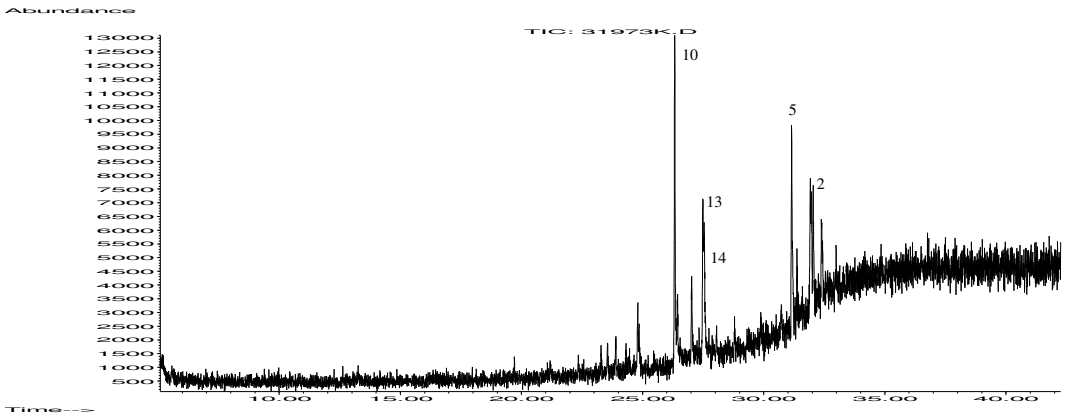


Figure 58. Total Ion Concentration of Fraction K of *Callitris glaucophylla* 31973.

31973 L

Fraction 31973 L did not show any volatile components in the GC/MS profile, but a big peak at a wavelength of 210 nm in the analytical HPLC.

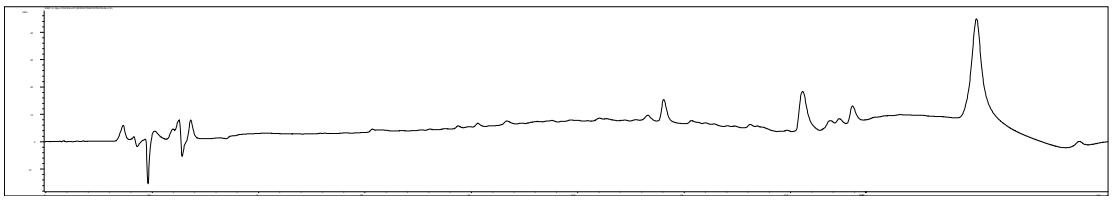


Figure 59. UV response of Fraction 31973 L at a wavelength of 210 nm on LC/MS.

4.3.2.2. Results of the pharmacological assays on 31973 fractions

4.3.2.2.1. ORAC results of 31973 fractions

The results of the antioxidant assay are shown in Table 45 and illustrated in Figure 60. No fraction showed a higher activity than the whole oil. Fractions C and G showed the highest values of all fractions, H, L, K, I and A showed the less. The high ORAC score of the whole oil could have possibly been a synergistic effect of all components. The synergistic effect would have also explained, why Fractions A and B are less antioxidant than C. There were not as much different components in it,

than in C, although most of the components were the same: The two selinenes (α-, and β), sandaracopimarinal and its derivatives were present in each of these fractions. In addition, B got an unknown compound in it, which appeared as an even bigger peak in Fraction C. The size of the peak of methyl geranate was much bigger in Fraction C than it is in B. It is possible that methyl geranate is responsible for the antioxidant activity. Fraction D was supposed to have a higher ORAC score. Its chemical profile was nearly identical to this of the whole 31973 oil, the composition was nearly the same. Also, guaiol and α-eudesmol have been reported before as antioxidant agents [27]. All in all, the antioxidant activity of Fractions C – F might be the nearly same, regarding the standard deviation. The presence of butylated hydroxytoluene explained the high TE-values of Fractions E and G [24]. The low value of Fraction H was doubtful. Fraction H contained BHT, as well as guaiol and both eudesmols. Fraction I was expected to be much higher because of its BHT and guaiazulene, which is already reported as an antioxidant agent [28], and fraction K should have been much higher too because of guaiol and both eudesmols.

Table 45. ORAC results of 31973 fractions.

	umolTE/g		
31973 oil	1247.67	±	116.77
31973 A	124.44	±	61.69
31973 B	249.05	±	62.26
31973 C	773.80	±	314.87
31973 D	526.82	±	174.74
31973 E	489.82	±	192.04
31973 F	589.34	±	194.02
31973 G	713.49	±	374.17
31973 H	9.65	±	5.74
31973 I	80.11	±	30.44
31973 J	268.64	±	107.22
31973 K	78.34	±	20.81
31973 L	53.92	±	23.74

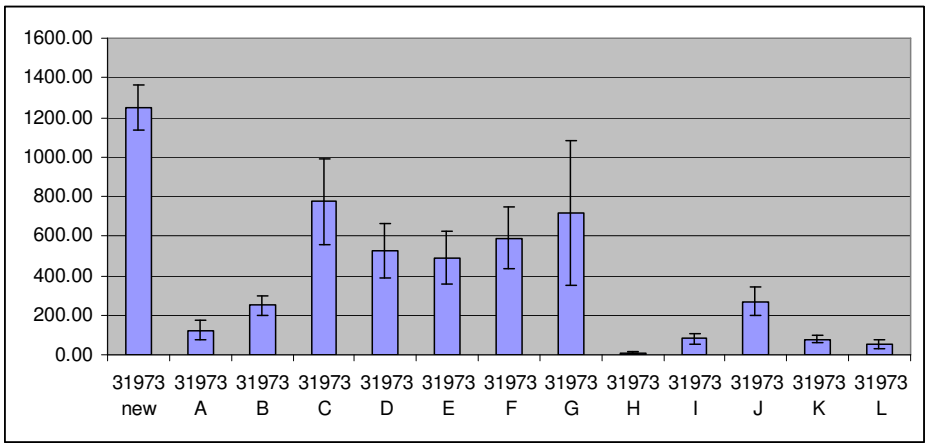


Figure 60. ORAC results (µmolTE/g) of 31973 fractions.

4.3.2.2.2. Cytotoxicity results of 31973 fractions

The cytotoxicity has been tested on P388D1 cells and HEP G-2 cells. The percent inhibition values are shown in Table 46 for P388D1 cells and in Table 47 for HEP G-2 cells. Samples got spilt and have been tested on three plates for P388D1 and on two plates for HEP G-2 cells. The figures show the plates with its control values below each other. Fraction B, containing methyl geranate, limonene, γ-gurjunene, β-selinene, α-selinene, a methyl ester of a γ-lactone and three unknown components with the m/z of 258, 256 and 258, showed high inhibition even in low concentrations on P388D1 cells (at a concentration of 3 µg/mL the inhibition is still 45.83%), but no trustful data could be obtained from HEP G-2 cells (inhibition values over 100%). However, the data can just be evaluated as preliminary because chlorambucil did not show any inhibition. Fractions C and D showed high values on both cells. On P388D1 cells Fraction C, containing methyl myrtenate and a methyl ester of a γ-lactone as principal components, still showed a percentage inhibition of 67.29 at a concentration of 25 µg/mL and Fraction D, with guaiol as highest peak, showed even higher inhibition with 86.64% at the same concentration. On HEP G-2 cells the inhibition of Fraction C is even higher than on the P388D1 cell line with 89%, but Fraction D showed a huge loss of activity from 88.91% to 23.47% when the concentration dropped from 50 to 25 µg/mL). Fraction F had the methyl ester of a γ-lactone as principal component and its abundance was much higher than in Fraction C, but on P388D1 cells the inhibition was less than the values from Fraction C and even lower on HEP G-2 cells. Fractions I and J showed less activity. Fractions H, K

and L did not show any inhibition on both cells and Fraction A showed a low inhibition of 43.36% at a concentration of 100 µg/mL on the HEP G-2 cells but no inhibition at all on the P388D1 cells. However, the positive controls did not give consistent results, so the data is doubtful and should just be evaluated as preliminary data. Chlorambucil showed no inhibition on the first plate of P388D1 and did not work on both plates of the assays carried out on the HEP G-2 cells.

Table 46. Cytotoxicity results on P388D1 cells (% inhibition) of 31973 fractions.

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil		no inhibition		
curcumin	100	77.63	87.32	82.48 ± 6.85
	50	4.77	3.06	3.91 ± 1.21
31973 A		no inhibition		
31973 B	200	98.91	99.32	99.11 ± 0.29
	100	98.85	98.77	98.81 ± 0.06
	50	98.75	98.62	98.69 ± 0.10
	25	98.35	98.52	98.44 ± 0.12
	13	96.55	98.17	97.36 ± 1.15
	6	93.63	83.01	88.32 ± 7.51
	3	72.82	18.84	45.83 ± 38.17

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil	600	10.18	30.17	20.17 ± 14.13
	19	26.34	29.27	27.80 ± 2.07
curcumin	100	98.98	99.03	99.00 ± 0.03
	50	56.20	55.37	55.78 ± 0.59
	25	30.62	34.61	32.62 ± 2.82
	6	3.36	19.23	11.30 ± 11.22
	3	1.74	25.64	13.69 ± 16.90
31973 C	200	99.75	99.77	99.76 ± 0.01
	100	99.33	99.29	99.31 ± 0.03
	50	90.13	85.79	87.96 ± 3.07
	25	56.03	78.54	67.29 ± 15.92
	13	51.30	76.16	63.73 ± 17.58
	6	37.09	68.14	52.61 ± 21.96
	3	10.50	37.29	23.90 ± 18.94
31973 D	200	99.32	99.41	99.36 ± 0.06
	100	76.35	79.80	78.08 ± 2.44
	50	44.76	52.14	48.45 ± 5.21
	25	78.74	94.54	86.64 ± 11.17
	13	25.91	37.06	31.48 ± 7.88
	6	5.43	43.72	24.58 ± 27.08
	3	1.77	7.23	4.50 ± 3.86
31973 E	212	42.93	62.72	52.83 ± 13.99
	106	48.51	83.70	66.11 ± 24.88
	53	22.39	23.77	23.08 ± 0.98
	13	16.48	30.79	23.64 ± 10.12
	3	7.16	18.90	13.03 ± 8.30
31973 F	188	71.43	73.10	72.26 ± 1.18
	94	29.31	47.31	38.31 ± 12.73
31973 G	200	98.76	99.05	98.91 ± 0.20

	100	84.82	88.51	86.66 ± 2.61
	50	47.20	59.82	53.51 ± 8.92
	25	8.36	39.98	24.17 ± 22.36
	13	10.27	15.83	13.05 ± 3.93
31973 H		no inhibition		
31973 I	200	60.34	61.71	61.03 ± 0.97
	100	15.58	5.78	10.68 ± 6.93
	25	35.51	8.22	21.86 ± 19.30
31973 J	200	22.11	22.67	22.39 ± 0.40
	25	49.56	93.36	71.46 ± 30.97
	6	4.62	4.55	4.58 ± 0.05

sample	**conc. (µg/mL)**	**% inhibition Rep I**	**Rep II**	**average**
chlorambucil	600	98.99		98.99 ± 0.00
	300	43.45		43.45 ± 0.00
	150	28.66		28.66 ± 0.00
	75	32.30		32.30 ± 0.00
curcumin	100	98.59		98.59 ± 0.00
	50	72.50		72.50 ± 0.00
	25	41.47		41.47 ± 0.00
	12.5	5.80		5.80 ± 0.00
31973 K		no inhibition		
31973 L		no inhibition		

Table 47. Cytotoxicity results on HEP G-2 cells (% inhibition) of 31973 fractions.

sample	**conc. (µg/mL)**	**% inhibition Rep I**	**Rep II**	**average**
chlorambucil		no inhibition		
curcumin	100	97.71	98.21	97.96 ± 0.36
	50	73.61	75.37	74.49 ± 1.25
31973 A	100	44.41	42.31	43.36 ± 1.48
	50	10.50	14.26	12.38 ± 2.66
31973 B	100	182.34	182.15	182.24 ± 0.13
	50	181.92	181.92	181.92 ± 0.00
	25	181.23	180.98	181.10 ± 0.18
	13	96.95	115.51	106.23 ± 13.13
	6	40.56	51.03	45.80 ± 7.41
	3	16.88	32.38	24.63 ± 10.96
	2	23.97	32.24	28.11 ± 5.84
31973 C	100	98.54	98.50	98.52 ± 0.03
	50	98.31	98.26	98.28 ± 0.04
	25	87.65	90.36	89.00 ± 1.92
	13	43.69	46.75	45.22 ± 2.17
	6	17.24	18.72	17.98 ± 1.05
	3	16.00	18.48	17.24 ± 1.75
	2	19.75	13.20	16.47 ± 4.63
31973 D	100	98.80	98.74	98.77 ± 0.04
	50	88.43	89.40	88.91 ± 0.68
	25	24.96	21.99	23.47 ± 2.10
	13	12.90	18.23	15.56 ± 3.77
	6	7.57	13.21	10.39 ± 3.99
	3	13.79	10.03	11.91 ± 2.65
	2	15.91	4.56	10.24 ± 8.03
31973 E	106	38.46	19.95	29.21 ± 13.09
	53	13.44	8.31	10.88 ± 3.63

	2	21.16	0.56	10.86 ± 14.57
31973 F	94	27.74	24.82	26.28 ± 2.07
	47	12.39	20.80	16.60 ± 5.95
	12	12.48	6.76	9.62 ± 4.05
	3	5.90	0.71	3.31 ± 3.67
	1	11.81	12.87	12.34 ± 0.75

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil		no inhibition		
curcumin	100	98.8652	96.8413	97.85 ± 1.43
	50	88.6667	81.7779	85.22 ± 4.87
	25	2.3919	1.21963	1.81 ± 0.83
31973 G	100	98.7286	98.5632	98.65 ± 0.12
	50	44.3306	48.2732	46.30 ± 2.79
	25	29.7548	30.4641	30.11 ± 0.50
	13	23.305	22.2784	22.79 ± 0.73
31973 H		no inhibition		
31973 I	100	15.4009	28.6011	22.00 ± 9.33
	50	8.04684	19.866	13.96 ± 8.36
31973 J	50	6.88974	7.06186	6.98 ± 0.12
31973 K		no inhibition		
31973 L		no inhibition		

4.3.3. Column subfractionation 31973 BC

Fractions B and C had a very similar GC/MS profile, so they were combined and fractionated further. The yield was 1.036 g. A glass column with a diameter of 2.5 cm and a length of 33 cm was used. Silica was used as stationary phase. The amount for a 5 – 10% loading was 55 g. The solvents used were hexane and diethylether, because these solvents were used to obtain fraction B and C at the first fractionation. Ethyl acetate was used at the end of the fractionation to make sure, that everything has come out of the column. Twenty-four fractions have been collected. The detailed fractionation scheme is shown in Table 48.

Table 48. Fractionation scheme of subfractionation on *Callitris glaucophylla* 31973 BC.

Fraction	mobile phase	volume (ml)	yield (mg)
1	90% hexane / 10% diethylether	120	0
2	90% hexane / 10% diethylether	120	0.1
3	80% hexane / 20% diethylether	120	293.5
4	80% hexane / 20% diethylether	120	57.9
5	70% hexane / 30% diethylether	120	113.9
6	70% hexane / 30% diethylether	120	49.6
7	60% hexane / 40% diethylether	120	25.4
8	60% hexane / 40% diethylether	120	18
9	50% hexane / 50% diethylether	120	17.2
10	50% hexane / 50% diethylether	120	12.1
11	40% hexane / 60% diethylether	120	10.3
12	40% hexane / 60% diethylether	120	6.3
13	30% hexane / 70% diethylether	120	5.5
14	30% hexane / 70% diethylether	120	3
15	20% hexane / 80% diethylether	120	2.5
16	20% hexane / 80% diethylether	120	2.2
17	10% hexane / 90% diethylether	120	2
18	10% hexane / 90% diethylether	120	1.6
19	100% diethylether	120	1.5
20	100% diethylether	120	0.8
21	50% diethylether / 50% ethylacetate	120	1
22	50% diethylether / 50% ethylacetate	120	0.9
23	100% ethylacetate	120	1.2
24	100% ethylacetate	120	0.3

4.3.3.1. Investigation of 31973 BC subfractions using GC/MS

Selected subfractions are figured, because many of them had exactly the same composition.

31973 BC 3

Table 49. Composition of Fraction 31973 BC 3.

N°	Compound	Retention Time	CAS #	KI	calculated KI
43	methyl geranate	21.05	2349-14-6	1324	1326
54	unknown, mw 180	23.84	-	-	1472
23	methyl ester of a γ-lactone	28.94	-	-	1738
27	sandaracopimarinal	35.36	3855-14-9	2184	2073

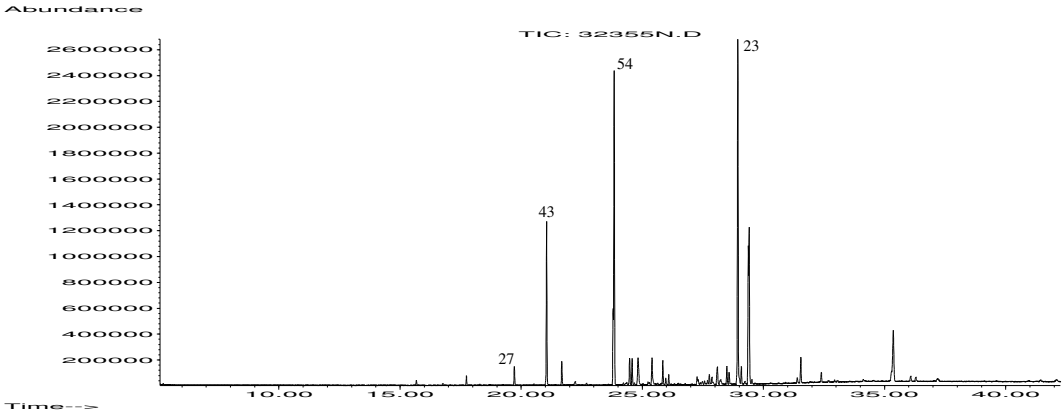


Figure 61. Total Ion Concentration of Fraction BC 3 of *Callitris glaucophylla* 31973.

31973 BC 4

Table 50. Composition of Fraction 31973 BC 4.

N°	Compound	Retention Time	CAS #	KI	calculated KI
54	unknown, mw 180	23.84	-	-	1472
55	unknown, mw 218	28.07	-	-	1693
56	unknown, mw 216	28.84	-	-	1733
4	columellarin	31.93	66873-37-8	1953	1894
34	6,7-dehydroferruginol	37.16	34539-84-9	2315	2167

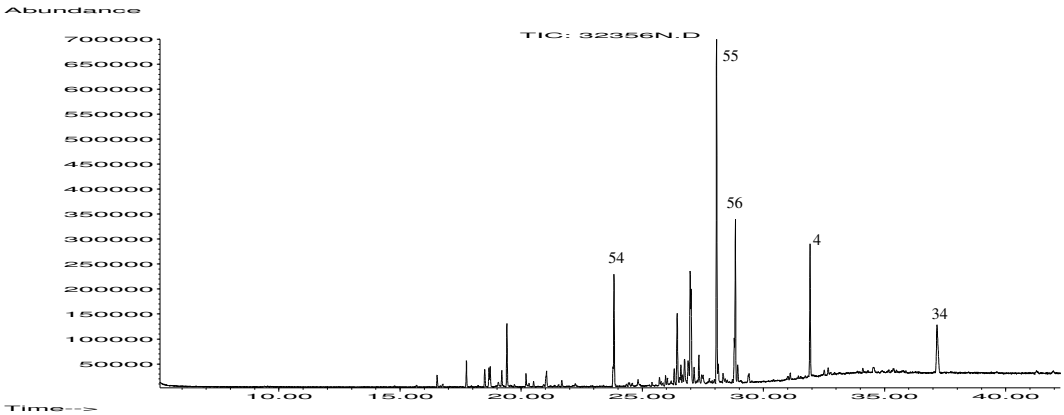


Figure 62. Total Ion Concentration of Fraction BC 4 of *Callitris glaucophylla* 31973.

