Agriculture **2014**, *4*, 308-316; doi:10.3390/agriculture4040308

OPEN ACCESS

agriculture

ISSN 2077-0472

www.mdpi.com/journal/agriculture

Article

Volatiles from the Rare Australian Desert Plant *Prostanthera centralis* B.J.Conn (Lamiaceae): Chemical Composition and Antimicrobial Activity

Timothy L. Collins *, Graham L. Jones † and Nicholas J. Sadgrove †

Pharmaceuticals and Nutraceuticals Group, Centre for Bioactive Discovery in Health and Ageing, University of New England, Armidale, NSW 2351, Australia; E-Mails: gjones2@une.edu.au (G.L.J.); nsadgrov@myune.edu.au (N.J.S.)

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: tcollins@myune.edu.au; Tel.: +6-143-162-3015.

External Editor: Muraleedharan G. Nair

Received: 5 November 2014; in revised form: 19 November 2014 / Accepted: 25 November 2014 / Published: 12 December 2014

Abstract: Hydrodistilled essential oils and dichloromethane (DCM) extracted volatiles were taken from cultivated specimens of *Prostanthera centralis*, endemic to central Australia. All volatiles were chemically characterised by Gas Chromatography-Mass Spectroscopy (GC-MS) with the use of authentic standards, followed by Nuclear Magnetic Resonance (NMR) Spectroscopy. The antimicrobial activity of the essential oils was measured against a range of Gram-negative and Gram-positive bacterial species using a micro-titre plate broth dilution assay. Twenty-two compounds were identified as components of the sweet smelling aromatic essential oil and DCM extracts, both showing a relatively high abundance of prostantherol. The volatiles extracted using DCM, differed only in the relative abundance of the major components and the lack of ledol and squamulosone. This study constitutes the first time ledol and squamulosone have been identified in a *Prostanthera* species. Antimicrobial assays showed moderate to high inhibitory activity against some Gram-positive bacteria and the yeast *Candida albicans*.

Keywords: *Prostanthera centralis*; essential oils; prostantherol; chemical composition; antimicrobial activity

1. Introduction

The genus *Prostanthera* (Lamiaceae) comprises approximately 100 species endemic to Australia [1]. *Prostanthera centralis* B.J.Conn is a shrub, 0.3–1 m in height, restricted to the Central Ranges bioregion, growing in gravelly sands and quartzite scree [2]. *P. centralis* is classified as "near threatened" in the Northern Territory and P3 (Poorly-known) in Western Australia.

Most species of *Prostanthera* yield appreciable amounts of essential oils upon hydrodistillation [3,4]. It has been documented that Aboriginal people utilised a few species of *Prostanthera* for medicinal and aromatherapeutic purposes, but there is no documented use of *P. centralis* [5–7]. In previous studies, components of essential oils produced from *Prostanthera* species included 1,8-cineole, maaliol and prostantherol [3,8], all of which have antimicrobial activity [8] while some of these components have anti-inflammatory and antinociceptive effects [9,10]. The most recent study of *Prostanthera*, which did not include *P. centralis*, helped to resolve many but not all of the phylogenetic relationships in the genus using nuclear and chloroplast sequence analysis [11]. In concert with these molecular studies, it has been shown that a chemotaxonomic approach, using chemical variability of essential oils, has the potential to contribute to the collective understanding of species limits and phylogenetic relationships in *Prostanthera* and other species [12,13].

The aim of the current study is to chemically characterise the hydrodistilled essential oil and solvent extracted volatiles from *P. centralis*, and to measure antimicrobial activity against a range of microorganisms. An understanding of volatiles from this species builds upon knowledge of the chemical variability (chemotaxonomy) of this genus and corroborates previous studies [3,12]. Furthermore, analysis of essential oils identifies possible antimicrobial or antifungal compounds with potential contemporary therapeutic use in topical ointments. In addition, characterisation of existing and novel components may provide chemical scaffolds for subsequent pharmaceutical development.

