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Investigations into the pituri plant: nicotine content, nicotine conversion to nornicotine, nicotine release and cytotoxicity of Australian native *Nicotiana* spp.

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Abstract

The Aboriginal population of Central Australia use endemic *Nicotiana* spp. to make a smokeless tobacco product known as pituri that they chew/suck for nicotine absorption. This thesis describes the relative abundance of nicotine alkaloids amongst Australian *Nicotiana* spp., with special focus on the molecular characteristics of nicotine to nornicotine conversion. The most popular chewed species, *N. gossei*, is investigated for nicotine release and cytotoxicity in comparison to similar products to gain insight into potential hazards to pituri users.

To analyse the alkaloids of *Nicotiana* leaves, a HPLC-UV method was developed to separate and quantify six closely related alkaloids (nicotine, nornicotine, anatabine, anabasine, myosmine, cotinine). A C18 column with a mobile phase of ammonium formate buffer (pH 10.5) separated the six alkaloids within 13 min with detection at 260 nm. Linearity, precision and reproducibility were satisfactory. The limit of quantification was 2.8 and 4.8 µg/mL for nornicotine and nicotine, respectively, and below 2 µg/mL for other alkaloids. This method quantifies more alkaloids and in less time than previously reported methods.

Tobacco alkaloids are responsible in formation of carcinogenic tobacco specific nitrosamines such as N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). To quantify NNN and NNK, a fast LC-MS/MS method was developed using a HILIC column with a triple quadrupole tandem mass spectrometry. The linearity, accuracy, recovery and repeatability for this method were satisfactory, with quantification limit of 2.6 and 4.3 ng/mL for NNN and NNK, respectively.

From 73 donated seedlots, plant growth under controlled environment conditions was successful for 24 of the 26 recognised Australian *Nicotiana* taxa. The quantification of alkaloids in leaves indicated nicotine as the abundant alkaloid in 15 taxa but nornicotine in 9. Nornicotine results from demethylation of nicotine, and is associated with negative effects on health. A group of cytochrome P450 genes that are mainly expressed during senescence or drying is involved in this conversion. The conversion loci were amplified in all studied 24 taxa, and sequenced in 6 selected species that contrast in conversion phenotype. Transcript accumulation of the responsible loci in fresh versus dried leaves of low or nonconverter *N. gossei, N. excelsior* and *N. benthamiana* maintained a steady level or a slight increase, but increased by 3 fold in cured leaves of the high converter *N. goodspeedii, N. velutina* and *N. cavicola*. This indicates the presence of functional loci that are triggered by curing only in high

converter species and poses a potential risk for chewers of these species due to their greater potential for nornicotine production.

The release of nicotine from the leaves of *N. gossei* was compared to that from a Swedish snus, the CORESTA reference smokeless tobacco (CRP2), and Nicabate chewing gum. A model buccal cavity system was developed and three different chewing conditions, performed manually were tested over 120 minutes: no chewing action, initial chewing and chewing at 15-minute intervals. To simulate the effect of alkaline wood ash, the effect of alkaline pH on the release of nicotine from *N. gossei* dry leaves was also evaluated. Samples of the dissolution media were analysed for alkaloids, and the media resulting from chewing every 15 minutes was quantified for NNN and NNK. The maximum cumulative nicotine release was produced by pressing every 15 minutes. Nicotine release from *N. gossei* dry leaves was not increased under alkaline condition. Nicotine release from smokeless tobacco products is faster and less dependent on chewing than that from Nicabate gum. *N. gossei* leaves showed similar release profile to snus, but contained higher nicotine and also released more nornicotine, anatabine, anabasine, and NNN and NNK into the media which can indicate potential health risks.

The *in vitro* toxicity of the aqueous extract from *N. gossei* leaves on human lung epithelium cell survival was compared with CRP2 and pure nicotine in an MTS assay. Results indicated remarkably lower survival of cells treated with extracts from *N. gossei* leaves and CRP2 than cells treated with nicotine at similar concentrations as present in the extracts. Nicotine in *N. gossei* leaves and CRP2 was not responsible for their cytotoxicity. Extracts contained other compounds, including NNN and NNK, which might be responsible for the higher toxicity.

In conclusion, Australian *Nicotiana* species used in pituri vary in their alkaloid compositions, with genetics driving some to contain higher nornicotine. These high-nornicotine species are more hazardous for chewing as pituri, especially when cured (dried). The nicotine release rate from *N. gossei* dry leaves, which is the main species used in pituri, is similar to Swedish snus and isn't increased by chewing. The extract from *N. gossei* dry leaves results in higher toxicity to human lung epithelium cells compared to nicotine at similar concentrations. Altogether, these results indicate the need for further investigation of pituri, its chemicals, and effects on health, as pituri remains a popular smokeless tobacco product within some Australian Aboriginal communities.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer reviewed papers

Moghbel N, Ryu B, Cabot PJ, Ratsch A, Steadman KJ. *In vitro* cytotoxicity of *Nicotiana gossei* leaves, used in the Australian Aboriginal smokeless tobacco known as pituri or mingkulpa. Toxicology Letters 2016, 254:45-51.

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Contributions by others to the thesis

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Table of Contents

Abstract ..... **i**

Declaration by author ..... **iii**

Publications during candidature ..... **iv**

Publications included in this thesis ..... **v**

Contributions by others to the thesis ..... **vii**

Statement of parts of the thesis submitted to qualify for the award of another degree ..... **vii**

Acknowledgements ..... **viii**

Keywords ..... **ix**

Australian and New Zealand Standard Research Classifications (ANZSRC) ..... **ix**

Fields of Research (FoR) Classification ..... **ix**

Table of Contents ..... **x**

List of Figures ..... **xiv**

List of Tables ..... **xvi**

List of Abbreviations used in the thesis ..... **xviii**

Chapter 1. Literature review **20**

1.1 Tobacco and health issues 21

1.2 Smokeless tobacco products 22

1.2.1 Postharvest processing of tobacco 22

1.2.2 Varieties of smokeless tobacco products used around the world 23

1.2.2.1 Pituri as a smokeless tobacco form used by Australian Aboriginals 25

1.2.2.2 Pituri chewing process 25

1.2.2.3 Psychoactive alkaloids source in pituri: *Nicotiana* spp. or *Duboisia hopwoodii?* 26

1.3 Nicotiana spp. 27

1.3.1 Australian *Nicotiana* species used as "Pituri" for chewing by Aboriginals 29

1.4 Chemistry of Nicotiana spp. 31

1.4.1 Alkaloids in *Nicotiana* spp. 31

1.4.2 Biochemistry and biosynthesis of alkaloids in *Nicotiana* spp. 32

1.4.2.1 Nicotine to nornicotine conversion 35

1.4.2.1.1 Biochemical and molecular characterization of N'-demethylation of nicotine 35
1.4.2.1.2 Cytochrome P450s in alkaloid biosynthesis 36
1.4.2.2 Conversion phenotype in *Nicotiana* species 37
1.4.2.3 Spatio-temporal distribution of nicotine to nornicotine conversion 38
1.4.3 Alkaloid levels and composition in different species 39
1.4.4 Alkaloid distribution and translocation in *Nicotiana* spp. 40
1.5 Chemical composition of smokeless tobacco products 41
1.5.1 Tobacco specific N-nitrosamines (TSNAs) 42
1.6 Nicotine delivery in smokeless tobacco products 44
1.6.1 Nicotine release from smokeless tobacco products 45
1.7 Toxicity of smokeless tobacco products 46
1.8 Rationale 47
1.9 Aims and hypotheses to be tested 48
Chapter 2. HPLC-UV and LC-MS/MS method development and validation for quantification of six alkaloids and two nitrosamines in tobacco plants and products 50
2.1 Introduction 51
2.2 Experimental 53
2.2.1 Chemicals 53
2.2.2 Instrumentation and chromatographic conditions 54
2.2.2.1 HPLC-UV 54
2.2.2.2 LC-MS/MS 54
2.2.3 Sample preparation 55
2.2.3.1 HPLC-UV 55
2.2.3.2 LC-MS/MS 55
2.2.4 Method validation 55
2.2.4.1 HPLC-UV 55
2.2.4.2 LC-MS/MS 56
2.3 Results and discussion 57
2.3.1 Method development 57
2.3.1.1 HPLC-UV 57
2.3.1.2 LC-MS/MS 58
2.3.2 Method validation 59
2.3.2.1 HPLC-UV 59
2.3.2.2 LC-MS/MS 61
2.4 Conclusion 62
Chapter 3. Chemical and molecular characterization of nicotine to nornicotine conversion in Australian *Nicotiana* species used as chewing tobacco 63
3.1 Introduction 64