31973 BC 5

Table 51. Composition of Fraction 31973 BC 5.

N°	Compound	Retention Time	CAS #	KI	calculated KI
10	guaiol	26.91	489-86-1	1600	1632
55	uk, mw 218	28.07	-	-	1693
45	amorpha-4,9-dien-14-al	28.84	394251-65-1	1707	1733
4	columellarin	31.93	66873-37-8	1953	1894

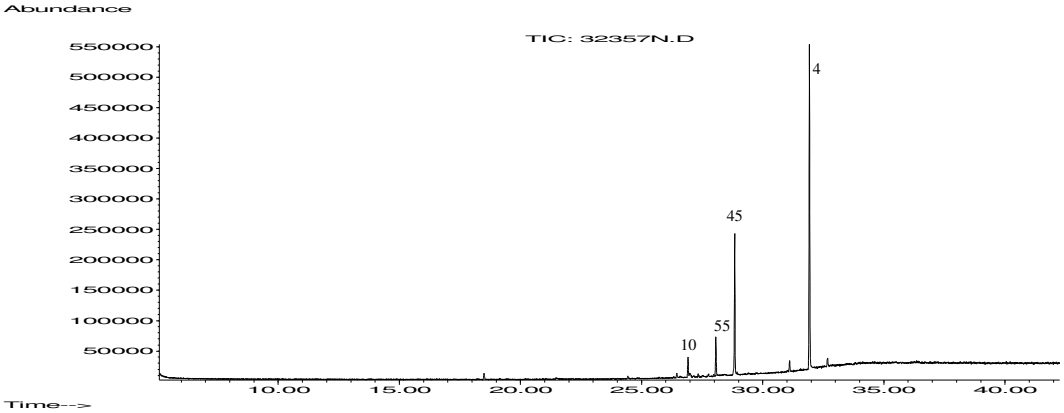


Figure 63. Total Ion Concentration of Fraction BC 5 of *Callitris glaucophylla* 31973.

31973 BC 7

Table 52. Composition of Fraction 31973 BC 7.

N°	Compound	Retention Time	CAS #	KI	calculated KI
9	citronellic acid	20.52	502-47-6	1313	1299
57	geranic acid	21.49	-	-	-
35	BHT	24.43	128-370	1515	1503

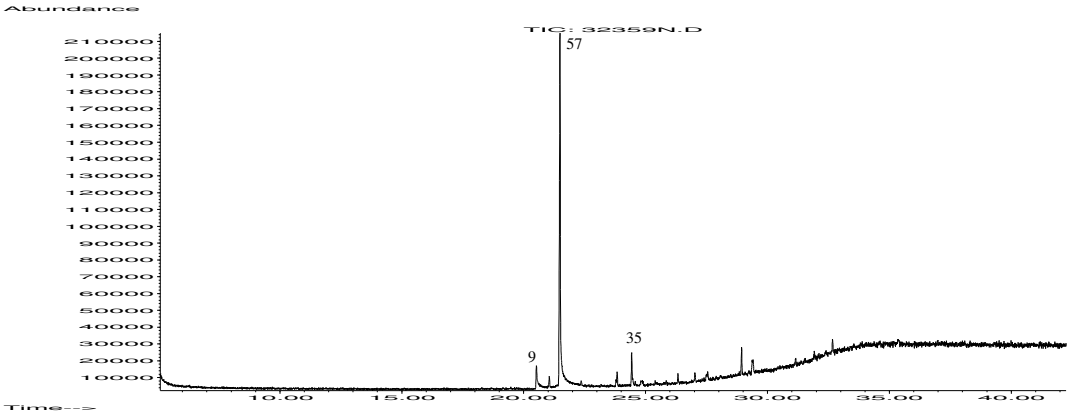


Figure 64. Total Ion Concentration of Fraction BC 7 of *Callitris glaucophylla* 31973.

31973 BC 9

Table 53. Composition of Fraction 31973 BC 9.

N°	Compound	Retention Time	CAS #	KI	calculated KI
9	citronellic acid	20.61	502-47-6	1313	1303
57	geranic acid	21.49	-	-	-
35	BHT	24.43	128-370	1515	1503

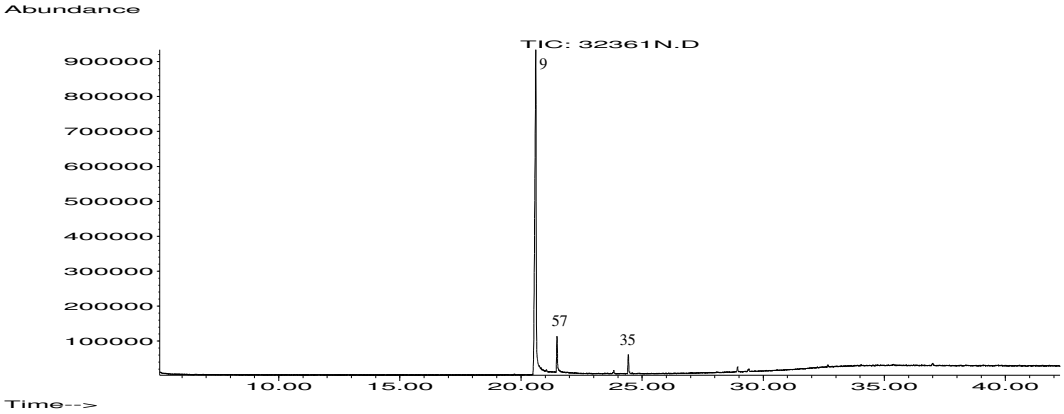


Figure 65. Total Ion Concentration of Fraction BC 9 of *Callitris glaucophylla* 31973.

31973 BC 14

Table 54. Composition of Fraction 31973 BC 14.

N°	Compound	Retention Time	CAS #	KI	calculated KI
9	citronellic acid	20.59	502-47-6	1313	1302
35	BHT (butylated hydroxy toluene)	24.43	128-370	1515	1503
58	BBP (benzyl butyl phthalate)	36.98	-	-	-

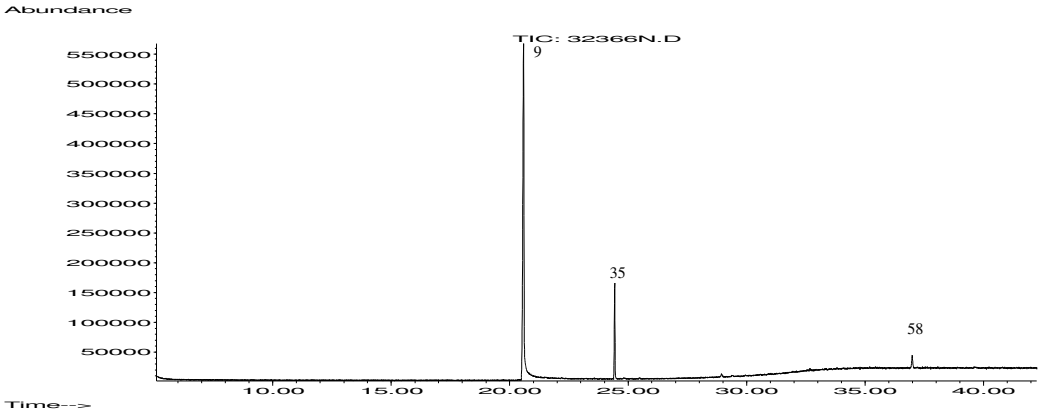


Figure 66. Total Ion Concentration of Fraction BC 14 of *Callitris glaucophylla* 31973.

31973 BC 16

Table 55. Composition of Fraction 31973 BC 16.

N°	Compound	Retention Time	CAS #	KI	calculated KI
9	citronellic acid	20.57	502-47-6	1313	1302
35	BHT (butylated hydroxy toluene)	24.43	128-370	1515	1503
58	BBP (benzyl butyl phthalate)	36.99	-	-	-

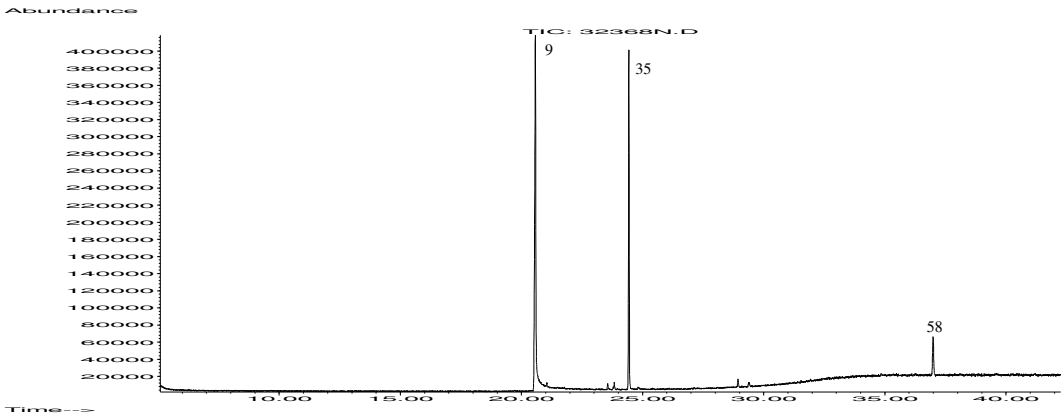


Figure 67. Total Ion Concentration of Fraction BC 16 of *Callitris glaucophylla* 31973.

4.3.3.2. Results of the pharmacological assays on 31973 BC subfractions

4.3.3.2.1. ORAC results on 31973 BC subfractions

The results of the ORAC assay on the subfractions of 31973 BC are shown in Table 56 and illustrated in Figure 68. Many fractions contained the same components, like Fractions 31973 BC 6 until 31973 BC 13. These fractions contained citronellic acid, gerianic acid and BHT. The abundance in the GC/MS profiles got less with every fraction. Fraction 7 showed high abundance and a high antioxidant activity of 1826.26 µmolTE/g. As the abundance decreased, the ORAC values decreased as well (Fraction 13 showed 847 µmolTE/g). All other subfractions did not have many volatile components in them and showed lower trolox equivalent values than the original Fraction 31973 BC.

Table 56. ORAC results of subfractions 31973 BC.

	umolTE/g
31973 B	249.05 ± 62.26
31973 C	773.80 ± 314.87
31973 BC 1	5.61 ± 0.15
31973 BC 2	4.54 ± 1.35
31973 BC 3	417.92 ± 86.19
31973 BC 4	812.19 ± 144.60
31973 BC 5	304.54 ± 28.23
31973 BC 6	1654.69 ± 217.30
31973 BC 7	1826.26 ± 373.29
31973 BC 8	1675.85 ± 365.28
31973 BC 9	1500.14 ± 517.15
31973 BC 10	1480.73 ± 339.20
31973 BC 11	1379.32 ± 473.47
31973 BC 12	820.20 ± 317.42
31973 BC 13	847.00 ± 329.97
31973 BC 14	1092.97 ± 290.44
31973 BC 15	313.78 ± 116.83
31973 BC 16	40.19 ± 16.30
31973 BC 17	477.94 ± 32.64
31973 BC 18	219.89 ± 69.42
31973 BC 19	571.91 ± 127.71
31973 BC 20	346.63 ± 24.39
31973 BC 21	44.51 ± 25.77
31973 BC 22	140.95 ± 25.00
31973 BC 23	16.81 ± 2.26
31973 BC 24	33.18 ± 1.34

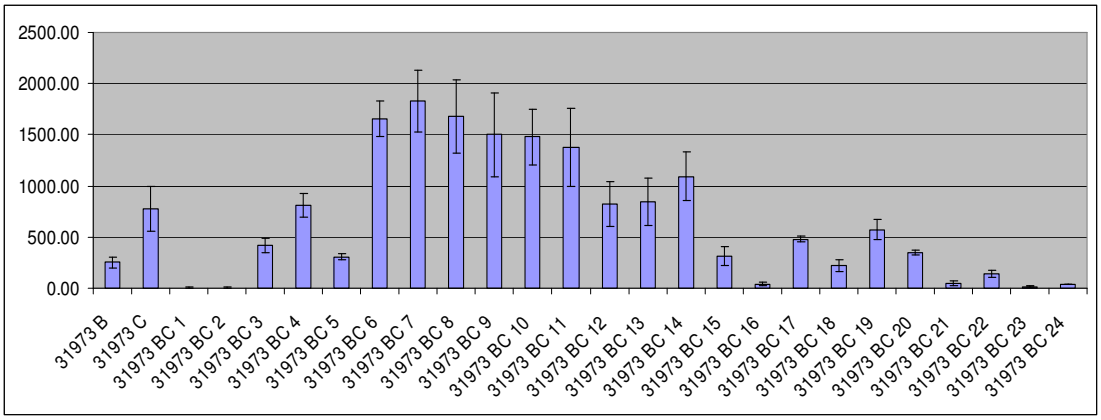


Figure 68. ORAC results (µmolTE/g) of subfractionation 31973 BC.

4.3.3.2.2. Cytotoxicity results of 31973 BC subfractions

The cytotoxicity assay was done on P388D1 cells. The percent inhibition values are shown in Table 57. The assay was repeated twice for all samples (Rep I and Rep II) except the controls chlorambucil and curcumin. Samples got spilt and have been tested on three plates. The values of chlorambucil and curcumin varied between the assay plates. Chlorambucil showed inhibition only at the highest concentration. Curcumin inhibition values varied between 61.04 and 82.82 % at a concentration of 50 µg/mL. The cytotoxicity data can be evaluated as preliminary. Fraction 31973 BC 4 showed the highest inhibition, even at a low concentration of 25 µg/mL it still showed a percentage inhibition of 65.09. Fractions 31973 BC 3, 5 and 6 showed low cytotoxic effects, even at high concentrations. All other fractions did not show any cytotoxic activity.

Table 57. Cytotoxicity results on P388D1 cells (% inhibition) of 31973 BC subfractions.

sample	conc. (µg/mL)	% inhibition Rep I	% inhibition Rep II	average
chlorambucil	600	95.12		95.12 ± 0.00
curcumin	100	97.72		97.72 ± 0.00
	50	61.04		61.04 ± 0.00
	25	5.75		5.75 ± 0.00
31973 BC 1		no inhibition		
31973 BC 2	200	41.56	27.16	34.36 ± 10.18
	100	23.31	19.62	21.46 ± 2.61
	50	28.79	20.77	24.78 ± 5.67
31973 BC 3	200	97.12	95.61	96.36 ± 1.06
	100	71.44	63.99	67.72 ± 5.26
	50	60.64	34.32	47.48 ± 18.61
	25	38.55	31.78	35.17 ± 4.78
31973 BC 4	200	99.41	99.31	99.36 ± 0.08
	100	98.92	98.99	98.95 ± 0.05
	50	71.77	86.63	79.20 ± 10.51
	25	65.00	65.18	65.09 ± 0.13
31973 BC 5	200	46.74	55.84	51.29 ± 6.43
	100	58.39	37.33	47.86 ± 14.89
	50	41.56	25.12	33.34 ± 11.63
	25	24.76	25.11	24.93 ± 0.24
31973 BC 6	200	67.36	58.12	62.74 ± 6.53
	100	44.56	46.23	45.40 ± 1.18
	50	24.88	19.76	22.32 ± 3.62
	25	43.17	9.55	26.36 ± 23.77
31973 BC 7	200	17.26	33.11	25.18 ± 11.21
	100	20.68	19.44	20.06 ± 0.88
31973 BC 8	200	34.09	29.28	31.68 ± 3.40
	100	13.98	5.08	9.53 ± 6.30
	50	7.21	0.10	3.66 ± 5.02

31973 BC 9		no inhibition	

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil	600	95.78		95.78 ± 0.00
curcumin	100	95.50		95.50 ± 0.00
	50	72.87		72.87 ± 0.00
	25	45.33		45.33 ± 0.00
	13	35.52		35.52 ± 0.00
31973 BC 10		no inhibition		
31973 BC 11	200	56.60	19.36	37.98 ± 26.33
31973 BC 12		no inhibition		
31973 BC 13	200	26.63	9.89	18.26 ± 11.83
31973 BC 14	200	0.20	31.58	15.89 ± 22.19
	100	4.34	20.48	12.41 ± 11.41
31973 BC 15	200	11.54	6.92	9.23 ± 3.27
31973 BC 16		no inhibition		
31973 BC 17	200	3.97	29.40	16.68 ± 17.98
31973 BC 18	200	31.12	35.01	33.06 ± 2.75
	100	34.58	2.80	18.69 ± 22.47
	50	18.93	9.83	14.38 ± 6.43

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil	600	95.79		95.79 ± 0.00
curcumin	100	97.16		97.16 ± 0.00
	50	82.82		82.82 ± 0.00
31973 BC 19		no inhibition		
31973 BC 20		no inhibition		
31973 BC 21		no inhibition		
31973 BC 22		no inhibition		
31973 BC 23		no inhibition		
31973 BC 24		no inhibition		

The yields of most of the obtained subfractions were too low for further fractionation, except subfraction 3 (293.5 mg) and 5 (113.9 mg), which were subfractionated by prep HPLC.

4.3.4. Preparative HPLC of subfraction 31973 BC 3

For getting a better separation of the subfraction 31973 BC 3, methanol was used instead of acetonitrile (Method Ruprep3) and the method was changed on the analytical HPLC as well (Ruth6). Again, eighty fractions have been collected, but unfortunately none of the fractions was pure enough. No pharmacological assays were made.

4.3.5. Preparative HPLC of subfraction 31973 BC 5

Sample 31973 BC 5 has been fractionated the same way as 31973 BC 3 (prepHPLC: Method Ruprep3 and analytical HPLC: Ruth6). Just one fraction seemed pure enough and the antioxidant activity was proven on the ORAC assay. The UV response of Fraction 11 is shown in Figure 69.

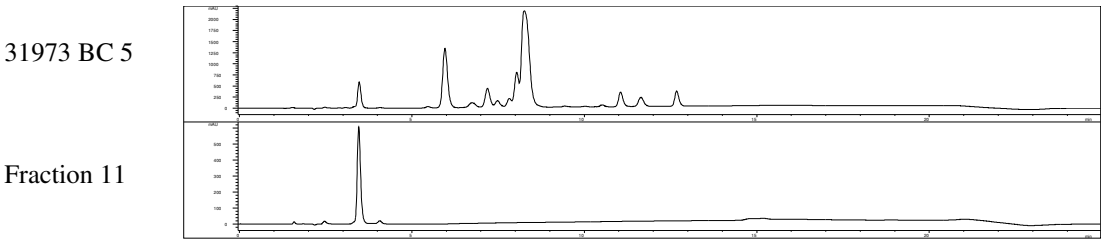


Figure 69. UV response at wavelength 210 nm (analytical HPLC) of 31973 BC 5 and Fraction 11.

4.3.5.1. Results of the pharmacological assays on 31973 BC subfractions

4.3.5.1.1. ORAC results of selected fraction of 31973 BC 5

The selected subfraction 31973 BC 5 11 showed very little antioxidant activity with a very high standard deviation (54.25 μmolTE/g ± 47.01).