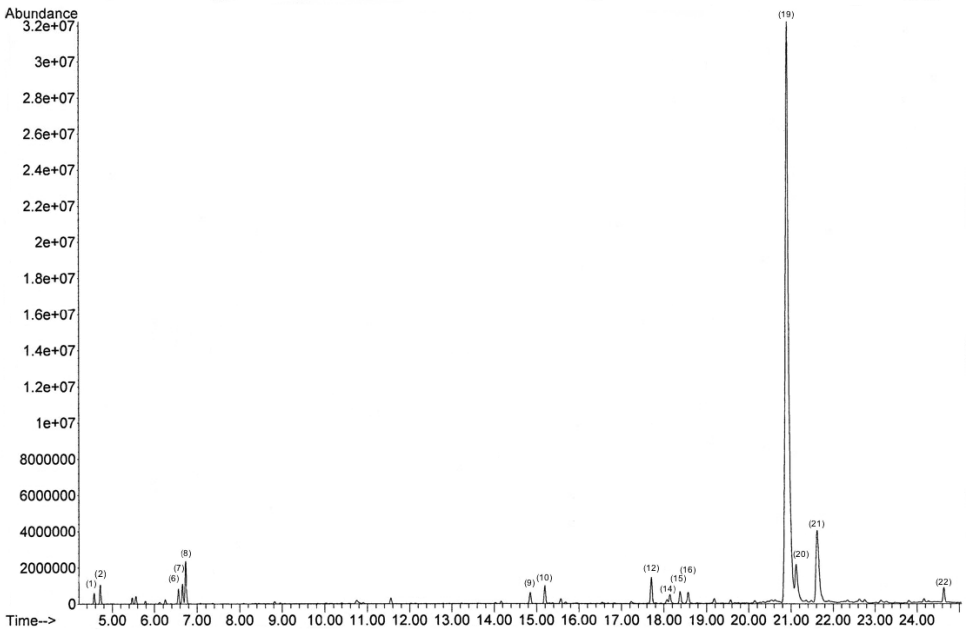
2. Results and Discussion

2.1. GC-MS Analysis

Hydrodistillation produced fifteen identified compounds (Figure 1) in 0.38 g of a pale yellow, viscous, aromatic oil, produced from 77 g of fresh leaves. Additional volatiles, together with those observed in the essential oil, were identified in the dichloromethane (DCM) extracted concrete (Table 1). The sesquiterpene alcohol prostantherol was the most abundant chemical entity of the essential oil (EO) and DCM extracts. Other compounds present in concentrations above 2.5% included: α-pinene, 1,8-cineole, alloaromadendrene, germacrene, ledol, and β-gurjunene, an analogue of prostantherol. Prostantherol was first identified and described from *P. aff. melissifolia* and *P. rotundifolia* and has the molecular formula of $C_{15}H_{26}O$ [8]. Similar unidentified sesquiterpenoid alcohols have been extracted from *P. aff. ovalifolia* and *P. aff. phylicifolia* [3]. α-Pinene and 1,8-cineole are common components of essential oils from

Prostanthera species as well as many other aromatic plants [3,9]. From all samples analysed, the sesquiterpene alloaromadendrene was measured in the range 3.0%–5.8%, similar to the concentration in the essential oil of *Cinnamomum osmophloeum* where it acts as an important *in vivo* antioxidant [14].

Figure 1. Chromatogram of fifteen identified compounds in essential oil from hydrodistillation of *Prostanthera centralis* leaves. Numbered peaks correspond to identified compounds in Table 1.



The present study constitutes the first time ledol has been confirmed in an essential oil from a *Prostanthera* species. The previous study by Lassak [3], on essential oils from such species, detected an unidentified sesquiterpene alcohol eluting just after globulol, which is most likely to be ledol. Ledol is a sesquiterpene alcohol found in many plant species including *Phebalium squameum* [15], recently revised to *Nematolepis squameum* Labill (Paul G. Wilson). In the current study, squamulosone was detected at low concentration in the hydrodistilled EO but not at all in the DCM extracts most likely due to at a concentration lower than our minimum peak area used in quantification. Squamulosone, a sesquiterpene ketone, shares the same structural skeleton to the alcohol ledol [16,17]. Squamulosone has previously only been identified in extracts and essential oils from *Phebalium squamulosum* (Rutaceae), where it was first described [17,18].

Samples DCM-1 and DCM-3 appear very similar quantitatively, with percentage yield of individual compounds closely matched throughout Table 1. These two samples may possibly be genetically identical (clones), as the plants were propagated vegetatively from material obtained from several individual plants (Timothy L. Collins, personal observation). Samples DCM-2 and DCM-4 appear to be quantitatively different, largely in relation to percentage yields of many compounds including 1,8-cineole, germacrene and prostantherol. Having said this, comparison of EO chemical entities within small population sizes can be problematic as concentrations can vary dependent upon the metabolic state of the plants related to season, soil type, *etc.* [19]. Natural variation of wild specimens with a variety of provenances may or may not demonstrate greater variability than that seen in Table 1.