3.2 Materials and methods 66
3.2.1 Plant Material 66
3.2.2 Chemical analysis 67
3.2.2.1 Chemicals and reagents 67
3.2.2.2 Alkaloid analysis 67
3.2.3 Molecular analysis 68
3.2.3.1 PCR amplification of CYP82E related genes 68
3.2.3.2 Sequencing the amplified CYP82E regions for selected species 68
3.2.3.3 Agarose gel electrophoretic analysis of CYP82E related genes transcript levels in fresh versus cured leaves of selected species 69
3.2.4 Statistical analysis 69
3.3 Results 69
3.3.1 Plant growth 69
3.3.2 Alkaloid profile 71
3.3.3 CYP82E genes in Australian Nicotiana spp. 73
3.3.3.1 Agarose gel electrophoretic analysis of CYP82E related genes transcript levels in fresh versus cured leaves of the selected high converter versus low or nonconverter species 74
3.4 Discussion 75

Chapter 4. *In vitro* evaluation of nicotine release from dry leaves of *Nicotiana gossei* used in pituri, the Australian Aboriginal smokeless tobacco 80

4.1 Introduction 81
4.2 Materials and methods 84
4.2.1 Materials 84
4.2.2 Simulation of a model buccal cavity 85
4.2.3 Chemical analysis 85
4.2.3.1 Nicotine and other alkaloids analysis with HPLC-UV 85
4.2.3.2 TSNAs analysis with LC-MS/MS 85
4.2.4 Statistical analysis 86
4.3 Results 86
4.4 Discussion 89
4.5 Conclusion 92

Chapter 5. The alkaloids chemistry and *in vitro* cytotoxicity of the *Nicotiana gossei* leaves used in Australian Aboriginal smokeless tobacco, 'pituri' compared to nicotine 93

5.1 Introduction 94
5.2 Materials and methods 96
5.2.1 Chemicals and reagents 96
5.2.2 Preparation of extracts 97
5.2.3 Quantification of nicotine and other alkaloids 97

5.2.4 Quantification of NNN and NNK 97
5.2.5 Cell culture, treatment and viability assay 98
5.2.6 Data analysis 99
5.3 Results 99
5.3.1 Chemical analysis of the extracts 99
5.3.2 Effect of nicotine and smokeless tobacco extracts on cell viability 100
5.4 Discussion 103
5.5 Conclusion 105
Chapter 6. Summary, conclusions and future research 106
6.1 Summary and conclusions 107
6.2 Future direction 112
References 116
Appendix 130

List of Figures

Figure 1-1 Pituri is prepared by mixing native tobacco leaves (A), with wood ash (B). An aboriginal woman chewing pituri (C) in Ernabella, South Australia. Photographs (A) and (B) taken by author. Photograph (C) published with consent from (National Library of Australia, 2016). 26
Figure 1-2 Pituri bag on display at the Queensland museum. Photograph taken by the author. 27
Figure 1-3 Nicotine alkaloid structures..... 32
Figure 1-4 Biosynthesis of nicotine and related alkaloids (Hakkinen et al., 2007) PMT, putrescine N-methyltransferase; MPO, N-methylputrescine oxidase; QPRTase, quinolinate phosphoribosyltransferase; LDC, lysine decarboxylase; CYP82E, Cytochrome P450s belonging to the 82E subfamily..... 34
Figure 1-5 Tobacco alkaloids, and the nitrosamines that can be formed from them. With the exception of NNA, all of these compounds are present in tobacco and tobacco smoke (Hoffmann et al., 1995). 43
Figure 2-1 Structure, molecular weight (MW, g/mol) and pKa values of A) the common tobacco alkaloids shown in order of HPLC elution and B) the two major carcinogenic TSNAs, NNN and NNK 52
Figure 2-2 Chromatograms depicting separation of alkaloids on a 150 × 3 mm, 3.5 µm particle size Zorbax Extend C-18 column and detection at 260 nm. The mobile phase (flow rate 0.8 mL/min) consisted of a gradient of acetonitrile and 15 mM ammonium formate in water (pH adjusted to 10.5 with triethylamine); the gradient elution was 9 to 10% acetonitrile from 0-5 min and then 10 to 13% acetonitrile from 5 to 13 min. (A) Standard solution of alkaloids and IS, (B) Extraction from *Nicotiana benthamiana* fresh leaves. 1. Cotinine; 2. Caffeine (IS); 3. Nornicotine; 4. Anatabine; 5. Myosmine; 6. Anabasine; 7. Nicotine. 58
Figure 3-1 Agarose gel electrophoretic photo of the CYP82E related fragment produced by 35 cycles of PCR amplification of DNA isolated from 1. *N. forsteri, 2. N. benthamiana, 3. N. excelsior, 4. N. gossei, 5. N. suaveolens (exigua), 6. N. goodspeedii, 7. N. simulans, 8. N. velutina, 9. N. cavicola, 10. N. amplexicaulis, 11. N. megalosiphon subsp. megalosiphon, 12. N. megalosiphon subsp. sessifolia, 13. N. truncate, 14. N. maritima, 15. N. occidentalis subsp. obliqua, 16. N. occidentalis subsp. Occidentalis, 17. N. occidentalis subsp. hesperis, 18. N. sp. 'Corunna', 19. N.*

heterantha, 20. N. rosulata subsp. rosulata, 21. N. rosulata var. ingulba, 22. N. rotundifolia, 23. N. burbidgae, 24. N. monoschizocarpa. 74
Figure 3-2 Agarose gel electrophoretic analysis and normalised band densitometry values of the CYP82E related fragment produced by 35 cycles of PCR amplification of cDNA isolated from A) fresh leaves B) Cured leaves (at 35° C for 6 hours) of 1. *N. gossei* 2. *N. excelsior* 3. *N. benthamiana*, 4. *N. cavicola*, 5. *N. goodspeedii*, 6. *N. velutina.* A 562 bp GAPDH fragment obtained by 35 cycles of PCR amplification of the cDNA for each sample is shown as an internal gene and for normalising the densitometric values that all were quantified using ImageJ software. The data points in graphs show mean ± std error of 3 independent experiments *: $p<0.05$ 75
Figure 4-1 Percent cumulative release of nicotine from (A) CRP2 (B) Snus (C) *N. gossei* dry leaves (D) N. gossei dry leaves in alkaline media adjusted to pH 9 (E) and Nicabate gum in 3 conditions of without pressing, initial pressing for 30 seconds and pressing for 30 seconds at every 15 minutes. The data points show mean ± std error of 3 independent experiments. 88
Figure 5-1 MTS assay results showing the effects of A) aqueous extract of *N. gossei* leaves used in pituri and B) aqueous extract of CRP2 on cell viability of human lung epithelial cells (A549). Applied concentrations of both extracts were 2, 10, 20, 50 mg/ml that were equivalent to approximately 0.05, 0.2, 0.5, 1-1.5 mg/ml of nicotine respectively. The data show the mean ± SEM of the 3 replicate experiments. 101
Figure 5-2 MTS assay results showing the effects of a concentration range of nicotine standard on cell viability of human lung epithelial cells (A549). Concentrations of 0.05 to 1.5 mg/ml covers the concentration range found in the 2-50 mg/ml of the pituri leaf and CRP2 extracts. Higher concentrations of 5 and 10 mg/ml were applied for calculation of IC50. The data show the mean ± SEM of the 3 replicate experiments. 102
Figure 5-3 Nicotine equivalent concentrations of extracts from pituri leaves and CRP2, and concentration of nicotine standard that resulted in 50% inhibition of growth in human lung epithelial cells (A549). The data show the mean ± SEM of the 3 replicate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$..... 102

List of Tables

Table 1-1 Smokeless tobacco products based on their mode of use with WHO regions in which they are used; AFRO: African region, AMRO: American region, SEARO: South-East Asian region, EURO: European region, EMRO: Eastern Mediterranean region, WPRO: Western Pacific region (International Agency for Research on Cancer, 2007) 24
Table 1-2 Sections classification of the *Nicotiana* genus, with species listed for the mainly Australian section, *Suaveolentes* (Knapp et al., 2004, Clarkson et al., 2004, Marks et al., 2011) 29
Table 1-3 Australian species of *Nicotiana* that have any report in relation to use as smokeless tobacco products (Latz et al., 1995, Symon, 2005, Peterson, 1979). 29
Table 1-4 Allele frequencies of CYP 82E genes among F2 plants (Pakdeechanuan et al., 2012) 37
Table 1-5 Subdivision of Australian species of *Nicotiana* in terms of total alkaloid, major alkaloid type and geographic distribution (Sisson and Severson, 1990) 40
Table 1-6 Carcinogens in smokeless tobacco products, and the types of cancer they may cause (Hoffmann et al., 2001, International Agency for Research on Cancer, 2004, Hecht, 2003) 42
Table 2-1 Mass spectrometry parameters used in MRM mode for two TSNAs 55
Table 2-2 System suitability data obtained for validation of the developed method with mobile phase pH 10.5. The values are the average or relative standard deviation (RSD) of six consecutive injections of a mixture of alkaloids and IS (caffeine) all at a concentration of 5 µg/mL using the developed method. 59
Table 2-3 Regression statistics, limit of detection (LOD) and limit of quantification (LOQ) for alkaloid standards. 60
Table 2-4 Method accuracy results obtained by calculating the relative standard deviation (RSD) value for the retention time (Rt) and average peak area ratio (Pa) of alkaloids to internal standard from six replicate injections within intra and inter-day runs in a mixture of alkaloids and IS (caffeine) all at a concentration of 5 µg/mL. 60
Table 2-5 Within-laboratory reproducibility results. Relative standard deviation (RSD) for the retention time (Rt) and peak area ratio (Pa) of six replicate injections of a mixture of alkaloids and IS at a concentration of 5 µg/mL for each, obtained from performing the developed method on two different HPLC systems within the lab. 61