4.4. *Callitris glaucophylla* 31973 Fraction 5

4.4.1. Overview

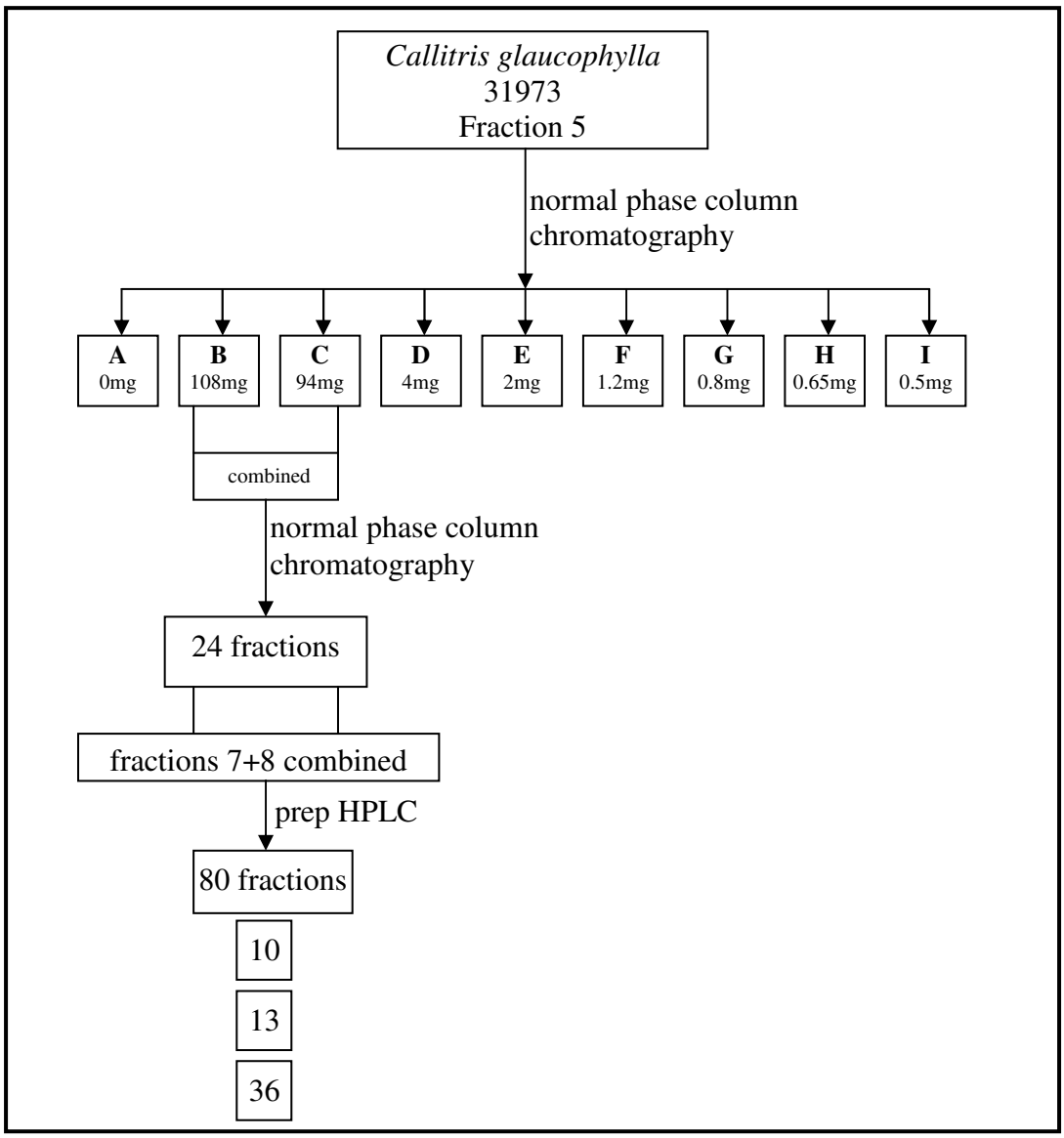


Figure 70. Schematic diagram for the isolation of compounds from *Callitris glaucophylla* 31973 Fraction 5.

4.4.2. Column fractionation of 31973 Fraction 5

The column had a diameter of 2.5 cm and a length of 33 cm. Column chromatography has been done using silica as stationary phase. The yield of Fraction 5, obtained from the *Callitris glaucophylla* 31973 oil, was 2 g. For getting a 5 – 10% loading, 50 g silica was used. Solvents used were hexane, diethylether and ethyl acetate, used in ascending order of polarity. A detailed scheme of the fractionation design is shown in Table 58.

Table 58. Fractionation scheme of *Callitris glaucophylla* 31973 Fraction 5.

Fraction	**Mobile phase**	**Volume (ml)**	**Yield (mg)**
A	100% hexane	120	0
B	50% hexane / 50% diethylether	120	108
C	50% hexane / 50% diethylether	120	94
D	25% hexane / 75% diethylether	120	4
E	25% hexane / 75% diethylether	120	2
F	100% diethylether	120	1.2
G	100% diethylether	120	0.8
H	50% diethylether / 50% ethylacetate	120	0.65
I	100% ethylacetate	120	0.5

4.4.2.1. Investigation of 31973 Fraction 5 fractions using GC/MS

Table 59. Composition of 31973 Fraction 5.

N^{o}	Compound	Retention Time	CAS #	KI	calculated KI
10	guaiol	25.89	489-86-1	1600	1599
14	α-eudesmol	26.58	473-16-5	1653	1641
11	10-epi-γ-eudesmol	27.05	15951-81-7	1623	1670
13	β-eudesmol	27.10	473-15-4	1650	1673

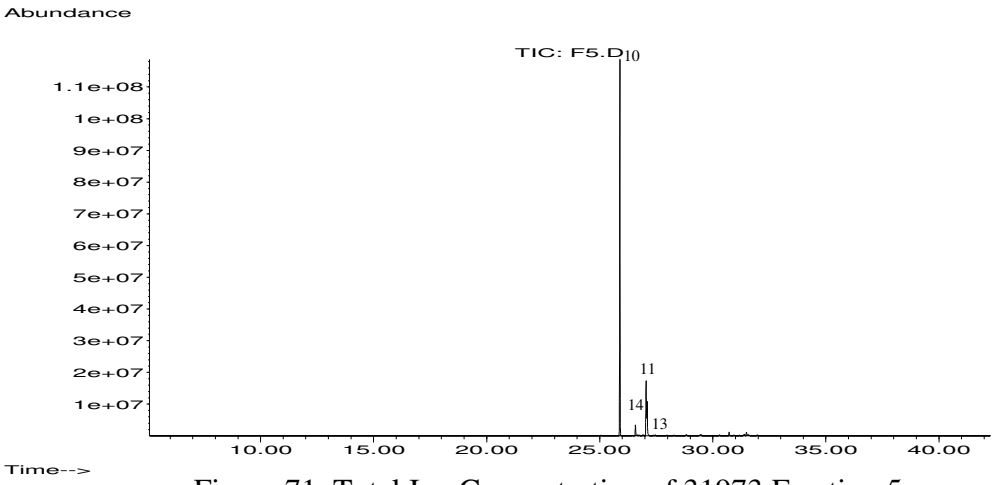


Figure 71. Total Ion Concentration of 31973 Fraction 5.

31973 Fraction 5 Fraction A

Fraction A did not show any volatile components in the GCMS profile, but one peak on the LC/MS at a wavelength of 210 nm.

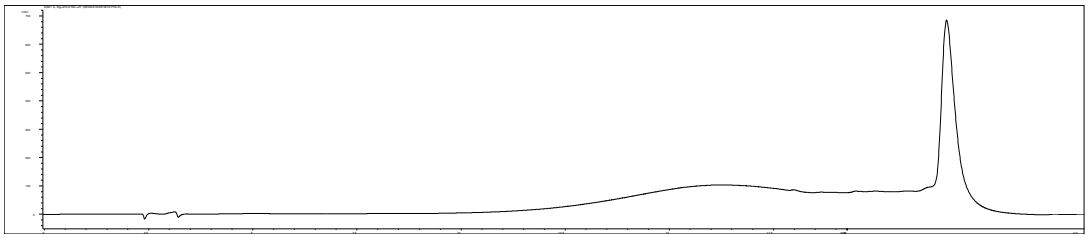
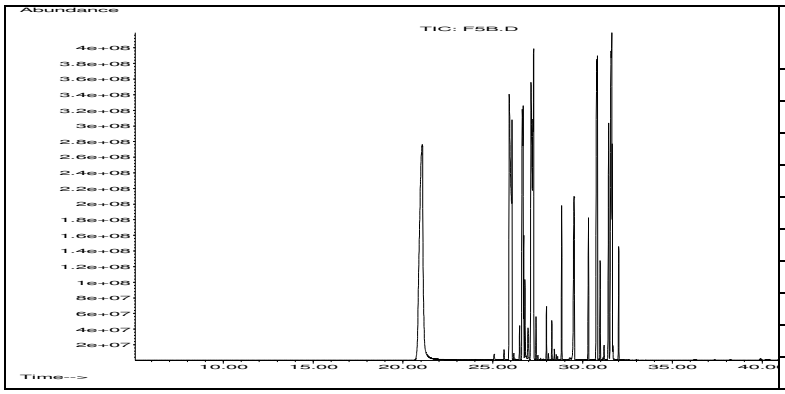


Figure 72. UV Response at a wavelength of 210 nm of 31973 Fraction 5.

All other fractions showed volatile components in the GC/MS, but the peaks could not be identified neither by comparison with fragmentation pattern nor by calculated KI.

31973 Fraction 5 Fraction B

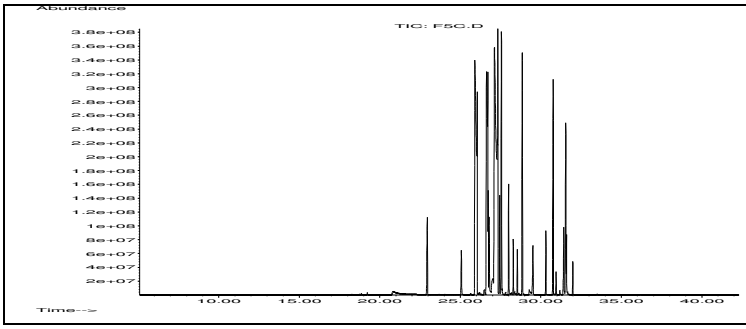
Table 60. Composition of 31973 Fraction 5 Fraction B.



Retention Time	calculated KI
22.95	1426
25.91	1580
26.63	1617
27.33	1654
28.85	1733
30.77	1833
31.55	1874
31.99	1892
31.62	1873
32.01	1893

31973 Fraction 5 Fraction C

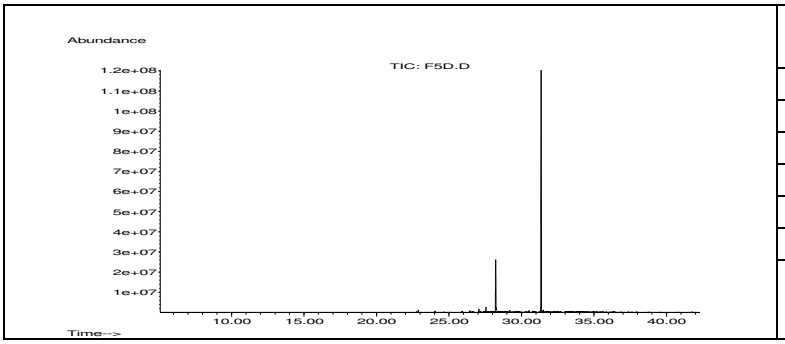
Table 61. Composition of 31973 Fraction 5 Fraction C.



Retention Time	calculated KI
22.95	1422
25.91	1576
26.63	1613
27.33	1650
28.85	1729
30.77	1829
31.55	1869
31.99	1892

31973 Fraction 5 Fraction D

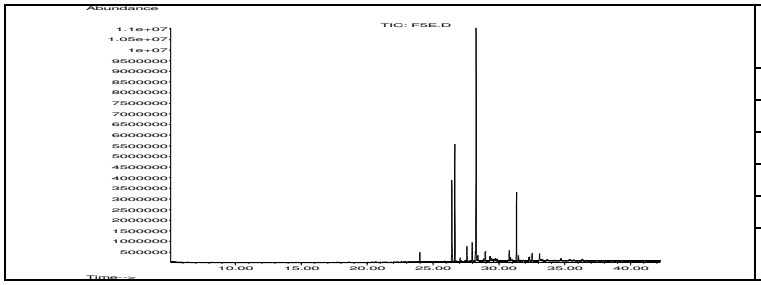
Table 62. Composition of 31973 Fraction 5 Fraction D.



Retention Time	calculated KI
28.22	1696
31.35	1859

31973 Fraction 5 Fraction E

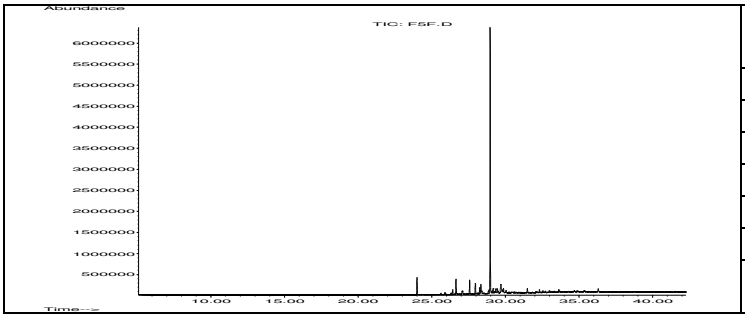
Table 63. Composition of 31973 Fraction 5 Fraction E.



Retention Time	calculated KI
24.00	1477
26.43	1603
26.65	1614
28.27	1699
31.34	1858

31973 Fraction 5 Fraction F

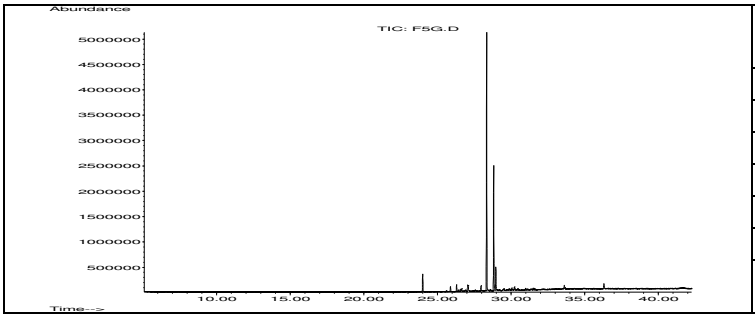
Table 64. Composition of 31973 Fraction 5 Fraction F.



Retention Time	calculated KI
24.00	1477
28.97	1735

31973 Fraction 5 Fraction G

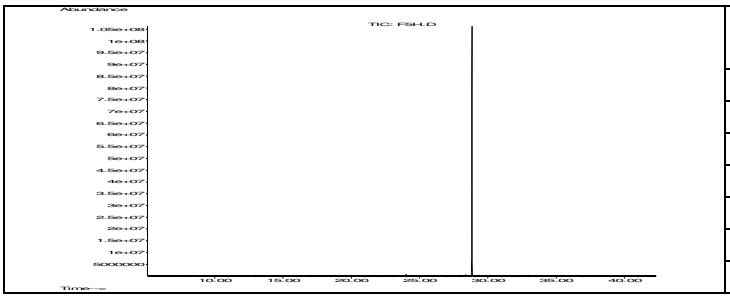
Table 65. Composition of 31973 Fraction 5 Fraction G.



Retention Time	calculated KI
24.00	1477
28.34	1702
28.82	1727

31973 Fraction 5 Fraction H

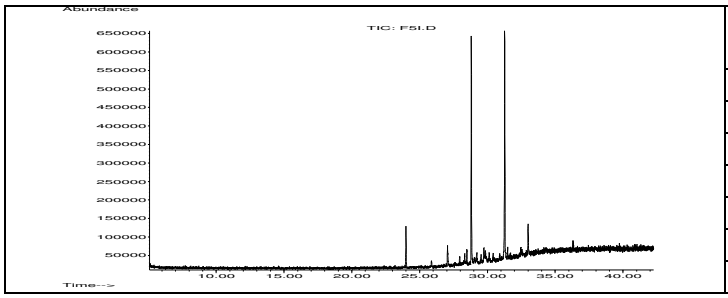
Table 66. Composition of 31973 Fraction 5 Fraction H.



Retention Time	calculated KI
28.82	1727

31973 Fraction 5 Fraction I

Table 67. Composition of 31973 Fraction 5 Fraction I.



Retention Time	calculated KI
24.00	1477
28.81	1727
31.38	1860
33.00	1945

4.4.2.2. Results of the pharmacological assays on 31973 Fraction 5 fractions

4.4.2.2.1. ORAC results of 31973 Fraction 5 fractions

The results of the antioxidant assay are shown in Table 68 and illustrated in Figure 73. Fractions B and C showed the highest values of all fractions and were fractionated further. Fractions D, E, and F showed the less activity and Fractions A, H and I showed very little effects.

Table 68. ORAC results of fractions of *Callitris glaucophylla* 31973 Fraction 5.

sample	ORAC value umolTE/g		
F5 A	45.00	±	30.43
F5 B	1759.44	±	322.04
F5 C	1359.45	±	282.48
F5 D	475.98	±	168.86
F5 E	280.44	±	10.45
F5 F	207.74	±	16.29
F5 G	166.32	±	17.26
F5 H	31.33	±	3.52
F5 I	122.32	±	13.44

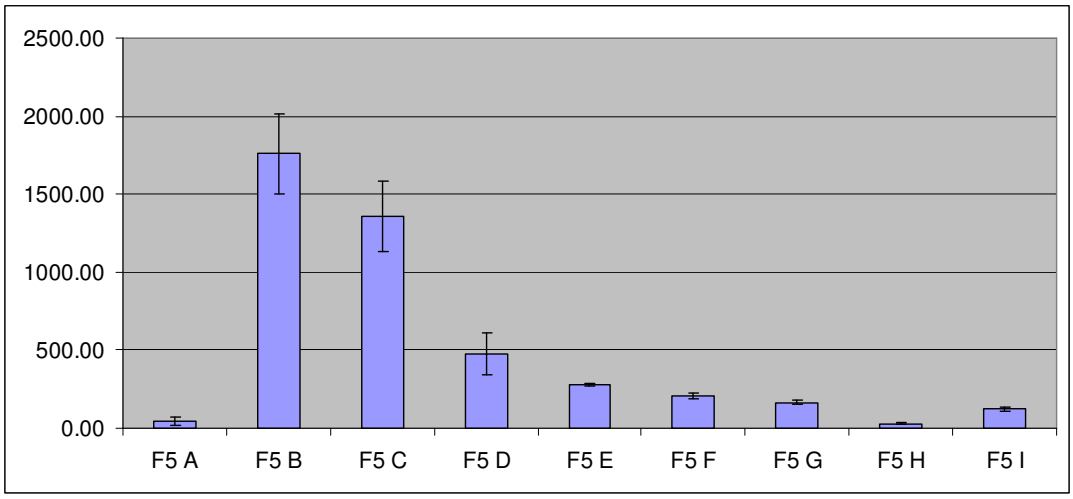


Figure 73. ORAC results (µmolTE/g) of fractions of *Callitris glaucophylla* 31973 Fraction 5.

4.4.2.2.2.Cytotoxicity results of fractions of 31973 BC fractions

The cytotoxicity assays were done on P388D1 cells. The percent inhibition values are shown in Table 69. The assay was repeated twice for all samples (Rep I and Rep II), except for the controls curcumin and chlorambucil. Samples got spilt and have been tested on two plates. The figures show the first plate with its control values first, followed by the second plate with its curcumin and chlorambucil values. Fraction B showed the highest activity among all fractions, followed by Fraction C. The

inhibition was high at high concentrations but lost cytotoxic activity as concentration decreased. Fractions D, E, F, G, H, and I just showed little cytotoxic activity, even at high concentrations. Fraction A did not show activity at all.

Table 69. Cytotoxicity results on P388D1 cells (% inhibition) of *Callitris glaucophylla* oils.