Table 1. Percentage chemical character determined by Gas Chromatography-Mass Spectroscopy (GC-MS) of essential oil (EO) from *Prostanthera centralis* and percentage chemical character of volatiles extracted from plants 1–4 using dichloromethane (DCM1–DCM4). Compounds are numbered sequentially according to rank by arithmetic index (AI) and the EO % yield (g/g) is also included. Published values of AI (Pub. AI) are from Adams [20].

No.	Compound	Extract Type		EO	DCM-1	DCM-2	DCM-3	DCM-4
		Yield g/g		0.38				
		AI	Pub. AI					
1	α-Thujene	928	924	0.5	1.2	1.7	0.9	0.6
2	α-Pinene	935	932	0.9	2.4	1.1	2.0	2.6
3	Sabinene	975	969	-	0.4	0.3	0.4	0.4
4	β-Pinene	980	974	-	0.5	0.3	0.5	0.6
5	Carene	1013	1011	-	-	0.4	-	-
6	p-Cymene	1026	1020	0.7	0.3	-	0.3	0.4
7	Limonene	1030	1024	1.0	0.9	0.5	0.8	1.0
8	1,8-cineole	1033	1026	2.2	7.1	4.3	7.7	11.1
9	α-Terpinyl acetate	1352	1346	0.7	0.4	0.3	0.5	0.5
10	Nerol acetate	1365	1359	1.1	0.7	0.7	0.6	0.6
11	α-Copaene	1380	1374	-	0.7	1.1	0.8	0.6
12	Alloaromadendrene	1466	1458	1.6	4.0	5.8	3.9	3.0
13	γ-Muurolene	1481	1478	-	0.7	1.2	0.6	0.4
14	Germacrene	1484	1485	0.6	2.1	5.5	1.3	-
15	Amorphene	1494	1495	0.8	2.6	4.9	2.0	1.2
16	Bicyclogermacrene	1501	1500	0.8	1.8	1.0	1.3	1.7
17	Premnaspirodiene	1510	1506	-	-	0.3	-	-
18	δ-Cadinene	1528	1522	-	1.0	2.2	0.6	-
19	Prostantherol	1602	1590	75.5	62.7	57.0	64.6	65.9
20	Ledol	1611	1602	3.9	-	-	-	-
21	β-Gurjunene	1638	1638	8.7	7.3	10.1	6.7	4.8
22	Squamulosone	1772	1771	1.0	-	-	-	-

Chemical characterisation of *P. centralis* shows that this taxon contains a relatively high abundance of prostantherol, a compound seen in only four other species of *Prostanthera*, as well as a relatively high yield of EO [3,8]. The relatively high abundance of prostantherol and trace amounts of the two esters, nerol acetate and α-terpinyl acetate, not only makes this oil rather pleasant and fruity smelling but also means it is novel to the genus.

Thus, the chemical character of EO from *P. centralis* clearly illustrates the widespread chemical variability in *Prostanthera*.

2.2. Antimicrobial Activity

Antimicrobial activity, as determined by a micro-titre plate broth dilution assay (Minimum Inhibitory Concentration: MIC), is presented in Table 2. In the context of other common essential oils, antimicrobial activity may be regarded as moderate to high against the Gram-positives *Staphylococcus aureus*, *S. epidermidis* and *Bacillus subtilis*, but less so against the Gram-negative bacteria, and only

moderate activity being recorded against the yeast *Candida albicans*. With some MICs as low as 0.13% v/v (approximate 0.1 mg/mL) against the Gram-positive species, this EO may be comparable with other moderate to highly antimicrobial oils including those from *Thymus vulgaris* or *Syzygium aromaticum* [21]. MIC values of Tetrcycline and Nystatin are within the expected range.

Table 2. Minimum Inhibitory Concentration (MIC) (Mean ± Standard Error of the Mean (SEM)) of *Prostanthera centralis* essential oil (*P. centralis* EO) against microorganisms in % v/v agar with positive controls in μg/mL ± SEM (Tetracycline and Nystatin).