Table 2-6 The LC-MS/MS validation parameters for the two major TSNAs, NNN and NNK. 61
Table 3-1 Sequences of the primers used for PCR amplification of CYP82E related genes designed from *N. tabacum* CYP82E4 mRNA (Accession: KC120817.1) and internal gene GAPDH designed from *N. benthamiana* glyderaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Accession: JQ256517.1). 68
Table 3-2 Sources of seeds used for growing the *Nicotiana* species for this study, with their corresponding herbarium voucher number, collection date, tetrazolium (V) and germination (G) results. 70
Table 3-3 Alkaloid content (mean ± se for 3 replicates) in freeze-dried fresh leaves of Australian *Nicotiana* spp. grown in a plant growth incubator under 16 h photoperiod provided with cool white fluorescent lamps, 23°C day and 21ºC night temperature, constant 70–90% relative humidity. Plants were watered daily as needed. Conversion rate was defined using the formula: [nornicotine content / (nornicotine content + nicotine content)] x 100. Species with less than 10% conversion rate are regarded to be low or nonconverters while those with 10-50% and more than 50% conversion rate are assigned as medium and high converters, respectively. 72
Table 3-4 Alkaloids (mean ± se for 3 replicates) of fresh versus cured (35°C for 6 hours in the dark) leaves for selected species of Australian *Nicotiana* spp. Significant differences ($p<0.05$) between fresh and cured leaves are indicated (*). 73
Table 4-1 Concentration (mg/g; mean ± se for 3 independent replicates) of nicotine and related alkaloids in smokeless tobacco products (fresh weight basis). For each alkaloid, mean values with the same superscript letter are not significantly different ($p<0.05$). 86
Table 4-2 Concentration of nicotine alkaloids and TSNAs (mean ± se of 3 replicates) released into 50 ml of simulated saliva in vessels with chewing at 15 minute intervals from smokeless tobacco products and Nicabate gum at 120 min. 89
Table 5-1 Alkaloid and TSNA composition (mean ± se for 3 independent replicates) of the Coresta reference smokeless tobacco product (CRP2 Moist Snuff) and dry leaves of *Nicotiana gossei*, along with freeze-dried aqueous extracts of CRP2 and dry leaves of *N. gossei*. ND: not detected. 99

List of Abbreviations used in the thesis

ANOVA	Analysis of Variance
BP	British Pharmacopoeia
cDNA	Complementary DNA
CORESTA	Cooperation Centre for Scientific Research Relative for Tobacco
CRP2	CORESTA reference smokeless tobacco product
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HCl	Hydrochloric Acid
HPLC-UV	High Performance Liquid Chromatography- Ultra Violet Detector
IARC	International Agency for Research on Cancer
IS	Internal Standard
LC-MS/MS	Liquid Chromatography- Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MRM	Multiple Reaction Monitoring
NAB	N′ Nitrosoanabasine
NAT	N′-Nitrosoanatabine
NCBI	National Centre for Biotechnology Information
NNK	4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone
NNN	N′-Nitrosonornicotine
NRT	Nicotine Replacement Therapy
NSW	New South Wales
NT	Northern Territories
PA	Peak Area

PAH	Polycyclic Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction
PREP	Potential Reduced Exposure Products
QLD	Queensland
RSD	Relative Standard Deviation
RT	Retention Time
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SA	South Australia
SLT	Smokeless Tobacco Product
TEA	Triethylamine
TF	Tailing Factor
TSNA	Tobacco Specific Nitrosamine
USP	United States Pharmacopoeia
VIC	Victoria
WA	Western Australia

Chapter 1. Literature review

1.1 Tobacco and health issues

Worldwide use of tobacco products is responsible for nearly 6 million deaths each year and, if this trend continues, it is expected to reach to 8 million by 2030 (World Health Organization, 2011). In developing countries, smoking is responsible for 30% of all cancer related deaths and this figure is 13% in developed countries, and 5.2% for Australia (Begg et al., 2007). Lung cancer is the most dominant cancer resulting from smoking (Hecht, 1999). Cigarette smoking is also associated with various other types of cancer including liver, pancreas, stomach, urinary bladder, and oesophagus (Sasco et al., 2004). Sixty-two compounds that are carcinogenic to laboratory animals have been shown to be present in cigarette smoke and 15 of them were proven to be carcinogenic to humans (International Agency for Research on Cancer, 2004).

Other tobacco products such as smokeless tobacco have been gaining greater interest as a substitute for cigarette smoking (Christen, 1980). Smokeless tobacco products have different health effects and not all of them have been studied for the health outcomes and diseases they may cause (Ebbert et al., 2004, Scientific Advisory Committee on Tobacco Products Regulation, 2010, Winstanley, 2012).

Short-term increase of blood pressure and heart rate is one of the health effects attributed to smokeless tobacco products use and users are probably more prone to the risk of dying from stroke and heart disease (International Agency for Research on Cancer, 2007, Paolo and Kurt, 2009). Also, significant effects on the soft and hard tissues of the mouth caused by smokeless tobacco products can lead to oral disease like bad breath, tooth decay, receding gums, lesions in the mouth (Carr and Ebbert, 2006, Christen, 1980, International Agency for Research on Cancer, 2007); hence, dentists are urging health professionals to warn people of the hazards of using smokeless tobacco products (Christen, 1980).

So far 28 carcinogenic compounds have been reported to be present in smokeless tobacco products (International Agency for Research on Cancer, 2007). Mouth cancer is the most prevalent cancer caused by using smokeless tobacco (Ebbert et al., 2004, Scientific Advisory Committee on Tobacco Products Regulation, 2010) and the risk of mouth cancer is reported to be the highest by using tobacco-betel, tobacco-lime and other mixtures of tobacco in India, Pakistan and South Asia and Toombak from the Sudan (Critchley and Unal, 2003, International Agency for Research on Cancer, 2007). Also, pancreas and oesophagus cancer have been reported to be caused by smokeless tobacco products (Secretan et al., 2009).

In pregnant women using smokeless tobacco products, there is a high risk of low birth weight, premature birth and preeclampsia (Ebbert et al., 2004, International Agency for Research on Cancer, 2007, Scientific Advisory Committee on Tobacco Products Regulation, 2010). In male users of smokeless tobacco products, there is an increase in number of abnormal sperm, while sperm count and semen volume decrease (International Agency for Research on Cancer, 2007, Paolo and Kurt, 2009).

In Australia, tobacco is a widely used legal drug and in 2003, 15,511 deaths have been reported as attributable to tobacco (Begg et al., 2007). It is a major risk factor for the development of diseases like coronary heart disease, stroke, peripheral vascular disease, several cancers, respiratory disorders and other diseases and condition. It has been reported that between 2003 and 2007, there have been nine deaths of Indigenous Australians, and 301 deaths of non-Indigenous Australians with direct cause of death related to tobacco use. Also 327 deaths of Indigenous Australians, and 7,582 deaths of non-Indigenous Australians in this period were indirectly linked to tobacco use in New South Wales, Queensland, Western Australia, South Australia and the Northern Territory (Australian Institute of Health and Welfare, 2011, Australian Institute of Health and Welfare, 2012).

1.2 Smokeless tobacco products

Smokeless tobacco products are used without burning and consumed orally (chewed, sucked) or nasally (inhaled) (International Agency for Research on Cancer, 2007, Winstanley, 2012). Depending on their composition and process of production, each smokeless tobacco product has different ingredients, and most of them have not been fully studied for the extent of their ingredients (Ebbert et al., 2004, Scientific Advisory Committee on Tobacco Products Regulation, 2010, Winstanley, 2012).