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil	600	95.79		95.79 ± 0.00
curcumin	100	97.16		97.16 ± 0.00
	50	82.82		82.82 ± 0.00
F5 A		no inhibition		
F5 B	200	99.09	98.64	98.86 ± 0.32
	100	87.68	83.89	85.78 ± 2.68
	50	55.28	42.57	48.92 ± 8.99
	25	31.54	10.16	20.85 ± 15.11
F5 C	200	98.93	98.60	98.77 ± 0.24
	100	67.59	89.33	78.46 ± 15.37
	50	30.72	33.11	31.92 ± 1.69
	25	6.71	37.61	22.16 ± 21.85

sample	conc. (µg/mL)	% inhibition Rep I	Rep II	average
chlorambucil	600	98.42		98.42 ± 0.00
	300	18.02		18.02 ± 0.00
curcumin	100	98.58		98.58 ± 0.00
	50	91.38		91.38 ± 0.00
	25	37.65		37.65 ± 0.00
	13	41.54		41.54 ± 0.00
F5 D	200	61.62	21.30	41.46 ± 28.51
	100	45.83	14.26	30.05 ± 22.33
F5 E	200	63.06	30.23	46.65 ± 23.21
	100	16.24	16.78	16.51 ± 0.38
F5 F	200	83.40	9.26	46.33 ± 52.42
	100	83.73	7.59	45.66 ± 53.84
	50	38.56	28.04	33.30 ± 7.44
	25	42.09	32.30	37.20 ± 6.92
F5 G	200	44.59	27.87	36.23 ± 11.82
	100	45.43	16.06	30.75 ± 20.77
	50	31.28	23.37	27.33 ± 5.60
	25	20.78	19.57	20.17 ± 0.86
F5 H	200	34.03	44.35	39.19 ± 7.30
	100	27.24	5.31	16.27 ± 15.51
	50	31.96	6.58	19.27 ± 17.94
	25	15.45	15.86	15.66 ± 0.29
F5 I	200	46.14	40.66	43.40 ± 3.88
	100	28.49	26.35	27.42 ± 1.52
	50	16.62	8.63	12.62 ± 5.65
	25	11.14	9.62	10.38 ± 1.08

4.4.3. Column subfractionation of 31973 Fraction 5 BC

Fractions B and C were combined and fractionated further. The yield was 1.96 g. A glass column with a diameter of 2.5 cm and a length of 33 cm was used. Silica was used as stationary phase. The amount for a 5 – 10% loading was 55 g. The solvents used were hexane and diethylether, because these solvents were used to obtain Fractions B and C at the first fractionation. Ethyl acetate was used at the end of the fractionation to make sure, that everything has come out of the column. Twenty-four fractions have been collected. The detailed fractionation scheme is shown in Table 70.

Table 70. Fractionation scheme of subfractionation on *Callitris glaucophylla* 31973 BC.

Fraction	**mobile phase**	**volume (ml)**	**yield (mg)**
1	90% hexane / 10% diethylether	120	0.2
2	90% hexane / 10% diethylether	120	0
3	80% hexane / 20% diethylether	120	8.4
4	80% hexane / 20% diethylether	120	2.9
5	70% hexane / 30% diethylether	120	41.6
6	70% hexane / 30% diethylether	120	208.5
7	60% hexane / 40% diethylether	120	350.2
8	60% hexane / 40% diethylether	120	200.5
9	50% hexane / 50% diethylether	120	47
10	50% hexane / 50% diethylether	120	20.8
11	40% hexane / 60% diethylether	120	11.4
12	40% hexane / 60% diethylether	120	10.6
13	30% hexane / 70% diethylether	120	8.3
14	30% hexane / 70% diethylether	120	6.6
15	20% hexane / 80% diethylether	120	4.1
16	20% hexane / 80% diethylether	120	4
17	10% hexane / 90% diethylether	120	3.1
18	10% hexane / 90% diethylether	120	2.4
19	100% diethylether	120	1.6
20	100% diethylether	120	9.6
21	50% diethylether / 50% ethylacetate	120	203.6
22	50% diethylether / 50% ethylacetate	120	315.6
23	100% ethylacetate	120	0.5
24	100% ethylacetate	120	0

4.4.3.1. Investigation of 31973 Fraction 5 BC subfractions using LC/MS

The subfractions 1 – 24 of *Callitris glaucophylla* 31973 Fraction 5 BC did not show any abundance in the GC/MS, so they were profiled by LC/MS and the obtained UV response is shown in Figure 74 below.

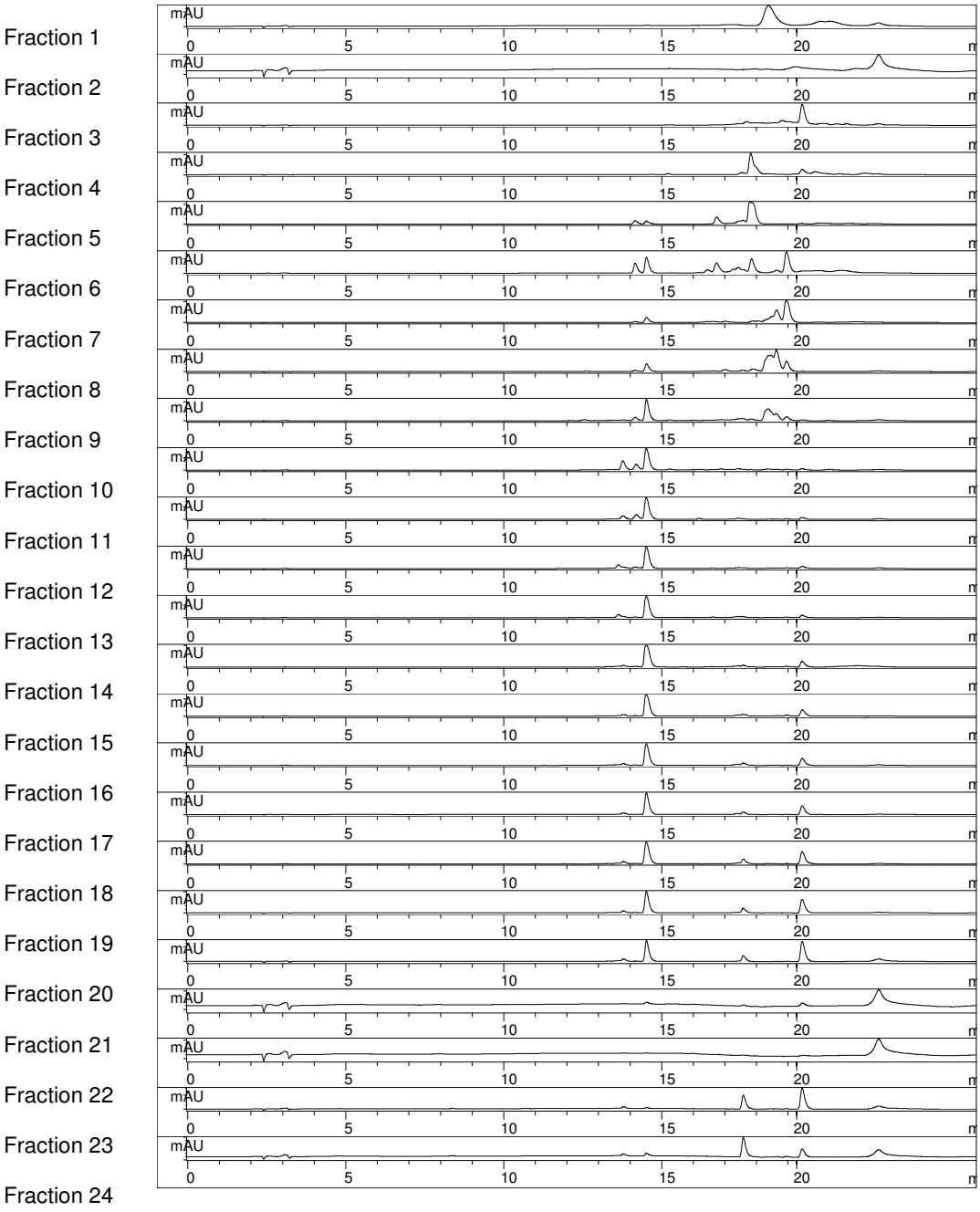


Figure 74. UV response at a wavelength of 210 nm of subfractions 1 – 24 of subfractions of *Callitris glaucophylla* 31973 Fraction 5 BC.

4.4.3.2. Results of the pharmacological assays on 31973 Fraction 5 BC subfractions

4.4.3.2.1. ORAC results of 31973 Fraction 5 BC subfractions

The results of the antioxidant assay are shown in Table 71 and illustrated in Figure 75. Subfractions 5, 6 and 7+8 showed very high antioxidant activity with the values 1229.06, 1520.37 and 1754.12 µmolTE/g. The values of Subfractions 9, 10-12, 13, 14, 15, 17-20 and 22 showed more activity than all other subfractions.

Table 71. ORAC values (µmolTE/g) of 31973 BC Fraction 5 subfractions.

	µmolTE/g
F5 BC 1	21.30 ± 1.21
F5 BC 2	17.40 ± 30.50
F5 BC 3	29.49 ± 5.11
F5 BC 4	9.58 ± 6.95
F5 BC 5	1229.06 ± 225.30
F5 BC 6	1520.37 ± 270.28
F5 BC 7+8	1754.12 ± 264.02
F5 BC 9	453.22 ± 170.45
F5 BC 10+11+12	358.60 ± 165.78
F5 BC 13	312.88 ± 144.82
F5 BC 14	217.23 ± 49.72
F5 BC 15	173.20 ± 27.66
F5 BC 16	0.17 ± 37.72
F5 BC 17+18+19+20	241.67 ± 111.19
F5 BC 21	-7.84 ± 54.13
F5 BC 22	677.28 ± 251.29
F5 BC 23	54.99 ± 15.86
F5 BC 24	33.72 ± 17.86

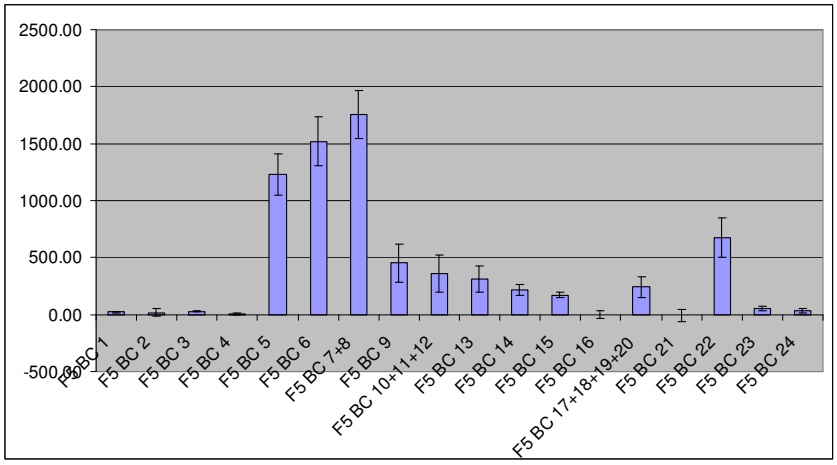


Figure 75. ORAC results of subfractions of 31973 Fraction 5 BC.

4.4.4. Preparative HPLC of subfraction 31973 Fraction 5 BC 7+8

Sample 31973 Fraction 5 BC 7+8 has been fractionated using the Ruprep3 method on the preparative HPLC and analysed on the analytical HPLC using the method Ruth6. Just three fractions seemed pure enough and were run on NMR. The chromatographic profile obtained from analytical HPLC is shown in Figure 76 below, followed by the UV data of selected fractions.

Fraction 5 BC 7+8

Fraction 10

Fraction 13

Fraction 36

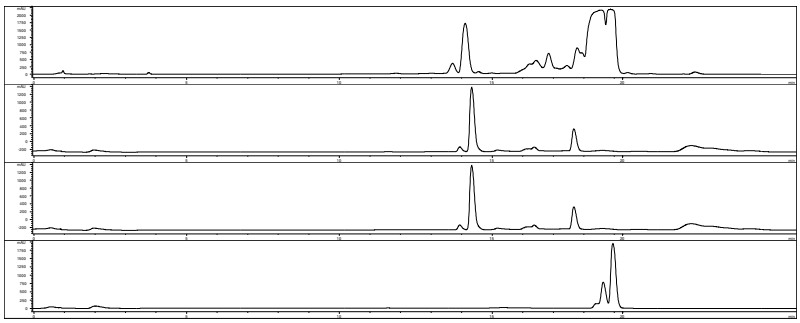


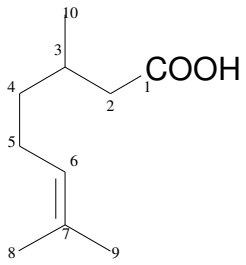
Figure 76. UV response at wavelength 210 nm (analytical HPLC) of 31973 Fraction 5 BC 7+8 and selected fractions.

4.4.4.1. Isolation of pure compounds

4.4.4.1.1. Isolation of citronellic acid

Citronellic acid **(9)** has been isolated from 31973 Fraction 5 BC 7+8 Fraction 13. Fraction 5 BC 7+8 Fraction 10 turned out to be citronellic acid too. Citronellic acid has been reported in *Callitris glaucophylla* before. The ^{1}H and ^{13}C data is shown in Table 72.

CAS:	2111-53-7
molecular formula:	$C_{10}H_{18}O_2$
molecular weight:	170.251
boiling point:	118°



(9)

Figure 77. Chemical structure of citronellic acid, 31973 Fraction 5 BC 7+8 Fraction 13.

Table 72. ^{1}H and ^{13}C-NMR spectral data of an isomer of citronellic acid **(9)**.

C/H Position	Chemical Shift, ppm 1H	13C
1	-	178.60
2	2.37, dd (15.00, 6.00) 2.17, dd (15.00, 8.30)	41.50
3	2.10, m (2H)	30.00
4	2.37, dd (15.00, 6.00) 1.27, m	36.90
5	2.10, m (2H)	25.60
6	5.10, m	124.40
7	-	131.90
8	1.70, s (3H)	25.90
9	1.61, s (3H)	17.90
10	0.99, d (3H) (6.60)	19.80

4.5. Anti-inflammatory assay on selected fractions

Considering the informations about anti-inflammatory properties of chemical classes in the literature, eight fractions got selected for the competitive ELISA-assay. These fractions were Fraction 31973 Fraction 5 BC 7+8 36, containing guaiol and bulnesol, Fraction 32352 C 53, containing 6,7-dehydroferruginol, Fraction 32352 B, containing a methyl ester of a γ-lactone, Fraction 32352 C 70, containing sandaracopimarinal, Fraction 32352 D 63, containing sandaracopimarinal, Fraction 32352 D41, containing dihydrocolumellarin, Fraction 31973 BC 5, containing columellarin and Fraction 31973 BC 8, containing citronellic and gerianic acid.

Unfortunately the assay did not show any results. Aspirin, the control, showed negative values. The data are not valid.

5. SUMMARY & CONCLUSION

The aim of the first part of this master thesis was to explore whether there are differences in the *Callitris glaucophylla* wood oil from different accessions or not. The oils obtained from three different accessions have been profiled by GC/MS and LC/MS to obtain MS and UV data and showed similarities but also differences in their composition. The oils from accessions 31973 and 32209 showed nearly the same components. Guaiol, a sesquiterpene, was the principal component in both of these oils. An overlapping peak of bulnesol and α- and β-eudesmol was also observed in both oils. The third detected eudesmol, 10-epi-γ-eudesmol, was found in all three oils and the two sesquiterpene lactones columellarin and dihydrocolumellarin appeared in all three wood oils as well. The oil from *Callitris glaucophylla* 32352 showed dihydrocolumellarin as principal compound. Another item of note is that guaiol was not observed in this oil, but a variety of other compounds like limonene, 1,8-cineole, methyl myrtenate, citronellic acid, β- and α-selinene, γ- and β-costol, sandaracopimarinal, a methyl ester of a γ-lactone and three unknown components, which could not be identified. It was also of interest how the composition of the oils changed when stored for one year at room temperature. That for, oils stored for one year have been profiled as well on GC/MS and LC/MS systems. The comparison showed that none of the three oils had changed a lot, except of relative peak intensities. However, the oils stored for one year showed different physical appearance: The colour of the oil had changed from mostly green – blue colours to ochre yellow tones or red tones. It was not possible to explain the change of colour because guaiazulene, which is responsible for the blue colour in water-steamed *Callitris* oils, could not be detected with GC/MS. Further on, two of the oils (32352 and 31973) have been fractionated using different chromatographic techniques such as normal phase glass column chromatography and preparative HPLC. The aim was to isolated new components. The fractions were tested for purity on the LC/MS and selected fractions which did not show contamination at wavelengths 210 and 280 were run on the NMR. Most of the selected fractions showed impurities but five components were isolated: sandaracopimarinol, sandaracopimaric acid, 18-Nor-8(14),15-pimaradien-4-ol, an isomer of 18-Nor-8(14),15-pimaradien-4-ol and citronellic acid. Pharmacological assays were done on

the crude oils and on the fractions as well. Antioxidant activity in the plant material was measured using the ORAC assay. The cytotoxicity assay was based on adenosine triphosphate (ATP). Unfortunately, the controls chlorambucil and curcumin often showed inconsistent results, so most of the cytotoxicity data should be seen as preliminary. Anti-inflammatory properties were tested with a competitive ELISA-assay. Unfortunately this test is not valid too because aspirin, the control, did not work on the plate.

6. RECOMMENDATION

The obtained cytotoxicity data should be seen as preliminary, so assays should be retested to get useful information about the pharmacological properties of *Callitris* oil. It was also disappointing that none of the tested fractions showed any anti-inflammatory activity. The components in the oils were expected to show activity. This assay should be repeated as well.

7. ZUSAMMENFASSUNG UND AUSBLICK

Die hauptsächliche Fragestellung dieser Diplomarbeit war es, ätherisches Öl aus dem Stamm und der Borke des *Callitris glaucophylla* Baumes chemisch zu charakterisieren. Dabei wurden von drei verschiedenen Sammelplätzen Hölzer wasserdampfdestilliert, um das Öl zu gewinnen. Die Öle wurden mittels GC/MS und LC/MS untersucht, um massenspektroskopische und UV-Daten zu erhalten. Es wurden sowohl Gemeinsamkeiten als auch Unterschiede zwischen den drei Sammelorten festgestellt. Den Ölen wurden Codenummern zugeordnet (31973, 32352, 32209). Die Öle 31973 und 32209 zeigten eine sehr ähnliche Zusammensetzung: Guaiol, ein Sesquiterpen, war Hauptkomponente in beiden Ölen. Ein überlappender Peak von Bulnesol, α- und β-Eudesmol wurde auch in diesen Ölen beobachtet. Das dritte ausgemachte Eudesmol, 10-Epi-γ-eudesmol, sowie die zwei Sesquiterpenlactone Columellarin und Dihydrocolumellarin wurde in allen drei Ölen beobachtet. Das ätherische Öl von 32352 zeigte Dihydrocolumellarin als Hauptinhaltsstoff. Interessant ist, dass Guaiol in diesem Öl gar nicht massenspektroskopisch auszumachen war, dafür aber eine Vielzahl anderer Komponenten vorkamen: Limonen, 1,8-Cineol, Methylmyrtenat, citronellic acid, α- und β-Selinen, γ- und β Costol, Sandaracopimarinal, der m/z zufolge ein Methylester eines γ–Lactons sowie drei unbekannte Komponenten, die nicht identifiziert werden konnten. Zusätzlich wurde der Frage nachgegangen, wie sich die Zusammensetzung der Öle ändert, nachdem sie für ein Jahr bei Raumtemperatur gelagert worden sind. Auch die gelagerten Öle wurden mittels GC/MS und LC/MS charakterisiert. Beim Überlappen der Chromatogramme wurde jedoch sichtbar, dass keines der Öle sich sehr verändert hatte. Lediglich die Peakintensitäten fielen mit der Lagerung. Diese Ergebnisse waren enttäuschend, denn physikalisch veränderten die Öle drastisch ihre Erscheinung: Die Farbe des Öles veränderte sich von satten Grün- und Blautönen in Gelb- bis Rotbrauntöne. Dieser Farbumschlag konnte nicht zufrieden stellend erklärt werden, da Guaiazulen, das laut Literatur für die blaue Farbe des wasserdampfdestillierten *Callitris*öls verantwortlich ist, nicht mittels GC/MS detektiert werden konnte. Weiters wurden die Öle 32352 und 31973 durch normalphasige Glassäulenchromatographie und präparative HPLC fraktioniert. Ziel war es, neue Komponenten aus den Fraktionen zu isolieren. Die Fraktionen wurden

auf Reinheit geprüft (LC/MS). Jene Fraktionen, die bei den Wellenlängen 210 und 280 nm keine zusätzlichen Peaks zeigten, wurden durch die NMR identifiziert. Die meisten Fraktionen zeigten starke Verunreinigungen, es konnten jedoch fünf Komponenten identifiziert werden: Sandaracopimarinol, sandaracopimaric acid, 18-Nor-8(14),15-pimaradien-4-ol, ein Isomer von 18-Nor-8(14),15-pimaradien-4-ol und citronellic acid. Pharmakologische Assays wurden auch durchgeführt, sowohl an den drei Ölen, als auch an den gewonnen Fraktionen. Antioxidative Aktivität wurde durch den ORAC assay getestet. Der Zytotoxizitätsassay basierte auf der Messung von Adenosintriphospats (ATP). Leider zeigten die Kontroll- und Referenzsubstanz Chlorambucil und Curcumin nicht immer eine Verringerung der Krebszellenanzahl. Dadurch sind diese Ergebnisse nur als vorläufig anzusehen. Antiinflammatorische Eigenschaften wurden mittels einem kompetitiven ELISA getestet. Auch hier zeigt die Positivkontrolle, Aspirin, keine Inhibition. Auch diese Ergebnisse sind nicht gültig.