	***P. centralis* EO**	**Tetracycline**	**Nystatin**
Staphylococcus aureus	0.13 ± 0.02	0.13 ± 0.22	-
Staphylococcus epidermidis	0.25 ± 0.02	0.13 ± 0.22	-
Salmonella typhimurium	1 ± 0.1	0.13 ± 0.22	-
Bacillus subtilis	0.13 ± 0.02	0.25 ± 0.54	-
Escherichia coli	2 ± 0.13	0.5 ± 1.0	-
Pseudomonas aeruginosa	3 ± 0.55	12 ± 4	-
Candida albicans	1.5 ± 0.06	-	4.5 ± 2

In another study, isolated prostantherol has shown relatively high activity against Gram-positive *Streptomyces scabies* and demonstrated inhibition of spore germination of the fungal plant pathogen *Cladosporium cucumerinum* [8]. Our results indicate that *P. centralis* itself has little value as an antibiotic *per se* but may be useful as an effective topical treatment for some human fungal and bacterial infections. In addition, this species may be useful as an antibacterial feed additive in the raising of livestock. Given the unusually high abundance of prostantherol in this species, suitable cultivars may be useful in commercial production of this as a potential pharmaceutical precursor. The *in vivo* role of prostantherol has not been determined.

An unfortunate consequence of British colonisation was the fragmentation of indigenous Australian medicinal knowledge. It is, therefore, now not clear if *P. centralis* was included in Aboriginal *materia medica*. As previously mentioned, no specific details of traditional use of *P. centralis* have been recorded. Our preliminary findings suggest possible therapeutic benefits. It is interesting however that collection notes record the co-occurrence of *Eremophila elderi* [22], a plant considered by the Pitjantjatjara senior traditional owners to be an important medicinal plant (Timothy L. Collins, personal observation), raising the possibility that *P. centralis* was overlooked as a medicinal plant in favour of more effective species. To expand on knowledge of therapeutic uses of the hydrodistilled EO, further pharmacological tests are necessary in the absence of ethnopharmacological indications. There are currently no indications of toxicity or indeed matters related to safe use of *P. centralis*. With the exception of prostantherol and squamulosone, the majority of EO components identified in the current study are well known and not considered dangerous at these lower concentrations [23]. However, safety evaluation of prostantherol and squamulosone has yet to be undertaken.

3. Experimental Section

3.1. Plant Collection, Hydrodistillation and Essential Oil Analysis

Leaf material and voucher specimens were collected from four cultivated plants at the Alice Springs Desert Park (accession number: D185139), under Northern Territory permit: 51404. Vouchers were lodged at the Noel Charles William (NCW), Beadle Herbarium (NE), University of New England, Armidale, NSW, Australia. For solvent extraction, approximately 2 g of leaves from each plant was diced into fragments and soaked in 30 mL DCM. DCM extracts were diluted 1:10 and analyses were undertaken using Gas Chromatography-Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance spectroscopy (NMR).

Due to small sample sizes, leaves from the four plants were pooled for hydrodistillation. Approximately 77 g of leaf was diced into fragments of average size 5 mm and placed into a 5 L round bottom flask filled with 2.5 L distilled H_2O. Distillation was run for 3 h. Upon standing, EO was separated from the hydrosol, collected and stored at −20 °C until analyses were performed.

3.2. GC-MS and NMR

GC-MS analyses were performed using an Agilent Technologies 7890A GC-System coupled with an Agilent 5975C mass selective detector (triple-Axis detector, Agilent Technologies, Wilmington, DE, USA). An autosampler unit (Agilent Technologies 7693-100 positions) held samples. Separation of 1 μL injections used a HP-5MS Agilent column (30 m × 250 μm × 0.25 μm). Operating conditions were as follows; injector split ratio 25:1, temperature 250 °C, carrier gas helium, 1.0 mL/min, constant flow. Column temperature, 50 °C (no hold), 5 °C per minute then at 280 °C hold 5 min. MS acquired at −70 eV using a mass scan range of *m/z* 30–400.

Primary identifications were performed by comparison of mass spectra with an electronic library database [24] and confirmed using arithmetic indices, calculated relative to *n*-alkanes, compared with values published in Adams [20]. Discrepancies in identification were resolved by comparison of mass spectra with spectra also published in Adams [20]. Quantification was achieved by GC-MS operating software, using data with a minimum peak area of 0.1%, by calculating the area under the curve.

The identity of Prostantherol was confirmed by matching the NMR carbon spectra (^{13}C; 75 MHz, $CDCl_3$) with published spectra [8] in a Bruker Avance 300 NMR spectrometer. Authentic squamulosone and authentic ledol were used as standards for confirmation of the same component in the hydrodistilled essential oil using GC-MS analysis.