1.2.1 Postharvest processing of tobacco

Tobacco plants used in smokeless tobacco products are from the genus *Nicotiana*, mainly *N. tabacum* and sometimes *N. rustica*, which are harvested and then cured (International Agency for Research on Cancer, 2007). Curing refers to treating harvested fresh *Nicotiana* leaves with regulated conditions of temperature and humidity to remove moisture from the leaves without sacrificing quality and to produce a stable product of suitable physical and chemical composition with desired colour and aroma (Abubakar et al., 2000, Touton, 1961). Curing should be done in a way that biological activities in the leaf continues, so moisture should be

removed slowly at the beginning and then this rate should increase to arrest the chemical reactions and complete curing (Abubakar et al., 2000).

For the commercial production of tobacco, after harvest the plants are immediately transferred to tobacco barns (kilns) to be cured. There are different designs of barns depending to the method of curing and type of tobacco:

- In air-curing, leaves are hung and dried over a period of four to eight weeks in well-ventilated barns. The smoke from this type of tobacco is light and sweet in flavour, and the level of sugar is low while there is high nicotine content. Examples are the tobacco used in cigar and burley tobacco used primarily for cigarette production (Bacon et al., 1952, Alfred, 1920).
- For fire-curing, which takes between three days to ten weeks, plants are hung in large barns and exposed to fires of hardwoods. Sugar is low in fire cured tobacco and nicotine is high. Fire curing is used for pipe tobacco, chewing tobacco, and snuff (Alfred, 1920).
- Flue-curing involves conveying flues of external fire to the curing barns that slowly raises the temperature and heat-cures the tobacco without exposing it to smoke. The process lasts for about a week. Cigarette tobacco is produced by this method and it is high in sugar and has medium to high levels of nicotine (Bacon et al., 1952, Alfred, 1920).
- In sun-curing, the plants are laid uncovered in the sun in order to dry. Both sugar and nicotine level is reported to be low and the sun-cured tobacco is mainly used in cigarettes (Alfred, 1920).

1.2.2 Varieties of smokeless tobacco products used around the world

The two main types of smokeless tobacco products are snuff and chewing tobacco (Ebbert et al., 2004) and there are a wide range of commercially available or native smokeless tobacco products used around the world (Table 1-1). Snuff is a cured and ground tobacco that can be held in the mouth and sucked or inhaled (in its dry form). Chewing tobacco consists of *Nicotiana* leaves chewed in combination with substances such as lime, betel leaf, areca nut (Winstanley, 2012).

Table 1-1 Smokeless tobacco products based on their mode of use with WHO regions in which they are used; AFRO: African region, AMRO: American region, SEARO: South-East Asian region, EURO: European region, EMRO: Eastern Mediterranean region, WPRO: Western Pacific region (International Agency for Research on Cancer, 2007)

Name and composition	WHO region
Oral use	
Betel quid (*paan* or *pan*): betel leaf, areca nut, slaked lime and tobacco; Chewed.	EMRO, SEARO WPRO
Chimó: tobacco, sodium bicarbonate, brown sugar, ashes from the Mamón tree, vanilla and anisette flavourings; kept in the mouth for almost 30 min and then spat out.	AMRO
Creamy snuff: ground tobacco mixed with aromatic substances like clove oil, glycerine, spearmint and camphor, salts, water; etc.; kept in the mouth; used for cleaning teeth.	SEARO
Dry snuff (*bajjar, tapkir/tapkeer, naffa, tenfeha, nufha)*: powdered fire cured, fermented tobacco; Used orally or nasally.	AFRO, AMRO EURO
Gul: powdered tobacco, molasses, etc.; applied to the teeth/ gums for cleaning	SEARO
Gudhaku: powdered tobacco mixed with molasses and applied to the teeth and gums.	SEARO
Gutka: roasted, sun-dried, chopped tobacco mixed with areca nut, slaked lime, catechu, flavourings and sweeteners; chewed/ sucked; saliva is spat out or swallowed.	SEARO
Iq'mik: fire-cured tobacco and punk ash (made from burning a woody fungus that grows on the bark of birch trees); mainly chewed.	AMRO
Khaini: sun-dried or fermented tobacco mixed with slaked lime paste or areca nut; rubbed in the palm of the hand, then held in the mouth and sucked or chewed.	SEARO
Khiwam or qimam: tobacco leaves boiled in the water (containing spices like saffron, cardamom, aniseed and additives such as musk) to make a paste; chewed.	SEARO
Loose-leaf: cigar tobacco sweetened with liquorice; sucked or chewed;	AMRO, EURO
Maras: sun-dried powdered tobacco mixed with the wood (oak, walnut or grapevine) ash, sprinkled with water to humidify; kept in the mouth for 4–5 min or while sleeping.	EURO
Mawa: crushed tobacco, sun-cured areca nut and slaked lime; chewed for 10-20 min.	SEARO
Mishri: powdered toasted tobacco leaves; applied to gums/ teeth as dentifrice.	SEARO
Moist snuff: cured tobacco in fine particles ('fine cut') or strips ('long-cut'); contain up to 50% moisture; a pinch (dip) or a pouch is sucked; saliva is spat out or swallowed.	AMRO EURO
Naswar or nass: sun-dried/ partially cured powdered tobacco mixed with ash, oil, flavouring and colouring agents or slaked lime; sucked under the tongue.	AFRO SMRO, EURO
Plug chewing tobacco: cigarette/ cigar tobacco mixed with liquorice / sugar, pressed into plug; contains 15% moisture; chewed/ held in the mouth; saliva swallowed or spat out.	AMRO
Red tooth powder: tobacco powder mixed with herbs and flavours; red in colour.	SEARO
Shammah: greenish-yellow mixture of powdered tobacco, lime, ash, black pepper, oils and flavours; placed in the mouth and sucked; debris are spat out.	EMRO EURO
***Snus (*Swedish moist snuff):** ground dry tobacco, aromatic substances, salts like sodium carbonate, humidifying agents, water and buffering agents such as sodium carbonate; a dip is placed between the gum and upper lip and kept for 11-14 h per day.	EURO
Tobacco chewing gum: developed under the brand name 'Fire'	WPRO
Tobacco tablets: ground tobacco (1.3 mg nicotine), mint / eucalyptus; melt in the mouth	AMRO
Toombak: dried and fermented tobacco mixed with sodium bicarbonate (4:1 ratio); 10 g ball forms (*saffa)* are held in the mouth; sucked for 10-15 min and then spit.	AFRO
Tuibur: tobacco smoke passed through water, the water is used as *Tuibur* (*hidakphu*) for gargling or sipping.	SEARO
Twist/roll chewing tobacco: twist or roll burely tobacco treated with tobacco leaf extract and flavours and twisted into rope-like strands that are dried.	AMRO
Zarda: tobacco leaves boiled with lime, spices until dry; coloured with vegetable dyes.	EMRO, SEARO
Nasal use	
Dry snuff: fire-cured, fermented tobacco in dry, powdered form; inhaled into the nostrils.	AFRO, EMRO EURO, SEARO
Liquid snuff: used nasally	AFRO

1.2.2.1 Pituri as a smokeless tobacco form used by Australian Aboriginals

Pharmacologically active components in plant material have always been the reason for them being chewed by indigenous populations of the Americas, Africa, the Indian subcontinent and the Asia-Pacific region including Australia (Latz et al., 1995). One example is the betel quid (Table 1-1) that has been reported to be used in the western pacific region including Australia (International Agency for Research on Cancer, 2007). Nicotine is the major pharmacologically active component in *Nicotiana* and in the Central Australian region a large group of Aboriginals chew a range of *Nicotiana* species for nicotine (Latz et al., 1995). 'Pituri' is the most common name, but other names such as 'mingkulpa' have been mentioned in the literature (Ratsch et al., 2010). The extent of pituri use is not well known, but a survey conducted 30 years ago reported that 25% of Aboriginal people, majority of which were female (38% women against 11% men), use chewing tobacco. According to this survey chewing was more popular in Central Australia with 41% of people practicing it (Fleming et al., 1991) and in a recent research around 30% of Aboriginal women giving birth in the major public hospital in Alice Springs were reported to use pituri either during their pregnancy or during lactation (Ratsch, 2011). Despite this, pituri was not listed in the WHO reports on smokeless tobacco types used around the world (International Agency for Research on Cancer, 2007).

1.2.2.2 Pituri chewing process

To prepare pituri, fresh or sun/fire-dried leaves of *Nicotiana* are broken into pieces often in sand ovens (Watson et al., 1983) and then mixed with burnt alkaline wood ash (Fig. 1-1 A-B) and chewed together to form into a 'quid' (Fig. 1-1 C). In terms of ingredients and preparation, pituri is similar to indigenous Alsakan smokeless tobacco iq'mik (International Agency for Research on Cancer, 2007, Hearn et al., 2013) and maras which is a smokeless tobacco used in Turkey (International Agency for Research on Cancer, 2007). Also, people of the Asian subcontinent have a similar practice by combining betel with lime before chewing (International Agency for Research on Cancer, 2004, Watson et al., 1983). The ash used in pituri is prepared by burning the wood from selected plants such as *Acacia* spp., *Grevillea s*pp. and *Eucalyptus* spp. (Aiston, 1937, Barr et al., 1988, Peterson, 1979). Among these, *Acacia salicina* has been mentioned in a report over 100 years ago as being a preferred source for producing a very alkaline wood ash (Higgin, 1903).