Zytotoxizitäts- sowie antiinflammatorische Assays sollten wiederholt werden. Die gewonnenen Daten sind nicht zu gebrauchen, da die Positivkontrollen und Referenzsubstanzen oft unerwartete Daten geliefert haben. Besonders unglaubwürdig sind die Ergebnisse des ELISA, denn laut Literatur sind viele Komponenten der ätherischen Öle des *Callitris glaucophylla* stark antiinflammatorisch. Nachgewiesene antiinflammatorische oder zytotoxische Aktivitäten könnten wertvolle Entdeckungen sein!

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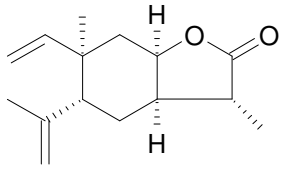
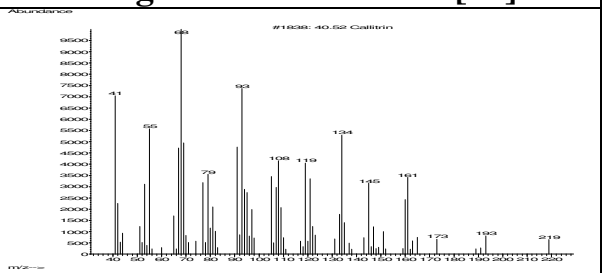
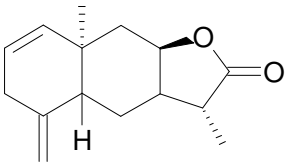
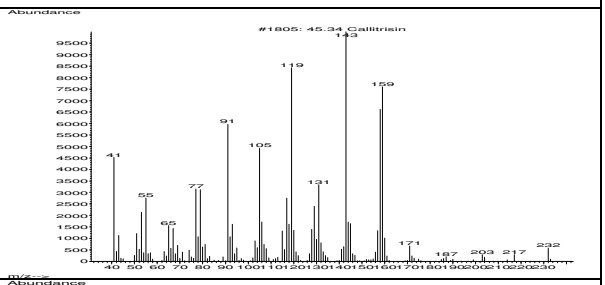
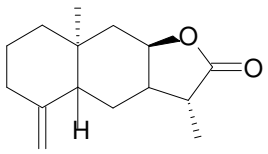
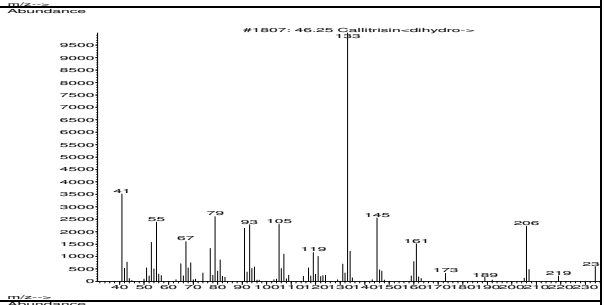
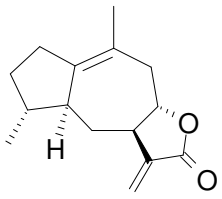
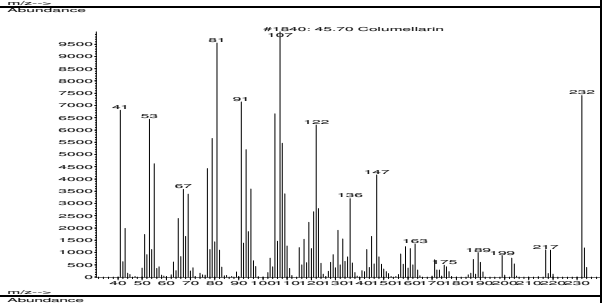
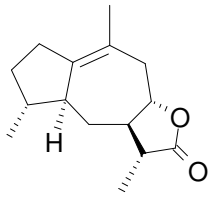
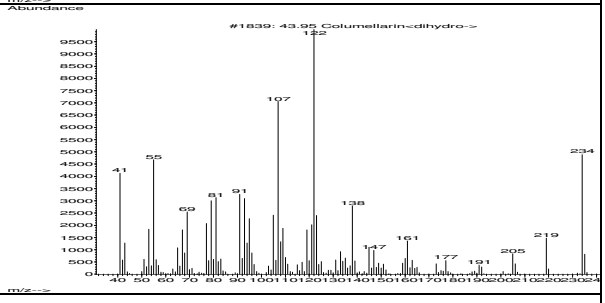
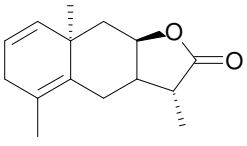
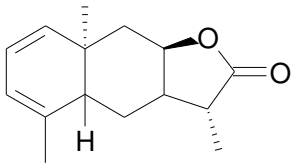
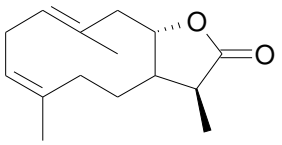
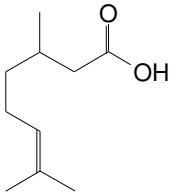
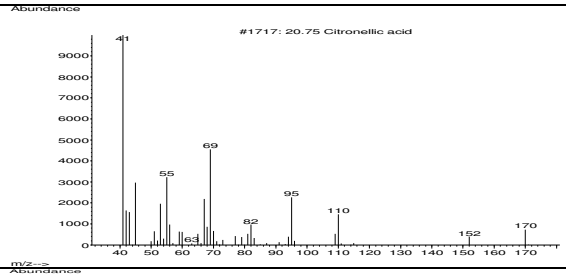
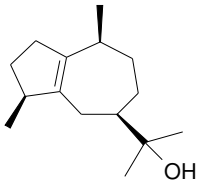
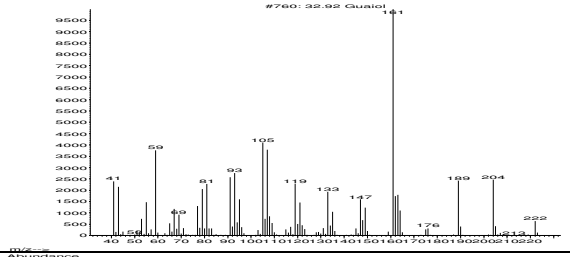
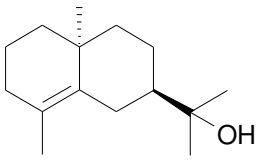
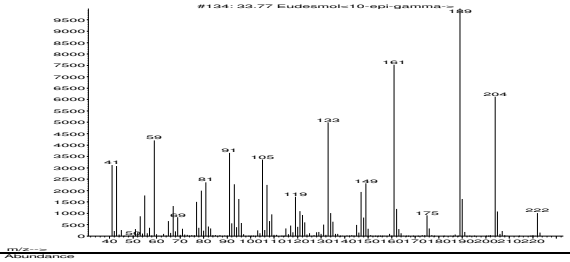
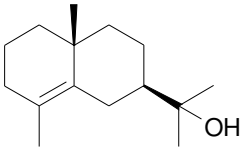
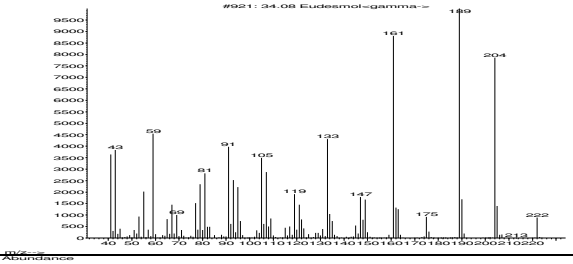
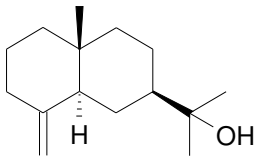
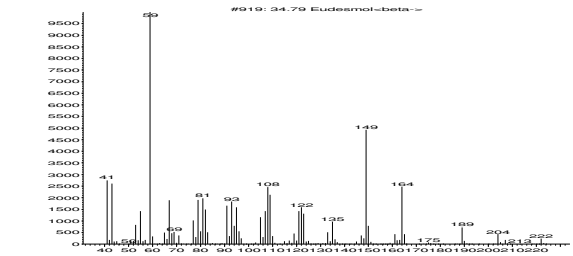
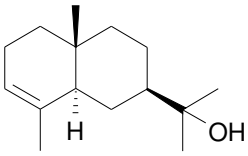
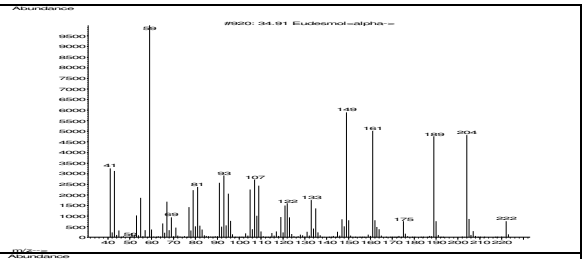
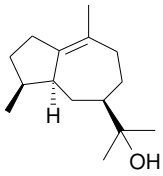
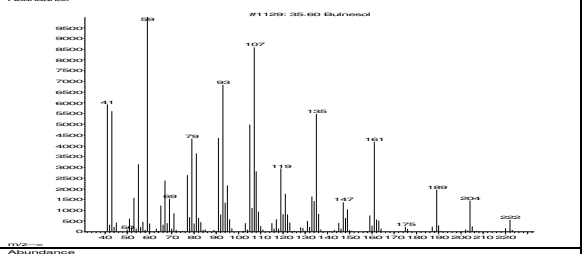
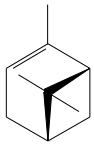
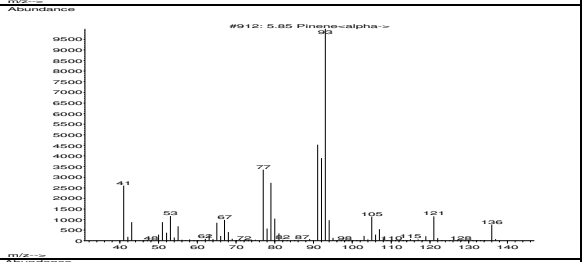
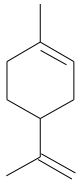
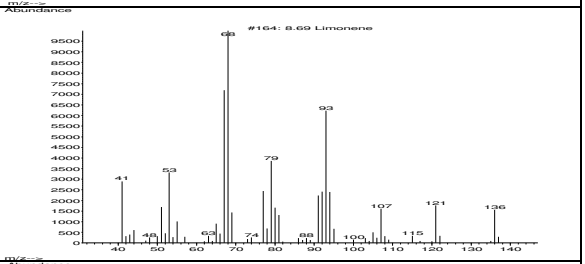
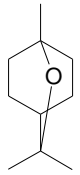
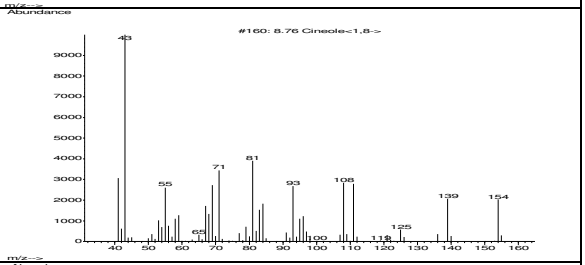
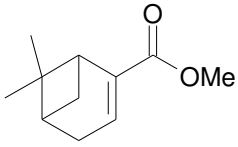
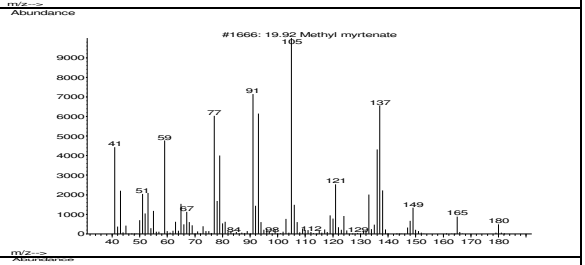
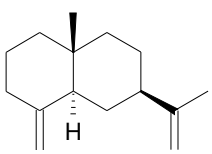
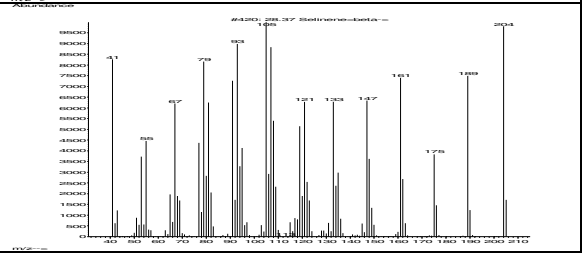
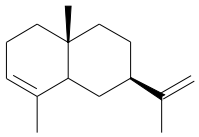
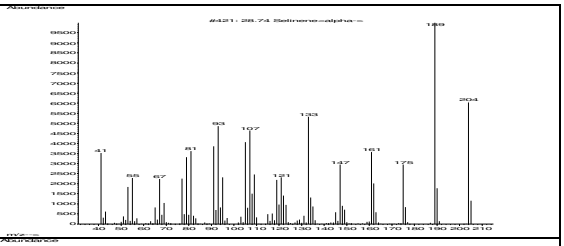
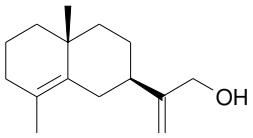
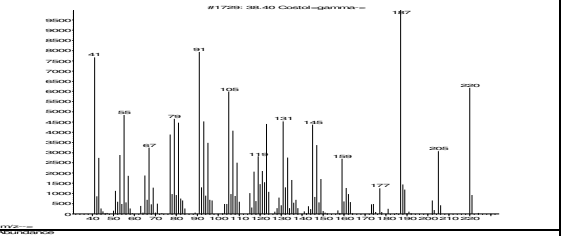
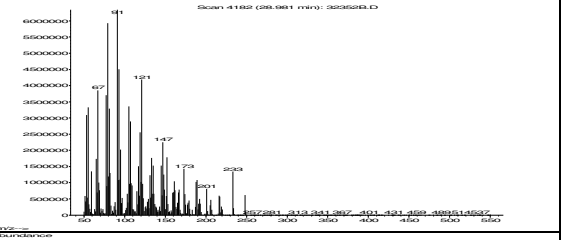
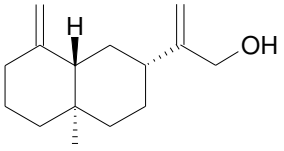
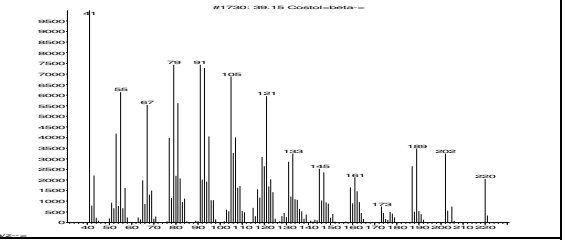
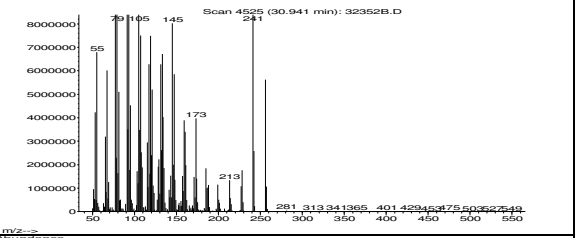
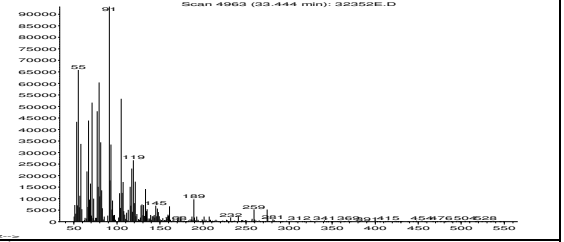
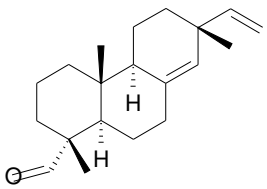
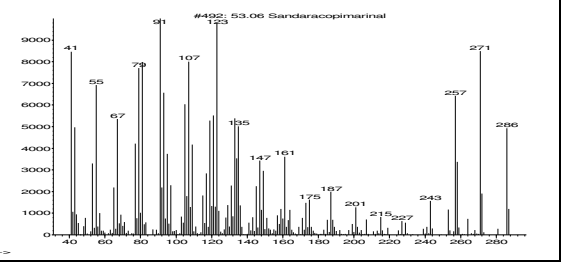
APPENDIX I