3.3. Antimicrobial Assays

In preparation for antimicrobial assays, working stocks of all species were maintained on nutrient agar (NA) with the exception of *Candida albicans*, which required Yeast Extract peptone agar (YEPA). All growth media were purchased from Oxoid (Thebarton, South Australia) and prepared according to the manufacturer's instructions.

MIC of the oils and extracts were determined using a micro-titre plate two-fold broth dilution method [25] with the following modifications. Where essential oil was used, emulsions were prepared

by vortexing a measured combination of oil and the appropriate broth with 0.15% w/w agar [26]. Most bacterial species were assayed in tryptone soya broth (TSB) containing 0.15% w/w agar, with the exception of the yeast *C. albicans*, which required YEP broth. Broth dilutions were performed in 96-well plates.

Inoculation was initiated by collecting colonies from fresh working stocks and dispensing into 0.9% w/v NaCl and diluting to match a 0.5 McFarland $BaSO_4$ Turbidity Standard [27] using a spectrophotometer at 600 nm (or 530 nm for *C. albicans*). To achieve a final inoculation concentration of 5×10^5 Colony Forming Units (CFU) the adjusted saline suspension was diluted into 40 volumes of the appropriate medium and 20 μL was used to inoculate 80 μL of media bringing the total volume to 100 μL and reducing the essential oil concentration to the appropriate target. Following inoculation the 96-well plates were sealed using parafilm and placed into an incubator at 37 °C for approximately 20 h before dispensing 40 μL of sterile 0.2 mg/mL p-iodonitrotetrazolium dye and examining for colour changes indicative of organism growth. Positive inhibition controls included tetracycline for bacterial growth and nystatin for *C. albicans*. Experiments were repeated 3 or more times and the results are reported as an average with standard error.

4. Conclusions

In the current study, essential oil analysis provided a means of examining both the chemotype and antimicrobial activity in this poorly studied plant. *P. centralis* has a unique essential oil character, with a high relative abundance of the sesquiterpene prostantherol, accompanied by 21 other identified chemical entities, including ledol and squamulosone. The EO demonstrated moderate to high inhibitory activity against some Gram-positive bacteria and the yeast *Candida albicans*. The pleasant smelling EO may be regarded as possibly useful in topical applications for the treatment of staphylococcal and possibly fungal infections. However, further pharmacological tests are yet to be undertaken, examining other possible therapeutic effects and more importantly for the evaluation of the safety of the EO in topical applications.

Acknowledgments

The authors would like to thank the Alice Springs Desert Park for permission to collect plant material. Additionally the authors would like to express gratitude for support analyzing chemical characters to Ben Greatrex from the University of New England, Armidale, Australia.