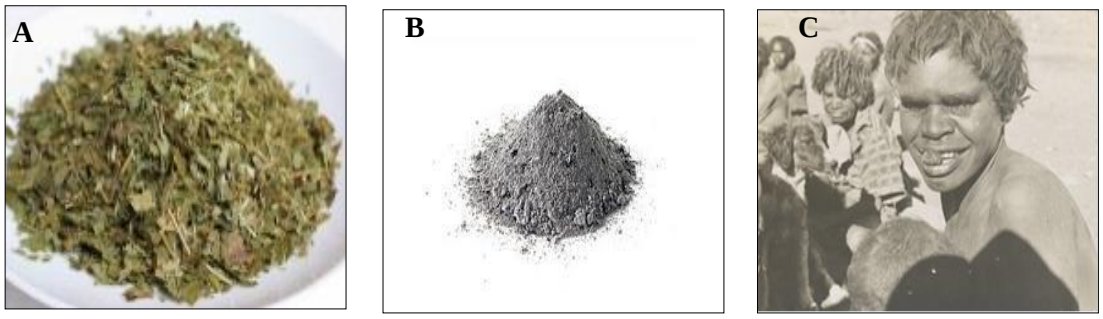


Figure 1-1 Pituri is prepared by mixing native tobacco leaves (A), with wood ash (B). An aboriginal woman chewing pituri (C) in Ernabella, South Australia. Photographs (A) and (B) taken by author. Photograph (C) published with consent from (National Library of Australia, 2016).

Pituri users hold the quids for long periods of time in their mouth and suck it rather than chewing it. This is to obtain fast absorption of the nicotine content through their mouth mucosa (Peterson, 1979, Winstanley, 2012). Chewers might share their quids with family or friends, and add more ash to revitalise the nicotine absorption. During breaks from oral use, the quid might be left on their skin in areas such as behind the ear, under a breast or accessories such as an arm or a head band. These approaches potentially allow nicotine absorption through the skin (Ratsch et al., 2010). Sometimes, a quid is kept in the mouth through the night which allows longer exposure to nicotine. Counting the number of chewers by simple observation likely results in an underestimation as the pituri quid may be held in a number of body locations and not easily visible.

1.2.2.3 Psychoactive alkaloids source in pituri: *Nicotiana* spp. or *Duboisia hopwoodii?*

The Aboriginal referral of the word "pituri" to a variety of plants they use as either the alkaloid source or the ash component in preparation of pituri has resulted in a confusion in the literature on the true identity of the plants used as the main component and source of psychoactive alkaloids in this smokeless tobacco product (Aiston, 1937). While some of the historical literature introduced pituri as *Duboisia hopwoodii* leaves (Keogh, 2011, Langley and Dickinson, 1890, Low, 1987, Watson et al., 1983), others attributed it as a species of Australian tobacco such a *N. gossei* or *N. excelsior* (Johnston and Cleland, 1934). It is most likely that both plants were used because both are reported to contain nicotine (Hicks and LeMessurier, 1935). What is currently used in the practice of chewing pituri by Aboriginals in Central Australia is some species of *Nicotiana* (Ladiges et al., 2011, Marks et al., 2011, Ratsch et al., 2010, Symon, 2005).

The knowledge of processing and preparing pituri (either *D. hopwoodii* or *Nicotiana* spp.) had a sacred ritual significance among Aboriginal peoples and was reported to be restricted to specific groups or tribes (Low, 1987, Watson et al., 1983). Pituri was packed and traded in specially woven crescent shaped bags (Fig. 1-2) (Watson et al., 1983).

Figure 1-2 Pituri bag on display at the Queensland museum. Photograph taken by the author.

1.3 Nicotiana spp.

The genus *Nicotiana*, with 86 naturally occurring species, is the sixth largest in the family *Solanaceae* and it is named after a sixteenth century French diplomat Jean Nicot, who popularized tobacco in Europe (Ladiges et al., 2011, Häkkinen and Oksman-Caldentey, 2004, Clarkson et al., 2004). It has been reported that this genus first originated in South America and subsequently spread to other continents (Aoki and Ito, 2000). *Nicotiana* has a natural distribution limited to the southern hemisphere (neotropical) and about 75% of species occur in the Americas and 25% of species occur in Australia (Knapp et al., 2004). Exceptions are *N. africana* which is the first (and only) species to be discovered in Africa (Namibia) and *N. fragrans* which is restricted to islands in South Pacific Ocean (Aoki and Ito, 2000, Clarkson et al., 2004).

Goodspeed described the section *Suaveolentes* in 1945 (Goodspeed, 1945). The origin of this group has not been clear and described as enigmatic (Goodspeed et al., 1954) and paradoxical (Chase et al., 2003). However, the section is retained in the current sectional classification of the genus (Knapp et al., 2004) and considered as monophyletic (Chase et al., 2003, Clarkson et al., 2004). Goodspeed et al. (1954) suggested that the progenitor of the Australian species came from relatives of the *Noctiflorae* and *Alatae* Chase et al. (2003) have supported the *Alatae* as the possible ancestor of this section, and also using chloroplast phylogeny, Clarkson et al. (2004) suggested an ancestor of section *Noctiflorae* as the maternal progenitor.

Members of the genus *Nicotiana* are widely used in traditional medicine in both South America and Australia. *N. tabacum* and *N. rustica*, known as cultivated tobaccos, are the most widely used drug plants in the world (Chase et al., 2003, Knapp et al., 2004). Floral and vegetative morphology in species of this genus is wide and there are species that grow to a few centimetres while others reach four or more metres (Clarkson et al., 2004). In 1954 using evidence from morphology, cytology, biogeography, and crossing experiments, Goodspeed provided a detailed history of the taxonomy of the genus. According to Goodspeed the base chromosome number of the genus is n=12 and he emphasized the role of doubling and hybridization in the evolution of the genus so that the haploid chromosome number variation from n=9 to n=24 is observed, but the basic chromosome number is n=12 (Goodspeed et al., 1954). As of 2004, there are 13 sections and 86 species recognised within *Nicotiana* (Table 1-2) (Clarkson et al., 2004, Knapp et al., 2004). Tobacco (*N. tabacum*), the species of primary economic importance, is an allotetraploid derived from two diploid ancestors, *N. tomentosiformis* and *N. sylvestris* (Goodspeed et al., 1954). Nicotine is the primary alkaloid accumulated in the cured leaves of tobacco, whereas both the ancestral species accumulate nornicotine (Sisson and Severson, 1990) because they have the conversion locus to convert nicotine to nornicotine. Gene silencing occurred after the polyploidization event in the evolution of polyploid species (Chakrabarti et al., 2007).

Table 1-2 Sections classification of the *Nicotiana* genus, with species listed for the mainly Australian section, *Suaveolentes* (Knapp et al., 2004, Clarkson et al., 2004, Marks et al., 2011)

***Nicotiana* section**	
Alatae	*Noctiflorae*
Nicotiana	*Paniculatae*
Polydicliae	*Repandae*
Petunioides	*Tomentosae*
Rusticae	*Trigonophyllae*
Sylvestres	*Undulatae*
Suaveolentes	
Species included in *Suaveolentes* section	
N. africana Merxm.	*N. monoschizocarpa* (P. Horton) Symon & Lepschi
N. amplexicaulis N.Burb.	*N. occidentalis* H.Wheeler ssp. *hesperis* (N.Burb.) P.Horton
N. benthamiana Domin	*N. occidentalis* H.Wheeler ssp. *obliqua* N. Burb.
N. burbidgeae Symon;	*N. occidentalis* H.Wheeler ssp. *occidentalis*
N. cavicola N.Burb.	*N. rosulata* (S.Moore) Domin ssp. *ingulba* (J.Black) P. Horton
N. forsteri Roem. & Schult (syn *N. debneyi* Domin)	*N. rosulata* (S.Moore) Domin ssp. *rosulata*
N. excelsior (J.Black) J.Black	*N. rotundifolia* Lindley
N. fatuhivensis F.Br.	*N. simulans* N.Burb.
N. fragrans Hook.	*N. sp.*'Corunna' Symon 17088
N. goodspeedii H.Wheeler	*N. suaveolens* Lehm. (syn *N. exigua* H.Wheeler)
N. gossei Domin	*N. truncata* Symon
N. heterantha Symon & Kenneally	*N. umbratica* N.Burb.
N. maritima H.Wheeler	*N. velutina* H.Wheeler
N. megalosiphon Van Heurck and Müll. Arg. ssp. *megalosiphon*	*N. wuttkei* J.Clarkson & Symon
N. megalosiphon Van Heurck & Mull. Arg. ssp. *sessifolia* P.Horton	

1.3.1 Australian *Nicotiana* species used as "Pituri" for chewing by Aboriginals

There are 26 Australian species and subspecies of *Nicotiana* (Table 1-3) that are contained in the section Suaveolentes, along with three other species in the Western Pacific including *Nicotiana fatuhivensis* F.Br. in the Marquesas Islands and *Nicotiana forsteri* Roem & Schult (synonymous with *Nicotiana debneyi* Domin) and *Nicotiana fragrans* Hook in New Caledonia and nearby islands (Ladiges et al., 2011, Marks et al., 2011). The Australian species are widely spread over the continent except for Tasmania. They are less common in the far north and there has not been any record of them from the Kimberley, Arnhem Land or Cape York Peninsula. *Nicotiana* does not occur in New Guinea or New Zealand and the main place of speciation has been in the warmer arid and semi-arid mainland of Australia (Burbidge, 1960, Symon, 2005).