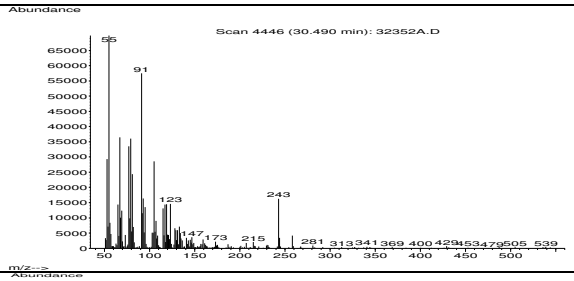
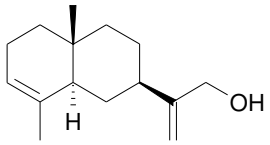
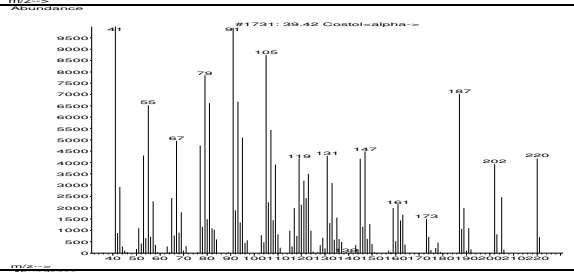
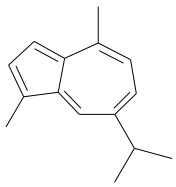
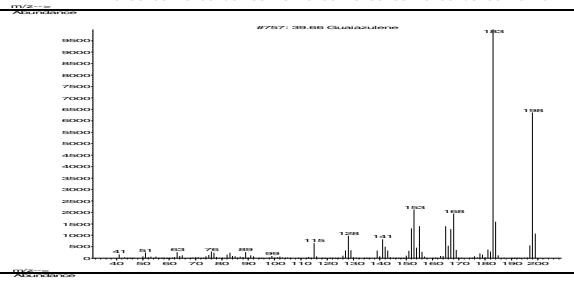
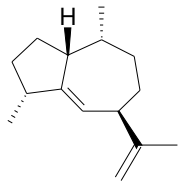
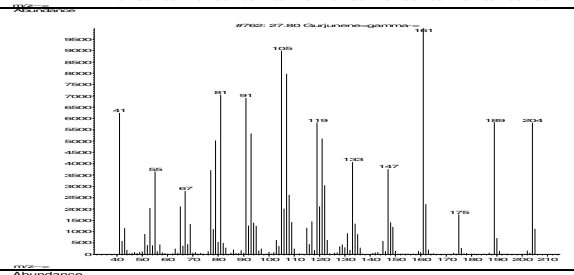
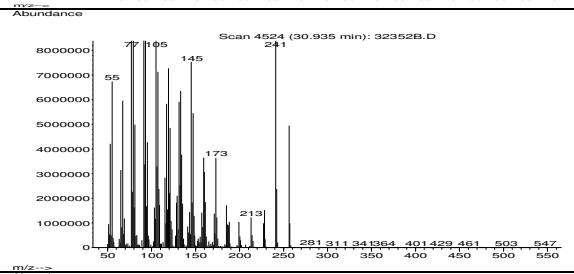
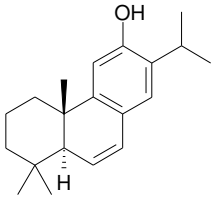
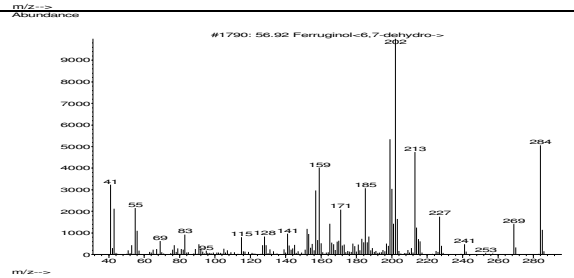
Table of molecular structure and fragmentation pattern [19] of observed components of *Callitris glaucophylla.*

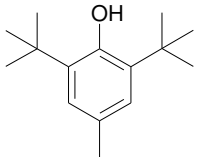
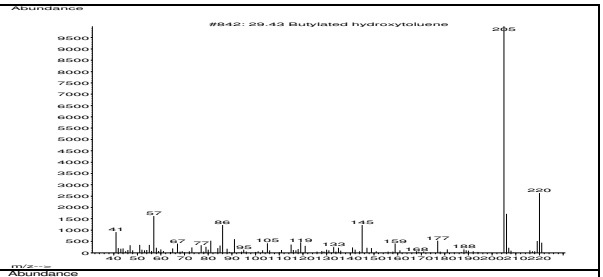
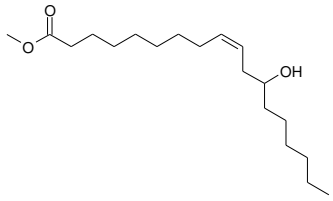
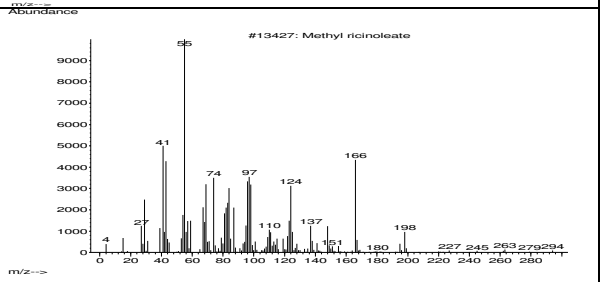
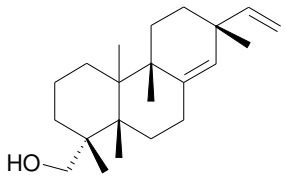
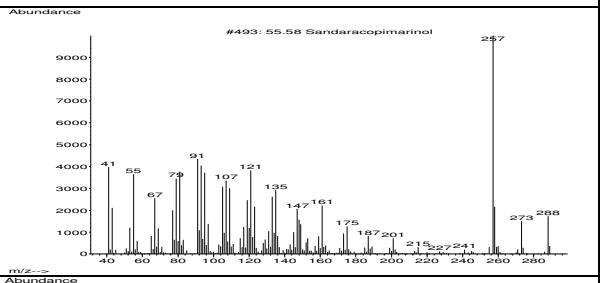
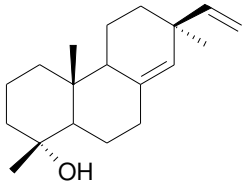
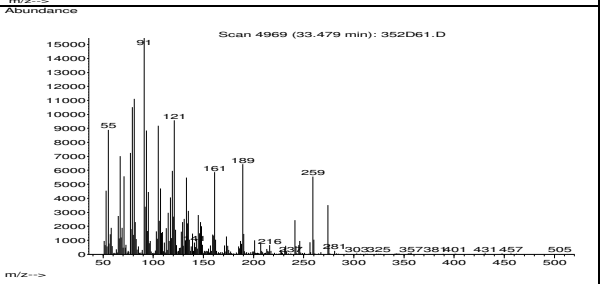
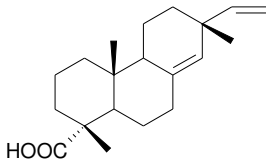
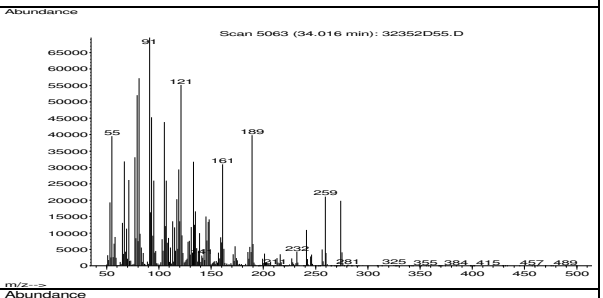
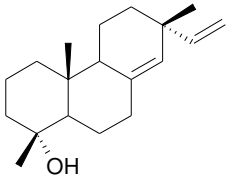
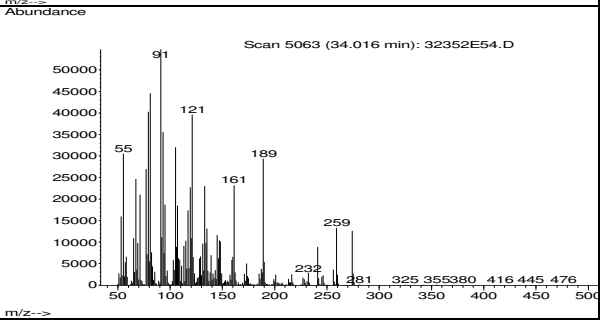
N°	Molecular Structure	Fragmentation Pattern [19]
1	CALLITRIN 	
2	CALLITRISIN 	
3	DIHYDROCALLITRISIN 	
4	COLUMELLARIN 	
5	DIHYDROCOLUMELLARIN 	
6	ISOMER OF CALLITRISIN 	---

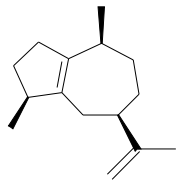
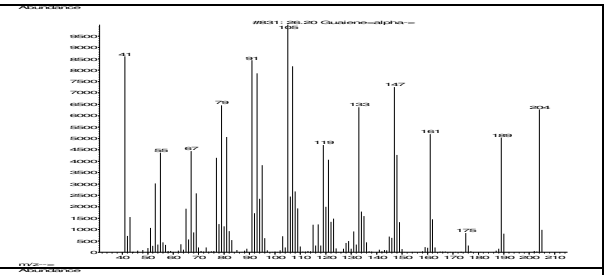
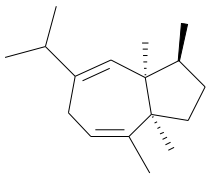
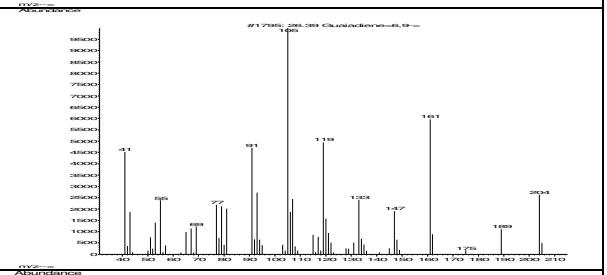
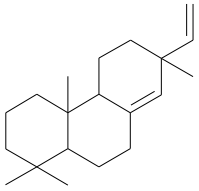
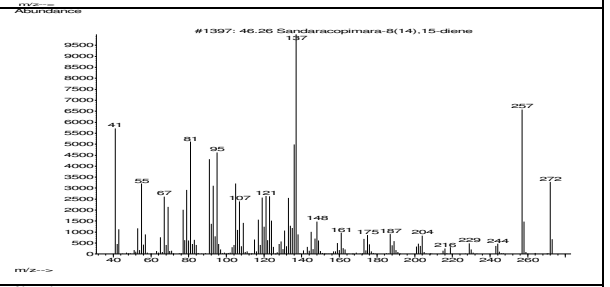
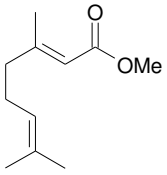
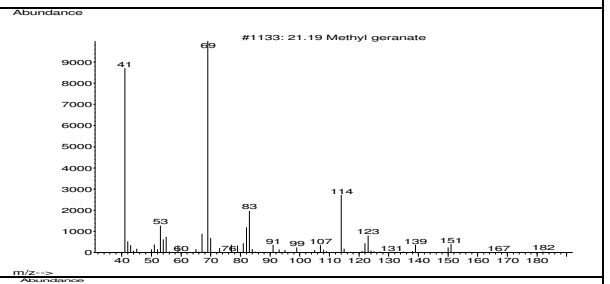
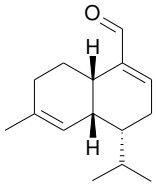
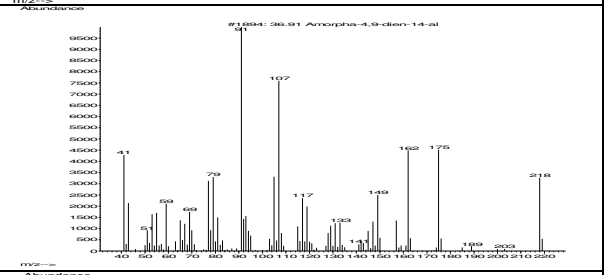
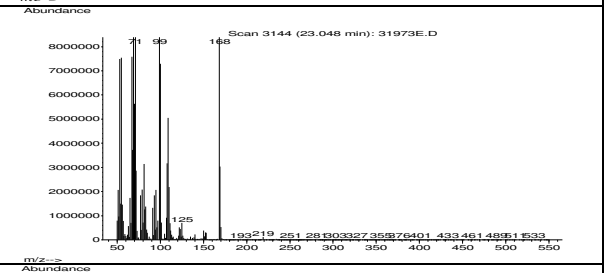
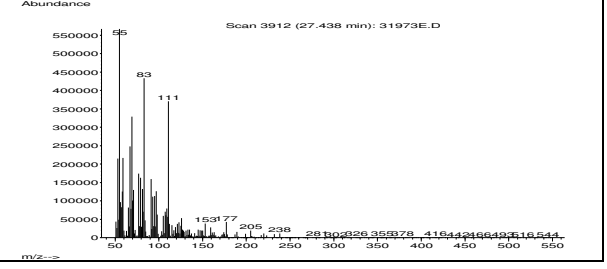
7	ISOMER OF CALLITRISIN 	---
8	ISOMER OF GERMACRANOLIDE 	---
9	CITRONELLIC ACID 	
10	GUAIOL 	
11	10-EPI-γ-EUDESMOL 	
12	γ-EUDESMOL 	
13	β-EUDESMOL 	

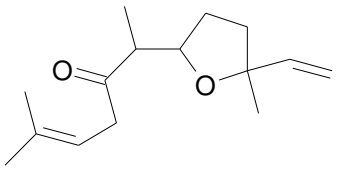
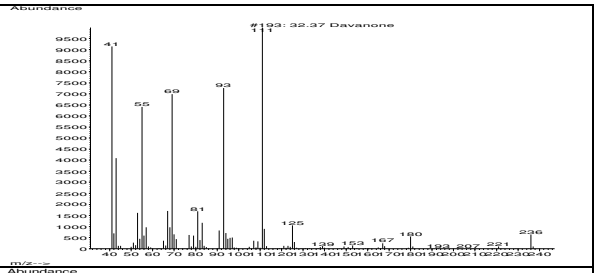
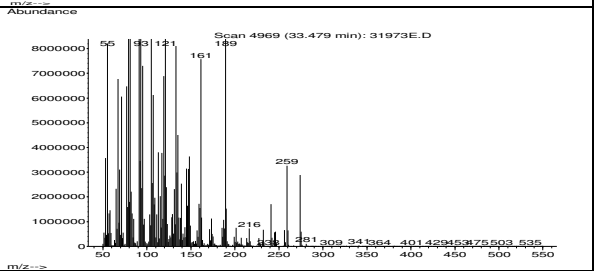
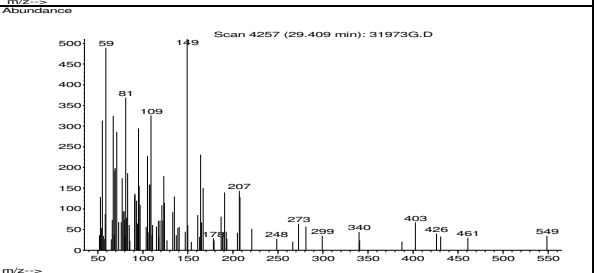
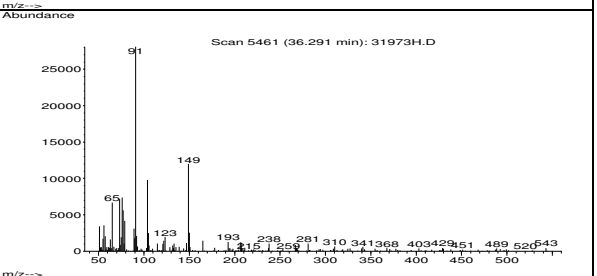
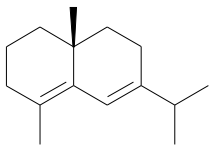
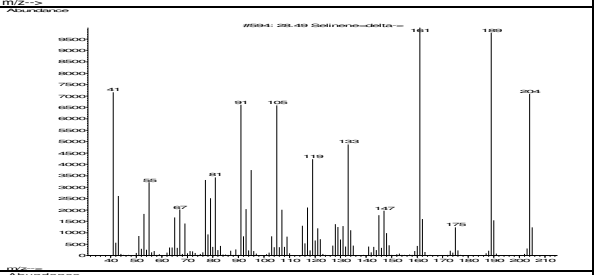
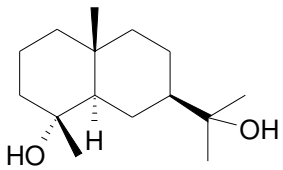
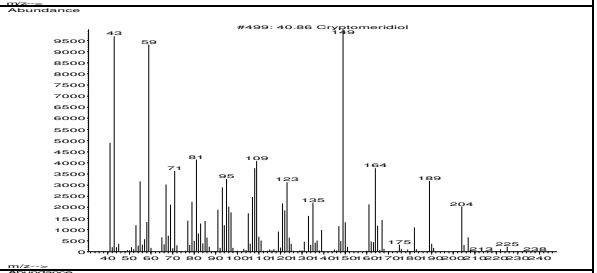
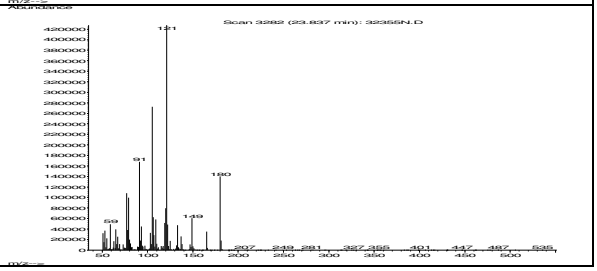
14	α-EUDESMOL 	
15	BULNESOL 	
16	α-PINENE 	
17	LIMONENE 	
18	1,8-CINEOLE 	
19	METHYL MYRTENATE 	
20	β-SELINENE 	

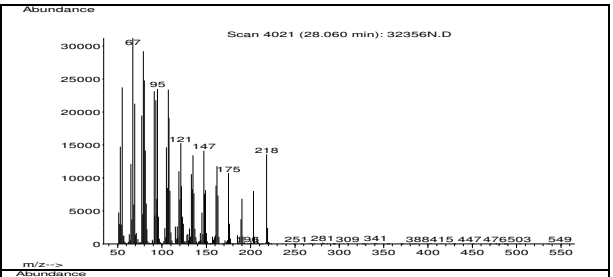
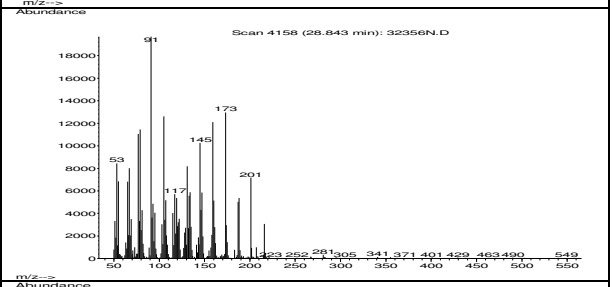
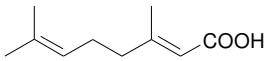
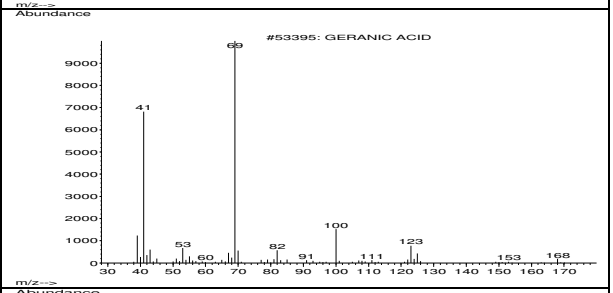
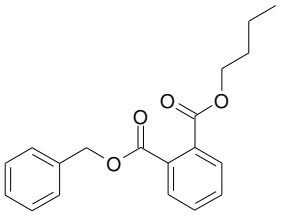
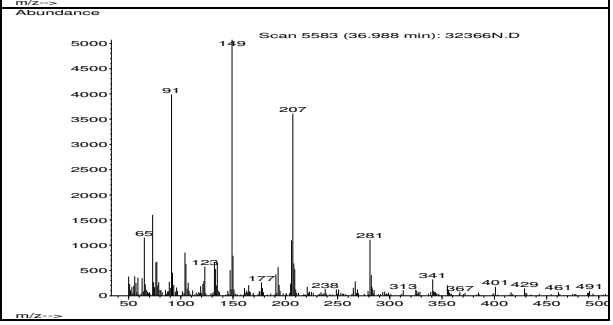
21	α-SELINENE 	
22	γ-COSTOL 	
23	METHYL ESTER OF A γ-LACTONE (mw 248) ---	
24	β-COSTOL 	
25	UNKNOWN (mw 256) ---	
26	UNKNOWN (mw 207) ---	
27	SANDARACOPIMARINAL 	

28	UNKNOWN (mw 281) ---	
29	α-COSTOL 	
31	GUAIAZULENE 	
32	γ-GURJUNENE 	
33	DERIVATIVE OF ISOPIMAROL ---	
34	6,7-DEHYDROFERRUGINOL 	

35	BUTYLATED HYDROXYTOLUENE 	
36	METHYL RICINOLEATE 	
37	SANDARACOPIMARINOL 	
38	18-NOR-8(14),15-PIMARADIEN-4-OL 	
39	SANDARACOPIMARINARIC ACID 	
40	ISOMER OF 18-NOR-8(14),15-PIMARADIEN-4-OL 	