Author Contributions

Timothy L. Collins undertook leaf-material collection, EO extraction and analysis, wrote the first draft, and contributed to the editorial process; Graham L. Jones contributed to the experimental design and editorial process; Nicholas J. Sadgrove undertook antimicrobial assays, supervised EO extraction and analysis, and contributed to the experimental design and editorial process.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Conn, B.J. IV. Subfam. Prostantheroideae. In *VII. Flowering Plants—Dicotyledons, Lamiales (except Acanthaceae Including Avicenniaceae)*; Kadereit, J.W., Ed.; Springer: Berlin, Germany, 2004; pp. 204–209.
2. Conn, B.J. A taxonomic revision of *Prostanthera* Labill. section Prostanthera (Labiatae). 1. The species of the Northern Territory, South Australia and Western Australia. *Nuytsia* **1988**, *6*, 351–411.
3. Lassak, E.V. New essential oils from the Australian flora. In proceedings of Annals of the 8th International Congress of Essential Oils, Cannes, France, 12–17 October 1980; pp. 409–415.
4. Gersbach, P.V. The essential oil secretory structures of *Prostanthera ovalifolia* (Lamiaceae). *Ann. Bot.* **2002**, *89*, 255–260.
5. Cribb, A.B.; Cribb, J.W. *Wild Medicine in Australia*; Fontana/Collins Pty Ltd.: Sydney, Australia, 1981.
6. Lassak, E.V.; McCarthy, T. *Australian Medicinal Plants*; Methuen Australia Pty Ltd.: Hawthorn, VIC, Australia, 1983.
7. Latz, P.K. *Bushfires and Bushtucker: Aboriginal plant use in central Australia*; IAD Press: Alice Springs, NT, Australia, 1995.
8. Dellar, J.E.; Cole, M.D.; Gray, A.I.; Gibbons, S.; Waterman, P.G. Antimicrobial sesquiterpenes from *Prostanthera aff. melissifolia* and *P. rotundifolia*. *Phytochemistry* **1994**, *36*, 957–960.
9. Santos, F.A.; Rao, V.S.N. Antiinflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. *Phytother. Res.* **2000**, *14*, 240–244.
10. Sah, S.P.; Mathela, C.S.; Chopra, K. *Valeriana wallichii* DC (maaliol chemotype): Antinociceptive studies on experimental animal models and possible mechanism of action. *Pharmacologia* **2012**, *3*, 432–437.
11. Wilson, T.C.; Conn, B.J.; Henwood, M.J. Molecular phylogeny and systematics of *Prostanthera* (Lamiaceae). *Aust. Syst. Bot.* **2012**, *25*, 341–352.
12. Conn, B.J. A taxonomic revision of *Prostanthera* Labill. section Klanderia (F.v. Muell.) Benth. (Labiatae). *J. Adel. Bot. Gard.* **1984**, *6*, 207–348.
13. Southwell, I.A.; Tucker, D.J. *Cis*-dihydroagarofuran from *Prostanthera* sp. aff. *ovalifolia*. *Phytochemistry* **1993**, *33*, 857–862.
14. Yu, C.-W.; Li, W.-H.; Hsu, F.-L.; Yen, P.-L.; Chang, S.-T.; Liao, V.H.-C. Essential oil alloaromadendrene from mixed-type *Cinnamomum osmophloeum* leaves prolongs the lifespan in *Caenorhabditis elegans*. *J. Agric. Food Chem.* **2014**, *62*, 6159–6165.
15. Lahey, F.; Lamberton, J. The occurrence of ledol in *Phebalium squameus* Labill. *Aust. J. Chem.* **1956**, *9*, 431–433.
16. Graham, B.; Jefferies, P.; Melrose, G.; Thieberg, K.; White, D. The stereochemistry of aromadendrene, globulol, and ledol. *Aust. J. Chem.* **1960**, *13*, 372–379.
17. Batey, I.; Hellyer, R.; Pinhey, J. The structure of squamulosone, a new sesquiterpene ketone from *Phebalium squamulosum*. *Aust. J. Chem.* **1971**, *24*, 2173–2177.

18. Sadgrove, N.J.; Telford, I.R.; Greatrex, B.W.; Jones, G.L. Composition and antimicrobial activity of the essential oils from the *Phebalium squamulosum* species complex (Rutaceae) in New South Wales, Australia. *Phytochemistry* **2014**, *97*, 38–45.
19. Sangwan, N.; Farooqi, A.; Shabih, F.; Sangwan, R. Regulation of essential oil production in plants. *Plant Growth Regul.* **2001**, *34*, 3–21.
20. Adams, R.P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4th ed.; Allured Publishing Corporation: Carol Stream, IL, USA, 2007.
21. Rusenova, N.; Parvanov, P. Antimicrobial activities of twelve essential oils against microorganisms of veterinary importance. *Trakia J. Sci.* **2009**, *7*, 37–43.
22. AVH, Australia's Virtual Herbarium. Specimen Search Results. Available online: http://avh.ala.org.au/occurrences/search?taxa=prostanthera+centralis#tab_mapView (accessed on 30 September 2014).
23. Adams, T.B.; Taylor, S.V. Safety evaluation of essential oils: A constituent-based approach. In *Handbook of Essential Oils: Science, Technology and Applications*; Can Başer, K.H., Buchbauer, G., Eds.; CRC Press: London, UK, 2010; pp. 185–208.
24. NIST, National Institute of Standards and Technology. Chemical Abstracts Service (CAS) Registry Number Search. Available online: http://webbook.nist.gov/chemistry/cas-ser.html (accessed on 7 August 2014).
25. Wiegand, I.; Hilpert, K.; Hancock, R.E.W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **2008**, *3*, 163–175.
26. Mann, C.M.; Markham, J.L. A new method for determining the minimum inhibitory concentration of essential oils. *J. Appl. Microbiol.* **1998**, *84*, 538–544.
27. McFarland, J. Nephelometer: An instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *J. Am. Med. Assoc.* **1907**, *14*, 1176–1178.