Table 1-3 Australian species of *Nicotiana* that have any report in relation to use as smokeless tobacco products (Latz et al., 1995, Symon, 2005, Peterson, 1979).

Species	Used for chewing	Reference
N. benthamiana Domin	Used as a chewing tobacco throughout its range	Latz (1995)
	Pintupi people use it	Peterson (1979)
	Alyawara people rank it below *N. ingulba*.	Latz (1974)
	A favoured species	Symon (2005)
	Used in WA (recorded *N. suaveolens* but as it doesn't grow there it is most likely *N. benthamiana*)	Reid and Betts (1977 in Symons, 2005)
N. cavicola N.Burb	Used mainly in Western Australia	Reid and Betts (1977 in Symons, 2005)
N. excelsior J.Black (J.Black)	Important plant for the Pitjantjatjara people.	Latz (1995)
	N. excelsior and *N. gossei* are the two most popular	Symon, (2005)
	Valued by Aborigines in the Musgrave Ranges area	Latz (1974)
	Use recorded in WA	Reid and Betts (1977 in Symons, 2005)
N. glauca	Introduced species, originates from South America, reputed to be chewed by Pitjantjatjara.	Latz (1995)
N. goodspeedii H.Wheeler	Tops and roots used by Aboriginal peoples at the eastern end of the Nullarbor Plain and to the south	Cleland (1957 in Symons, 2005)
N. gossei Domin	The most important plant in Central Australia	Latz (1995)
	N. excelsior and *N. gossei* are the two most popular	Symon, (2005)
	N. gossei is preferred as a stronger variety	Peterson (1979)
	The most potent plant, highly prized by Aborigines	Latz (1974)
N. megalosiphon Van Heurck & Mull. Arg. ssp. *megalosiphon*	Was used by Aborigines, low on the list of preferences.	Latz (1974)
	Very rarely used by the Alyawara	Peterson (1979)
	Rarely used	Latz (1995)
N. occidentalis H.Wheeler ssp. *occidentalis*	Rarely used	Latz (1974), (1995)
N. rosulata (S.Moore) Domin ssp. *ingulba* (J.Black) P. Horton	subsp. *ingulba:* important to Pintupi for availability	Latz (1995)
	N. ingulba is ranked second to *N. gossei* by the Southern Walpiri and Alyawara people.	Peterson (1979)
	N. ingulba is common in Central Australia	Latz (1974)
	N. ingulba is in Central Australia and *N. rosulata* is more southern and western.	Symon (2005)
N. simulans N.Burb.	Rarely used	Latz (1974), (1995)
	Ranked after *N. gossei* and *N. ingulba* by the Southern Walpiri	Peterson (1979)
N. sauveolens Lehm.	Used by humans, not clear whether Aboriginal.	Lee (1925 in Symon, 2005)
N. velutina H. Wheeler	'Shunned by Aborigines'	Latz (1974)
	Rarely if ever used by Aboriginal people	Latz (1995)
	Very rarely used by the Alyawara	Peterson (1979)

Australian species of *Nicotiana* are mostly annuals, or in exceptionally good seasons they may survive for a second year. In southern parts there are a few short-lived perennial species (Latz et al., 1995, Peterson, 1979). Supply of leaves varies from year to year, for example *N. velutina* may cover sand hills in one year but then not be present in a subsequent year (Symon, 2005). The two popular species for chewing, *N. excelsior* and *N. gossei* which grow in the Central Australian ranges, may have a little more reliable supply, but supply of the species *N. ingulba* from the sand plains is very dependent on irregular rains, especially after bushfires (Symon, 2005).

1.4 Chemistry of Nicotiana spp.

Plant defence and response to environmental stress largely depends on secondary metabolites. These are often useful for making natural products, medicines, cosmetics and food additives (Häkkinen and Oksman-Caldentey, 2004, Kidd et al., 2006). *Nicotiana* species (*Solanaceae*) contain a variety of secondary metabolites, the most important groups of which are alkaloids, phenolic compounds, and terpenoids (Keinanen et al., 2001). Among them, alkaloids have been long studied for their role in plant defence mechanisms and use as medicines (Häkkinen and Oksman-Caldentey, 2004).

1.4.1 Alkaloids in *Nicotiana* spp.

Alkaloids are a diverse group of secondary metabolites in plants and mainly involved in defence against herbivores and pathogens. There are more than 12,000 different types of alkaloids. The majority of the alkaloids are heterocyclic compounds containing secondary or tertiary nitrogen derived from a relatively small group of amino acids in one of the rings (Cane et al., 2005, Kidd et al., 2006).

Alkaloids in *Nicotiana* have been studied for many years. Production of these metabolites is controlled genetically and alkaloid levels can vary dramatically throughout growth in response to environmental conditions (Keinanen et al., 2001). Alkaloids have important roles in the life and reproductive strategies of their producing plants (Cane et al., 2005, Kidd et al., 2006). These secondary metabolites mediate many of the biological interactions between the *Nicotiana* genus with its environment, including a longstanding use by humans (Keinanen et al., 2001). Many alkaloids are toxic and protective against herbivores. Plant tissue damage induces alkaloid production as a defence against herbivore attack in *Nicotiana* (Baldwin and Schmelz, 1994).

All species of the genus *Nicotiana* (L.) have the characteristic feature of producing pyridine alkaloids; but the number and abundance of the different alkaloids are highly variable within the genus (Sisson and Severson, 1990). While nicotine is considered to be the most abundant alkaloid (~95%) in *Nicotiana* species, it has been reported that there are many other pyridine alkaloids in various *Nicotiana* plants, and in the cured *Nicotiana* leaves, and all of them are structurally related to nicotine. Nornicotine, anabasine, anatabine, myosmine and isonicoteine are structurally related alkaloids which are found in several wild *Nicotiana* species and cultivars of commercial tobacco (*Nicotiana tabacum*) (Fig. 1-3) (Lisko et al., 2013). The four

well studied and major alkaloids in *Nicotiana* species are nicotine, nornicotine, anabasine, and anatabine (Siminszky et al., 2005).