41	α-GUAIENE 	
42	6,9-GUAIADIENE 	
43	SANDARACOPIMARA-8(14),15-DIENE 	
44	METHYL GERANATE 	
45	AMORPHA-4,9-DIEN-14-AL 	
46	UNKNOWN (mw 168) ---	
47	UNKNOWN (177) ---	

48	DAVANONE 	
49	UNKNOWN (mw 274) ---	
50	UNKNOWN (mw 164) ---	
51	UNKNOWN (mw 150) ---	
52	δ-SELINENE 	
53	CRYPTOMERIDIOL 	
54	UNKNOWN (mw 180) ---	

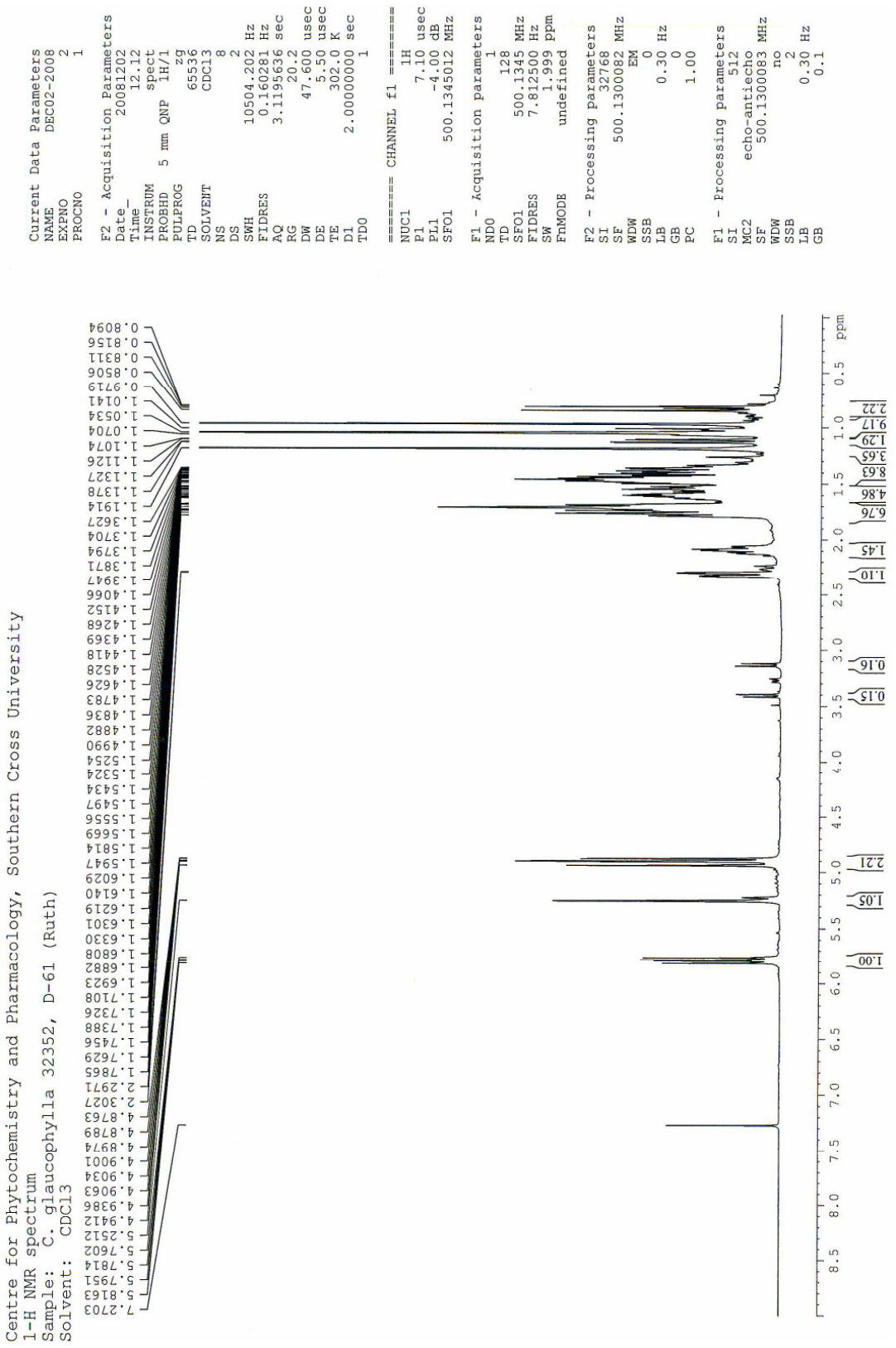
55	UNKNOWN (mw 218) ---	
56	UNKNOWN (mw 216) ---	
57	GERANIC ACID 	
58	BUTYL BENZYL PHTHALATE (BBP) 	

Adams, Robert P., Identification of Essential Oil Components by Gas Chromatography / Mass Spectrometry, 4th Edition, Allured Publishing Corporation, 2007of

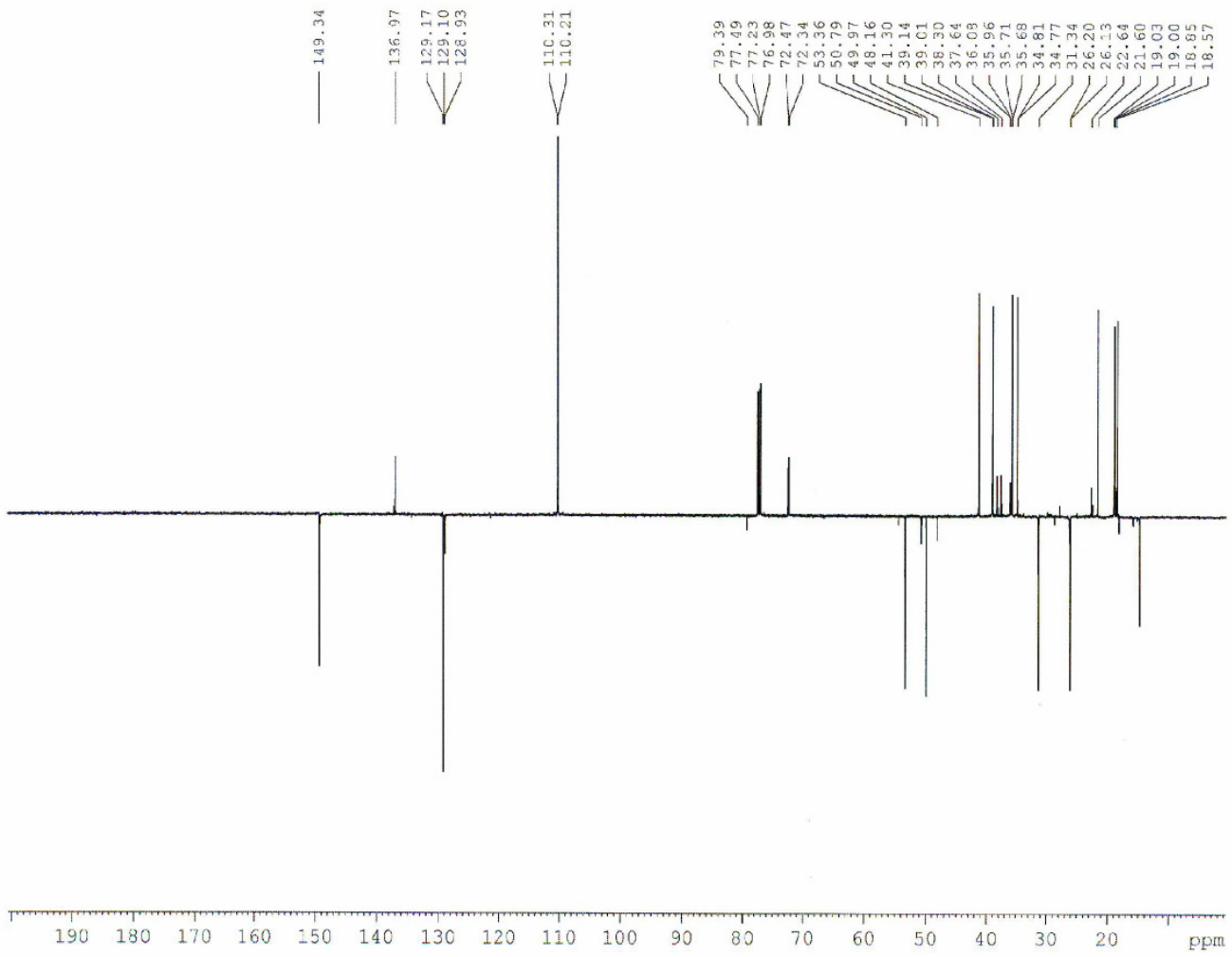
CN: chemical name used by Chemical Abstracts Service

APPENDIX II

NMR spectra of isolated compounds.



Centre for Phytochemistry and Pharmacology, Southern Cross University
JMOD Experiment
Sample: C. glaucophylla 32352, D-61 (Ruth)
Solvent: CDCl3

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PROCNO                        1

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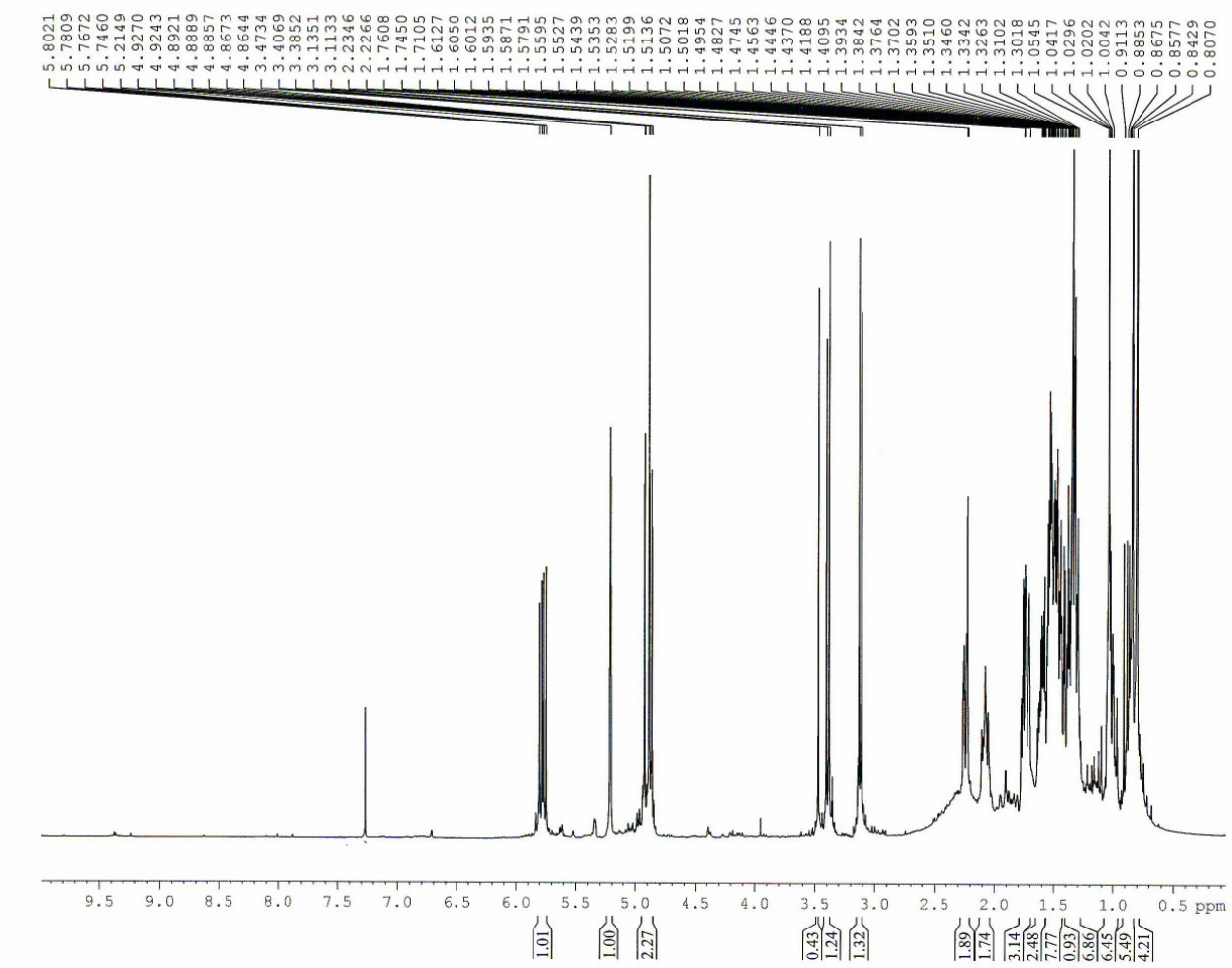
PULPROG                    jmod
TD                        65536
SOLVENT                    MeOH
NS                        20000
DS                            2
SWH                   30303.031 Hz
FIDRES                0.462388 Hz
AQ                    1.0813940 sec
RG                       9195.2
DW                       16.500 usec
DE                       20.00 usec
TE                       302.0 K
CNST2                155.0000000
CNST11                 1.0000000
D1                     1.00000000 sec
d20                    0.00645161 sec
DELTA                  0.00001401 sec
TD0                           1

======== CHANNEL f1 ========
NUC1                        13C
P1                       11.00 usec
p2                       22.00 usec
PL1                      -5.00 dB
SFO1                125.7715724 MHz

======== CHANNEL f2 ========
CPDPRG2                 waltz16
NUC2                        1H
PCPD2                    80.00 usec
PL2                      -4.00 dB
PL12                     18.50 dB
SFO2                500.1325000 MHz