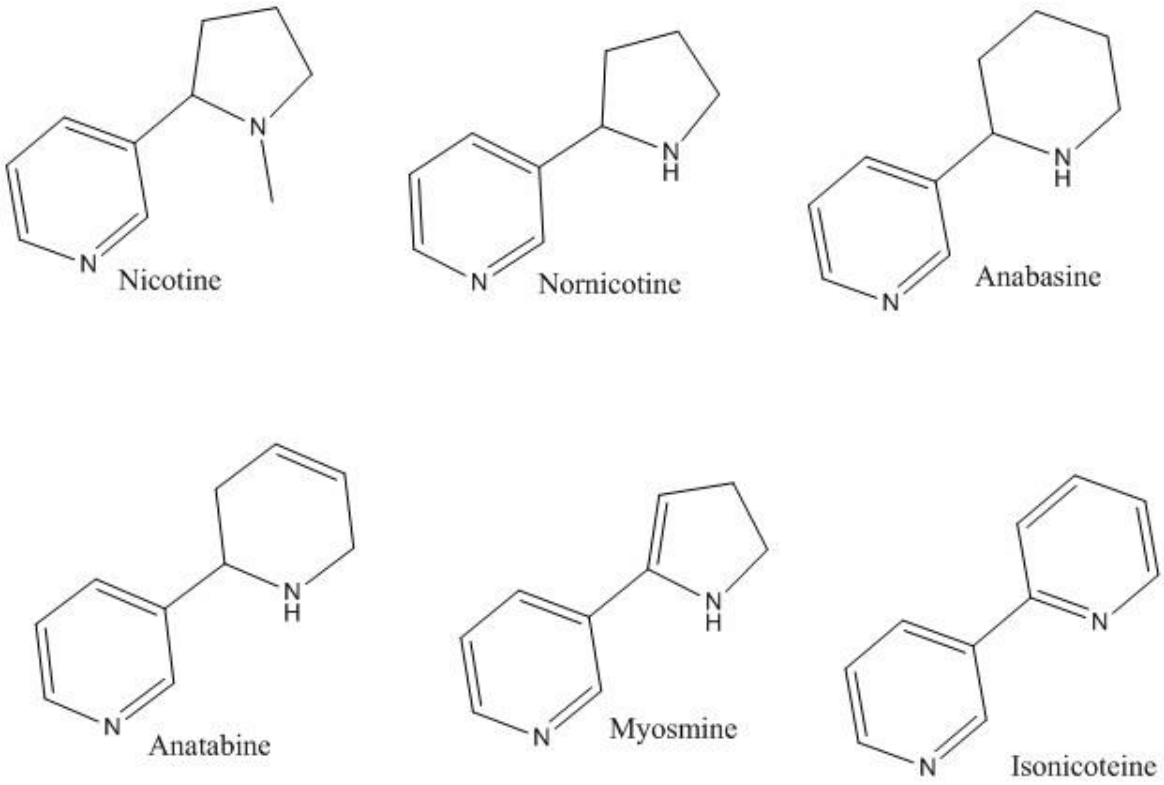


Figure 1-3 Nicotine alkaloid structures

1.4.2 Biochemistry and biosynthesis of alkaloids in *Nicotiana* spp.

The biosynthesis of all nicotine alkaloids (Fig. 1-4) needs pyrrolidine ring formation that is derived from putrescine. For biosynthesis of alkaloids, putrescine, that also serves as the precursor of other polyamines (Pegg and McCann, 1982), is converted to *N*-methylputrescine by the action of putrescine *N*-methyltransferase (PMT). In the alkaloid biosynthetic pathway, PMT catalyses the first committed step driving the flux of nitrogen from polyamine biosynthesis to alkaloid biosynthesis (Facchini, 2001, Robins et al., 1994). It is the rate-limiting enzyme in nicotine synthesis and its transcripts are found only in the roots (Chintapakorn and Hamill, 2003).

The next step is oxidization of *N*-methyl putrescine to N-methylaminobutanal that is critical for alkaloid biosynthesis and is catalysed by methylputrescine oxidase (MPO) (De Luca and St Pierre, 2000, Mizusaki et al., 1971, Hakkinen et al., 2007). Then, through a spontaneous chemical rearrangement, N-methylaminobutanal is cyclized to form the 1-methyl-D1-

pyrrolium cation which subsequently condenses with nicotinic acid or its derivative to form nicotine. Aspartate and glycerol generate quinolinate, and quinolinate phosphoribosyltransferase (QPRTase) catalyses nicotinic acid formation via the quinolinate cycle. In the biosynthesis of all the pyridine alkaloids nicotinic acid is considered as a key component (Ghosh, 2000, Chintapakorn and Hamill, 2003, Facchini, 2001).

Nicotine is the precursor for the biosynthesis of some other major alkaloids in *Nicotiana* species. Demethylation of nicotine results in production of nornicotine (Hakkinen et al., 2007) and nornicotine can convert to myosmine (Griffith et al., 1960). Anatabine is generated through a dimerization reaction of a nicotinic acid metabolite (Hakkinen et al., 2007).

The oxidation of cadaverine to Δ'–piperideine, which upon condensation with nicotinic acid forms anabasine is also catalyzed by MPO. Anabasine formation also competes for MPO with nicotine formation and hence, MPO regulates the proportion of nicotine and anabasine in the genus *Nicotiana* (Facchini, 2001, Walton et al., 1988).

Nicotine, with the chemical name 3-(1-methyl-2-pyrrolidyl) pyridine, is the main and best studied alkaloid in cultivated tobacco (*N. tabacum* L.). It is found in all species of the genus *Nicotiana* and constitutes approximately 0.6–3.0% of leaf dry weight (Keinanen et al., 2001, Zhang et al., 2012). It is a colourless to pale oily yellow liquid, which is miscible with water in its unionised form and turns brown on exposure to light and air (Lu and Ralapati, 1998). Nicotine easily penetrates the skin. It is strongly chromophoric in the UV region and is directly detected at about 260 nm (Lu and Ralapati, 1998). Free base nicotine burns at a temperature below its boiling point, and its vapours combust at 308 K (35°C; 95°F) in air despite a low vapour pressure. Because of this, most of the nicotine is burned when a cigarette is smoked; however, enough is inhaled to cause pharmacological effects (Vollhardt and Schore, 2011).

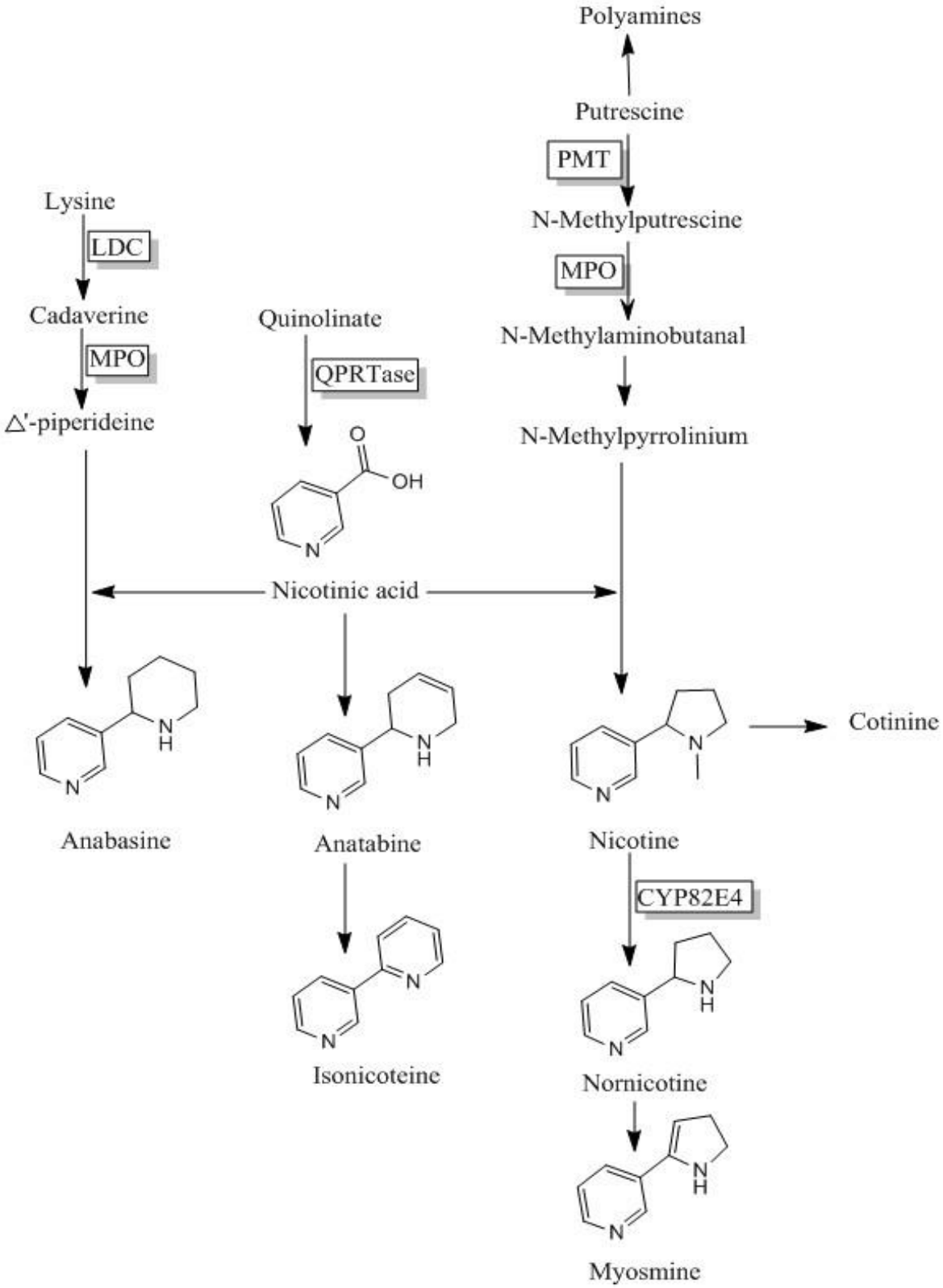


Figure 1-4 Biosynthesis of nicotine and related alkaloids (Hakkinen et al., 2007) **PMT**, putrescine N-methyltransferase; **MPO**, N-methylputrescine oxidase; **QPRTase**, quinolinate phosphoribosyltransferase; **LDC**, lysine decarboxylase; **CYP82E**, Cytochrome P450s belonging to the 82E subfamily.