F2 - Processing parameters
SI                        65536
SF                  125.7577616 MHz
WDW                          EM
SSB                           0
LB                        1.00 Hz
GB                            0
PC                        1.00
```

Centre for Phytochemistry and Pharmacology, Southern Cross University
1-H NMR spectrum
Sample: C. glaucophylla 32352, D-63 (Ruth)
Solvent: CDCl3

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PROCNO                 1

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PULPROG             zg
TD                65536
SOLVENT           CDCl3
NS                   64
DS                    2
SWH           10504.202 Hz
FIDRES         0.160281 Hz
AQ            3.1195636 sec
RG                 20.2
DW               47.600 usec
DE                5.50 usec
TE               302.0 K
D1           2.00000000 sec
TD0                    1

======== CHANNEL f1 ========
NUC1                  1H
P1                 7.10 usec
PL1               -4.00 dB
SFO1         500.1345012 MHz

F1 - Acquisition parameters
ND0                    2
TD                   128
SFO1          500.1325 MHz
FIDRES       46.949928 Hz
SW              12.016 ppm
FnMODE        undefined

F2 - Processing parameters
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SF          500.1300082 MHz
WDW                   EM
SSB                    0
LB                0.30 Hz
GB                     0
PC                1.00

F1 - Processing parameters
SI                   512
MC2         echo-antiecho
SF          500.1300083 MHz
WDW                   no
SSB                    2
LB                0.30 Hz
GB                   0.1
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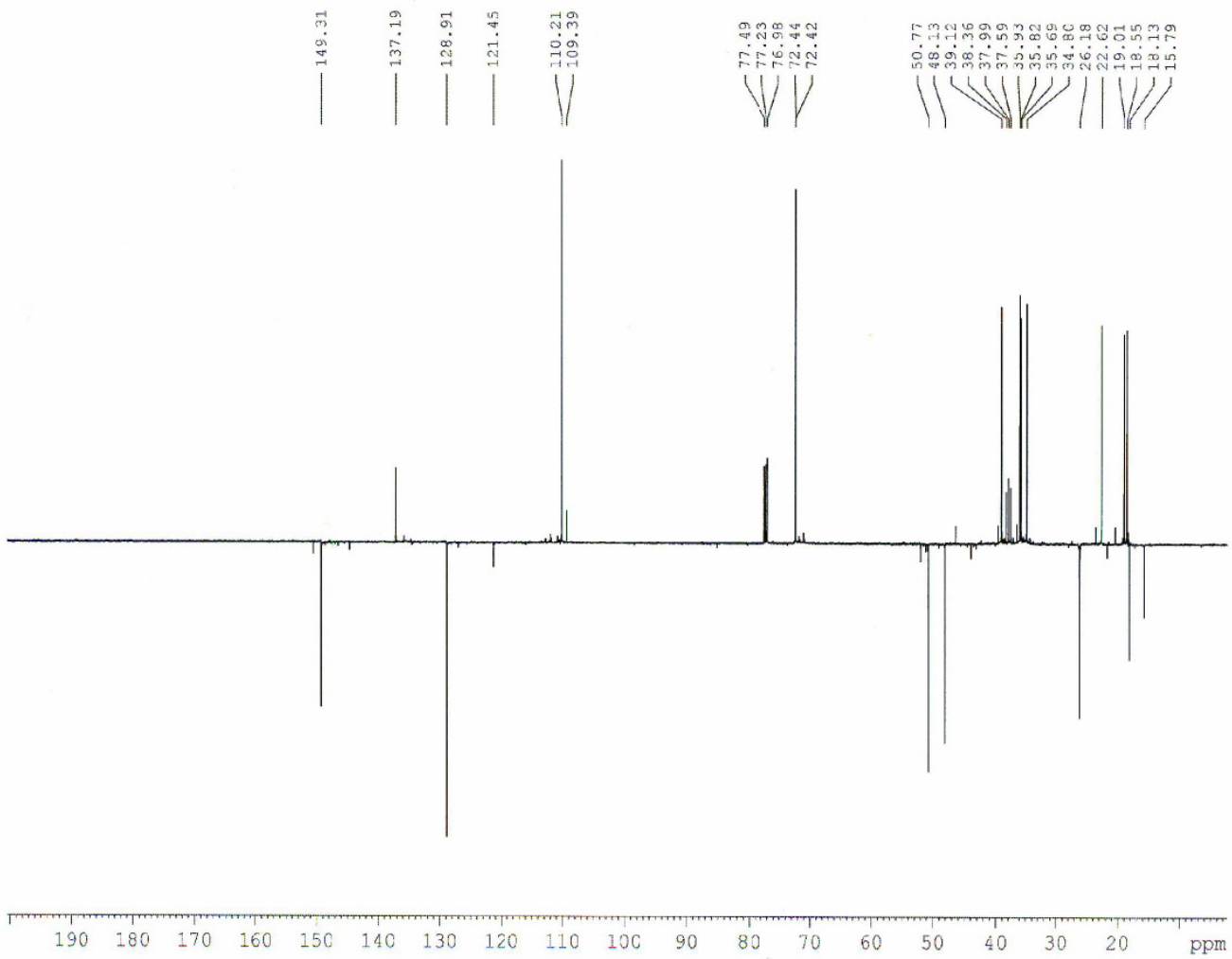
Centre for Phytochemistry and Pharmacology, Southern Cross University
JMOD Experiment
Sample: C. glaucophylla 32352, D-63 (Ruth)
Solvent: CDCl3



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PROCNO 1

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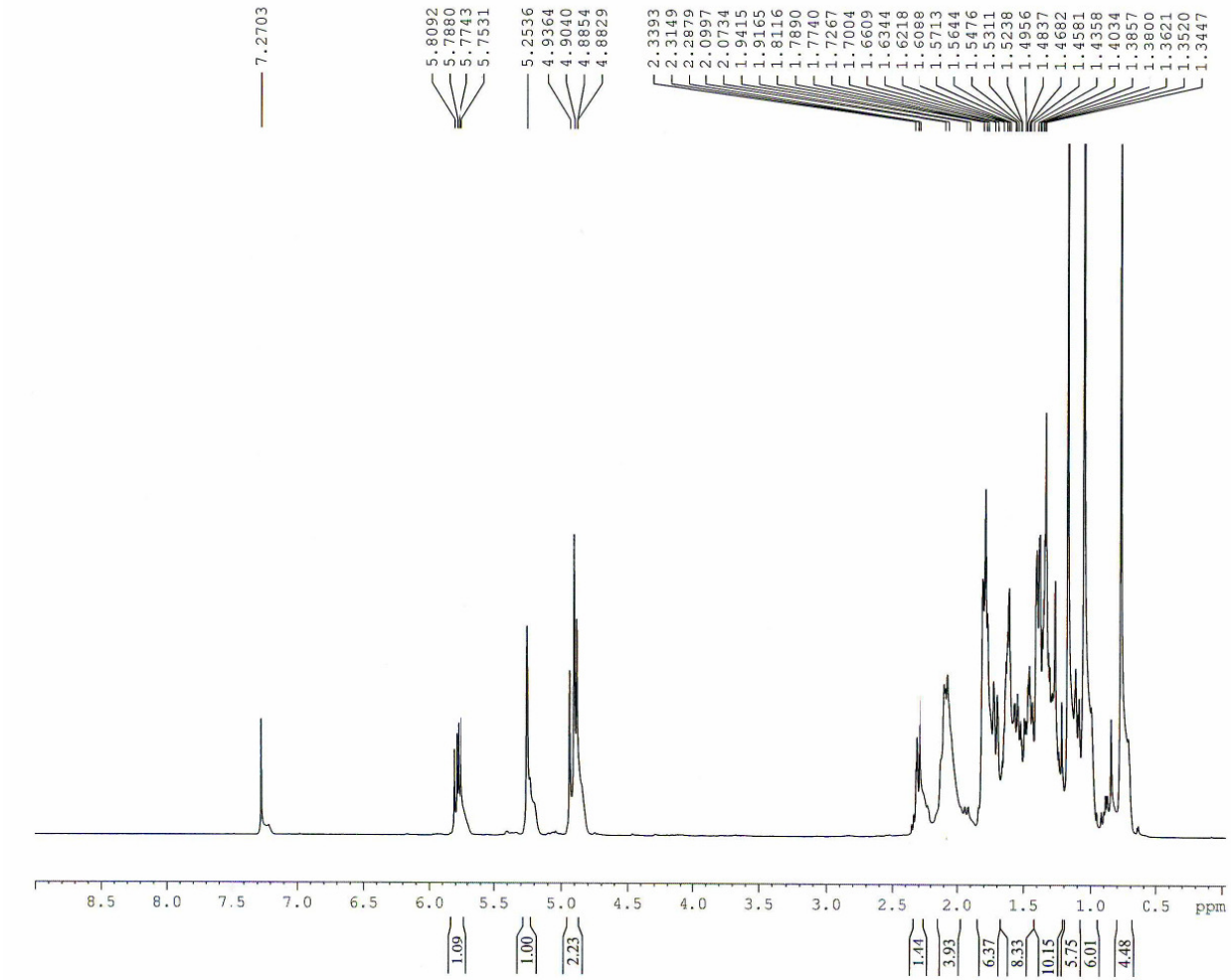
PULPROG jmod
TD 65536
SOLVENT MeOH
NS 15097
DS 2
SWH 30303.031 Hz
FIDRES 0.462388 Hz
AQ 1.0813940 sec
RG 9195.2
DW 16.500 usec
DE 20.00 usec
TE 302.0 K
CNST2 155.0000000
CNST11 1.0000000
D1 1.00000000 sec
d20 0.00645161 sec
DELTA 0.00001401 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 11.00 usec
p2 22.00 usec
PL1 -5.00 dB
SFO1 125.7715724 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -4.00 dB
PL12 18.50 dB
SFO2 500.1325000 MHz

F2 - Processing parameters
SI 65536
SF 125.7577631 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00

Centre for Phytochemistry and Pharmacology, Southern Cross University
1-H NMR spectrum
Sample: C. glaucophylla 32352, E-54 (Ruth)
Solvent: CDCl3

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PROCNO                 1

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PULPROG              zg
TD                65536
SOLVENT           CDCl3
NS                   64
DS                    2
SWH           10504.202 Hz
FIDRES         0.160281 Hz
AQ            3.1195636 sec
RG                 35.9
DW               47.600 usec
DE                5.50 usec
TE               302.0 K
D1            2.00000000 sec
TD0                    1

======== CHANNEL f1 ========
NUC1                  1H
P1                 7.10 usec
PL1               -4.00 dB
SFO1        500.1345012 MHz

F1 - Acquisition parameters
ND0                    2
TD                   128
SFO1          500.1325 MHz
FIDRES        46.949928 Hz
SW               12.016 ppm
FnMODE         undefined

F2 - Processing parameters
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SF          500.1300097 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00

F1 - Processing parameters
SI                   512
MC2          echo-antiecho
SF          500.1300083 MHz
WDW                QSINE
SSB                    2
LB                  0.30 Hz
GB                   0.1
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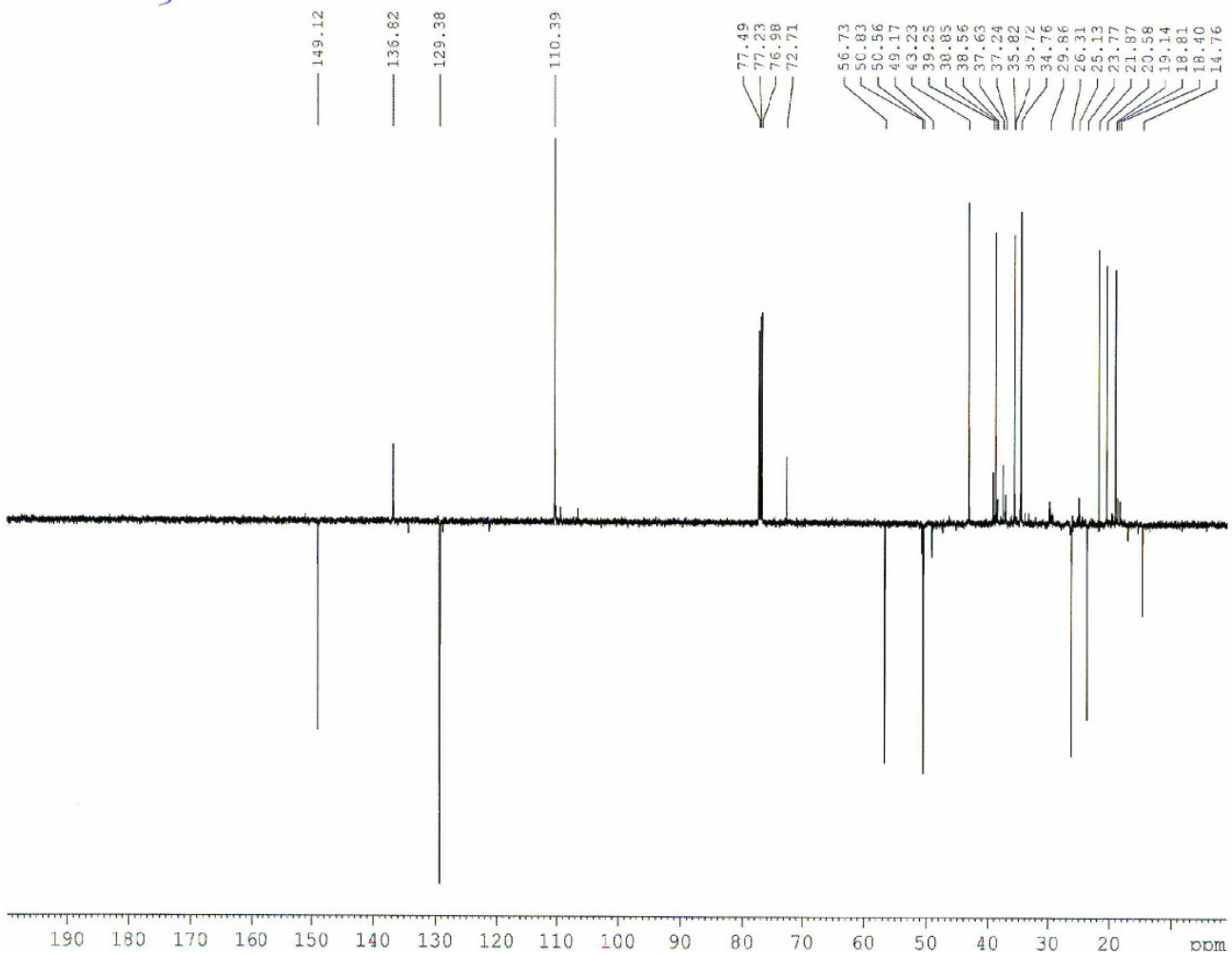
Centre for Phytochemistry and Pharmacology, Southern Cross University
JMOD Experiment
Sample: C. laucophylla 32357, E-54 (Rudd)
Solvent: MeOH



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EXPNO 5
PROCNO 1

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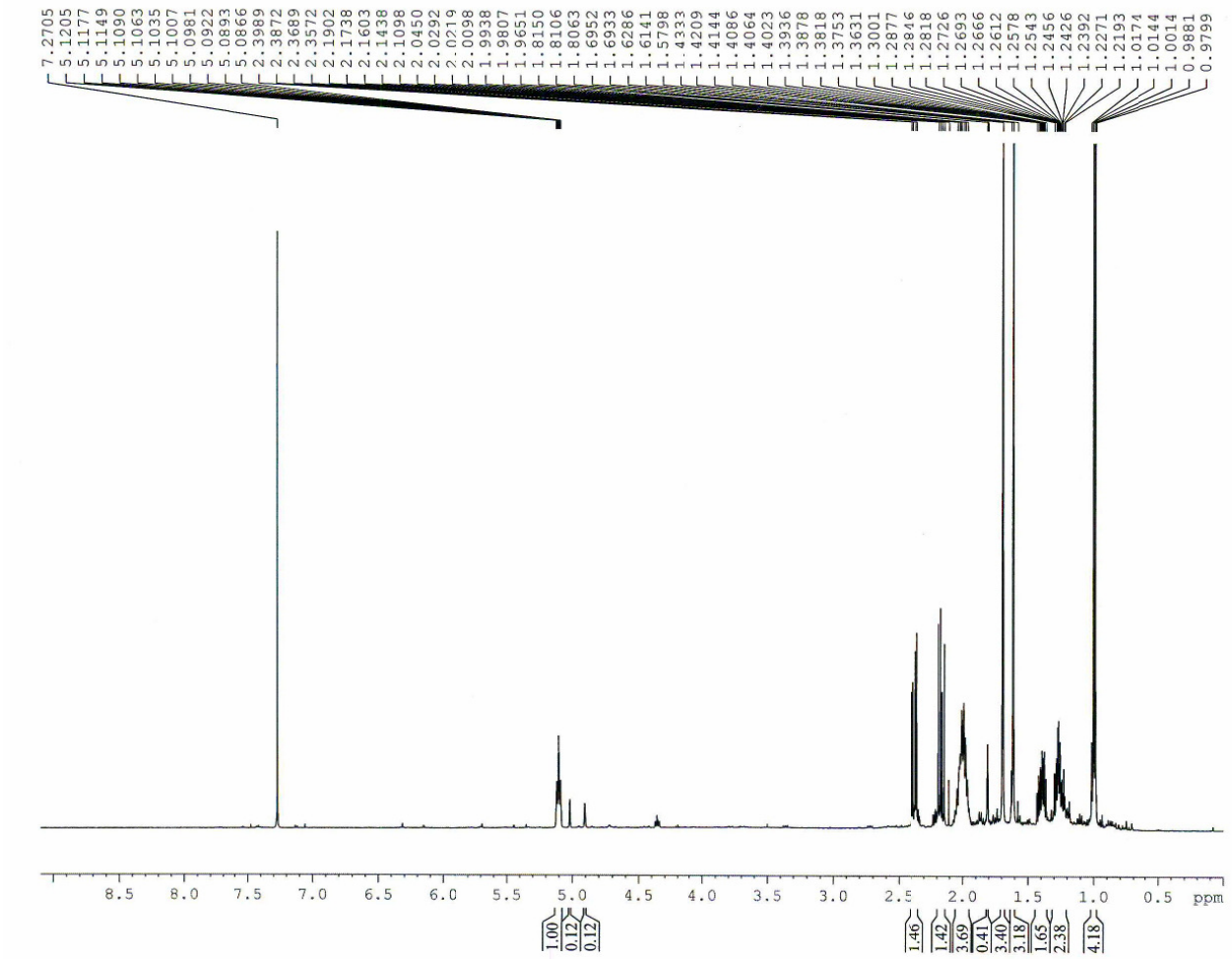
PULPROG jmod
TD 65536
SOLVENT MeOH
NS 20000
DS 2
SWH 30303.031 Hz
FIDRES 0.462388 Hz
AQ 1.0813940 sec
RG 9195.2
DW 16.500 usec
DE 20.00 usec
TE 302.0 K
CNST2 155.0000000
CNST11 1.0000000
D1 1.00000000 sec
d20 0.00645161 sec
DELTA 0.00001401 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 11.00 usec
p2 22.00 usec
PL1 -5.00 dB
SF01 125.7715724 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -4.00 dB
PL12 18.50 dB
SF02 500.1325000 MHz

F2 - Processing parameters
SI 65536
SF 125.7577607 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00

Centre for Phytochemistry and Pharmacology, Southern Cross University
1-H NMR spectrum
Sample: C. glaucophylla-F5-BC-13 (Ruth)
Solvent: CDCl3

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PULPRCG              zg
TD                65536
SOLVENT           CDCl3
NS                    8
DS                    2
SWH           10504.202 Hz
FIDRES         0.160281 Hz
AQ            3.1195636 sec
RG                  128
DW               47.600 usec
DE                5.50 usec
TE               302.0 K
D1            2.00000000 sec
TD0                    1

======== CHANNEL f1 ========
NUC1                  1H
P1                 7.10 usec
PL1               -4.00 dB
SFO1        500.1345012 MHz

F1 - Acquisition parameters
ND0                    1
TD                   128
SFO1          500.1345 MHz
FIDRES        7.812500 Hz
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FnMODE       undefined

F2 - Processing parameters
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GB                     0
PC                  1.00

F1 - Processing parameters
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WDW                   no
SSB                    2
LB                 0.30 Hz
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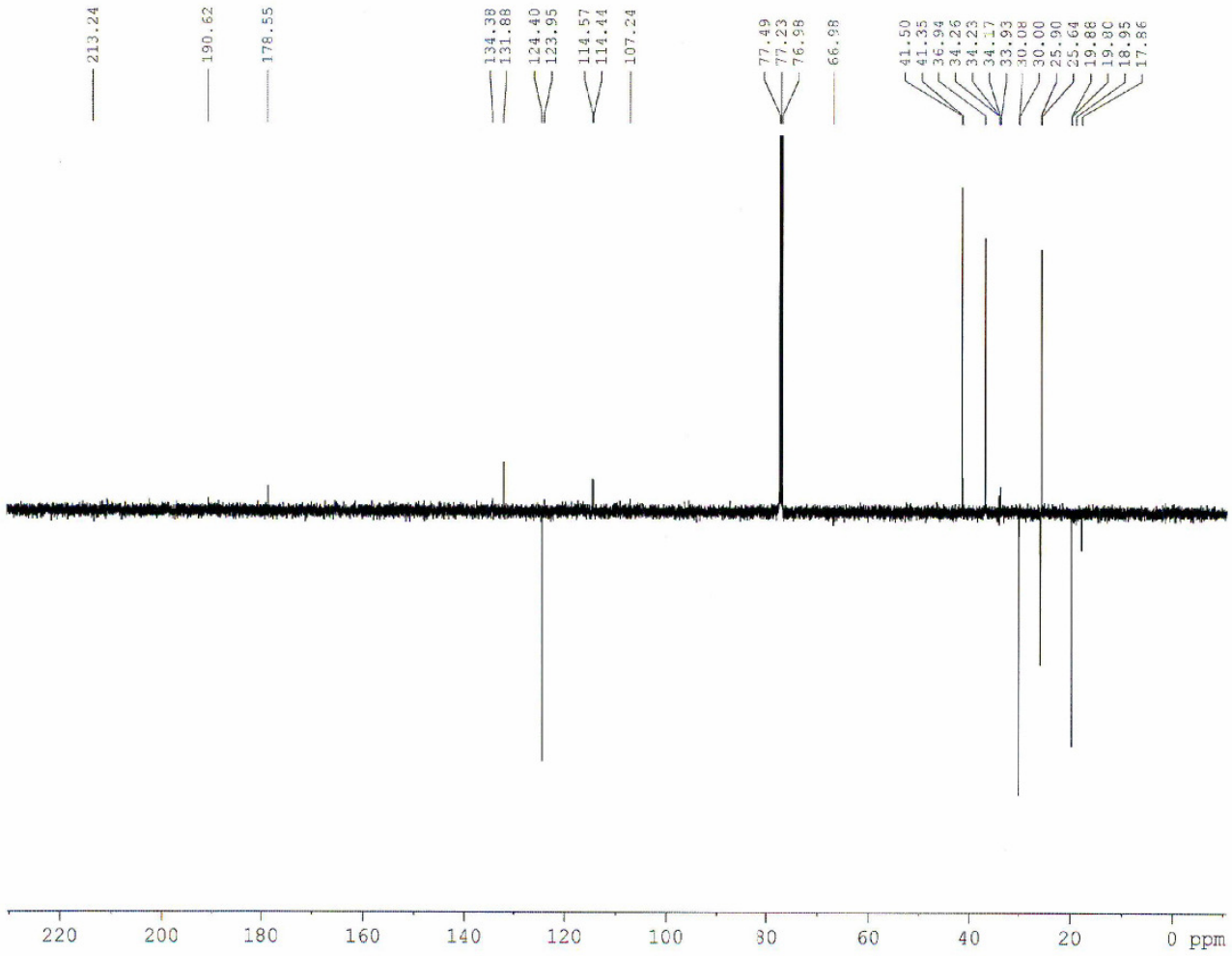
Centre for Phytochemistry and Pharmacology, Southern Cross University
JMOD Experiment
Sample: C. glaucophylla-F5-BC-13 (Ruth)
Solvent: CDCl3

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PROCNO                 1

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PULPROG            jmod
TD                65536
SOLVENT            MeOH
NS                25000
DS                    2
SWH           30303.031 Hz
FIDRES        0.462388 Hz
AQ            1.0813940 sec
RG               9195.2
DW              16.500 usec
DE              20.00 usec
TE              302.0 K
CNST2       155.0000000
CNST11        1.0000000
D1            1.00000000 sec
d20           0.00645161 sec
DELTA         0.00001401 sec
TD0                   1

======== CHANNEL f1 ========
NUC1                13C
P1              11.00 usec
p2              22.00 usec
PL1             -5.00 dB
SF01       125.7715724 MHz

======== CHANNEL f2 ========
CPDPRG2         waltz16
NUC2                1H
PCPD2           80.00 usec
PL2             -4.00 dB
PL12            18.50 dB
SF02       500.1325000 MHz

F2 - Processing parameters
SI                 65536
SF         125.7577593 MHz
WDW                   EM
SSB                    0
LB              1.00 Hz
GB                     0
PC              1.00
```

Curriculum Vitae

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Familienstand:	ledig

Studium und Ausbildung

09/08-02/09	**Diplomarbeit** an der Southern Cross University, Lismore, NSW, Australien
10/02–Dato	Diplomstudium **Pharmazie** an der Universität Wien
9/98-6/02	**Bundesoberstufenrealgymnasium Bad Hofgastein**; Matura mit gutem Erfolg

Arbeitserfahrung

9/2009	Posterpräsentation der Diplomarbeit beim **40th International Symposium On Essential Oils** in Savigliano (Italy)
9/2007–10/2007	**Internationales Forum Gastein**, Kongresshostess für das "Europäisches Gesundheitsforum Gastein" (http://www.ehfg.org).
08/2005–09/2005	**Kurapotheke Bad Hofgastein**, Ferialpraktikantin.
11/2005–11/2005	**Mundipharma**, Hostess am Wiener Schmerztag im Rathaus.

Sprachen

Deutsch	Muttersprache
Englisch	Fließend in Wort und Schrift
Französisch	Grundkenntnisse