1.4.2.1 Nicotine to nornicotine conversion

In tobacco research, nicotine to nornicotine conversion has a vital importance. This is because it has been reported that nornicotine affects cigarette smoke quality in a negative way (Roberts, 1988). It has also some hazardous health outcomes and serves as a precursor for carcinogenic nitrosamine N'-nitrosonornicotine (Siminszky et al., 2005, Bush et al., 2001).

After nicotine, nornicotine is usually the next most abundant alkaloid in *Nicotiana* plants, comprising about 3-5% of the total alkaloid content of fresh leaves (Saitoh et al., 1985). In senescing leaves, a large percentage of the nicotine may be metabolized into nornicotine. The term “nonconverters” is used for plants that accumulate nicotine as their primary alkaloid in both the green and senescing leaves, while *Nicotiana* species that convert a large portion of their nicotine content to nornicotine during senescence and curing are called “converters” (Chakrabarti et al., 2007, Gavilano and Siminszky, 2007, Siminszky et al., 2005).

N'-demethylation of nicotine to nornicotine has been first reported more than half a century ago. It is believed that this process depends on some general cellular property, such as oxidation-reduction potential, rather than interaction between nicotine and a specific dealkylating or alkyl transferring enzymes. Even feeding of different nicotine homologs and analogs (e.g., l-nicotine, d, l-nicotine and d, l-N'-ethylnornicotine) to the *N. glutinosa* leaves results in nornicotine production (Dawson, 1951). Nornicotine is a more toxic alkaloid than nicotine (Katz et al., 2005). The proven role of nornicotine in age-related macular degeneration (AMD) (Brogan et al., 2005, Dickerson and Janda, 2002), one of the major causes of blindness in the world, and its causing of periodontal diseases (Katz et al., 2005) makes it a more undesirable alkaloid in tobacco plants and products (Brogan et al., 2005). Therefore, efforts have been invested in breeding and genetic studies to understand the conversion phenomenon because the nicotine-accumulating tobacco is more desirable in terms of smoking quality as compared to the nornicotine accumulating tobacco (Mann et al., 1964).

1.4.2.1.1 Biochemical and molecular characterization of N'-demethylation of nicotine

Theoretically conversion of nicotine to nornicotine is possible through two different ways: transmethylation (though there is not much experimental support for it) or oxidative demethylation (Bose et al., 1956, Stepka and Dewey, 1961). Nicotine demethylation is associated with increased uptake of oxygen (Il'in and Serebrovskaya, 1958). The methyl group of nicotine is oxidised to CO_2 in conversion of nicotine to nornicotine, and probably this methyl

group is oxidised to the level of formaldehyde, then transferred to a carrier molecule and then further oxidized to CO_2 (Stepka and Dewey, 1961). NADPH is the preferred reducing element and when the concentration of NADPH is subsaturated NADH enhances demethylation (Donaldson and Luster, 1991). The molecular identity of the conversion factor in *Nicotiana* has been of great importance. A single dominant locus has been reported to be involved in the conversion process in *Nicotiana* (Yamanaka et al., 2005). A major breakthrough was the identification of a group of cytochrome P450 genes, encoding the active nicotine demethylase enzymes in *Nicotiana* (Siminszky et al., 2005).

1.4.2.1.2 Cytochrome P450s in alkaloid biosynthesis

Cytochrome P450 names begin with CYP (which stands for cytochrome and pigment), followed by a number representing the family (proteins having > 40% sequence similarity constitute a family), followed by a letter representing subfamily (proteins with >55% sequence identity remain in the same subfamily). The subfamily designation is followed by a number representing the individual gene within a subfamily

In plants, biosynthesis of lots of metabolites involves P450s activity (Kim and Tsukaya, 2002). In the pyridine alkaloid synthesis, nornicotine is formed from nicotine by nicotine N-demethylase involving P450 which belongs to the CYP82E subfamily monooxygenase. Nornicotine is accumulated to high levels in some tobacco (*Nicotiana tabacum*) cultivars and many wild *Nicotiana* species (Chakrabarti et al., 2008, Chelvarajan et al., 1993, Pakdeechanuan et al., 2012, Siminszky et al., 2005).

This group of P450 genes (CYP82E2, CYP82E3 and CYP82E4) was found to be differentially regulated between converter and nonconverter *Nicotiana* (Siminszky et al., 2005). In human liver microsomes, nicotine N-demethylation is catalysed by the P450 subfamilies CYP2A and CYP2B (Yamanaka et al., 2005). There are at least five CYP82E-related genes in *Nicotiana*: CYP82E4, CYP82E5 and CYP82E10 encode functional nicotine N-demethylases (Siminszky et al., 2005, Gavilano and Siminszky, 2007, Lewis et al., 2010) whereas two others CYP82E2 and CYP82E3 encode inactive enzymes (Chakrabarti et al., 2007, Gavilano and Siminszky, 2007).

Inhibition of the nicotine to nornicotine conversion happens as the result of silencing of CYP82E gene subfamily. Tetcyclasis, which is an inhibitor of cytochrome P450, and carbon monoxide inhibited nicotine demethylation (Chelvarajan et al., 1993, Donaldson and Luster, 1991); while light of 450 nm reverses this inhibition (Donaldson and Luster, 1991). Therefore

these findings suggest a role of CYP82E4 in N-demethylation of nicotine to nornicotine that can be used to reduce nornicotine content of tobacco varieties, which is desirable for improving smoking quality and harm reduction of tobacco products.

1.4.2.2 Conversion phenotype in *Nicotiana* species

N. tabacum is the result of hybridization of diploid *Nicotiana* species closely related to modern *N. sylvestris* and *N. tomentosiformis* (Chakrabarti et al., 2007, Gavilano and Siminszky, 2007). In converter *N. tabacum* conversion happens only in the cured / senesced leaves, whereas *N. tomentosiformis* has both the green and senescing leaf conversion phenotype. That is because *N. tomentosiformis* has both active CYP82E3 and CYP82E4 which control conversion in green and senescing leaves, respectively. In contrast in *N. tabacum* a W330C amino acid substitution inactivates CYP82E3 and a CYP82E4 is also transcriptionally silenced (Gavilano et al., 2007).

Pakdeechanuan and Shoji (2012) examined the relationship between phenotype (nornicotine accumulation) and genotype (CYP82E). They studied the nornicotine phenotype in *N. alata* and *N. langsdorffii* and the molecular mechanisms by which the nicotine N-demethylase genes in *N. langsdorffii* are inactivated. They also provided genetic evidence that nornicotine formation in wild *Nicotiana* species is governed by the expression of functional CYP82E genes (Pakdeechanuan et al., 2012). Using gas liquid chromatography, they quantified the amount of alkaloids in the roots of 10-week-old plants of *N. alata* and *N. langsdorffii*, and their F1 hybrid and F2 progeny, and developed a formula to calculate the conversion rate. They divided the nornicotine content by the sum of the nicotine and nornicotine content. Based on that they classified F2 plants into 3 categories: nonconverters (no nornicotine detected), medium converters (<50% conversion) and high converters (conversion rates more than 50%). Then they scored allele frequencies of CYP82E genes for each group (Table 1-4).

Table 1-4 Allele frequencies of CYP 82E genes among F2 plants (Pakdeechanuan et al., 2012)

Phenotype	n	NalaE1	NalaE2	NalaE3	NalaE4	NlanE1	NlanE2
Non-converter	26	0	0	0	0	26	26
Medium converter	73	71	70	69	24	60	54
High converter	10	9	10	9	6	0	0
Total	109	80	80	78	30	86	80
	(100%)	(73.4%)	(73.4%)	(71.6%)	(27.5%)	(78.9%)	(73.4%)

n: Number of plants containing the indicated CYP82E alleles; NalaE: NalaCYP82E; NlanE: NlanCYP82E

According to their results, 26 plants were nonconverters and lacked all four of the *N. alata* CYP82E genes, but instead had both CYP82E genes of *N. langsdorffii*. In 73 plants which were medium converters, two or more *N. alata* CYP82E genes were always present, and NlanCYP82E1 and NlanCYP82E2 were also found in most plants. In 10 plants which were high converters two or more *N. alata* CYP82E genes were present, but no NlanCYP82E1 and NlanCYP82E2 were detected. Their genetic data indicates that in these two diploid *Nicotiana* species (*N. alata* and *N. langsdorffii*), the clustered CYP82E genes are located at the identical locus of the corresponding chromosome (Pakdeechanuan et al., 2012).

They concluded that whereas the *N. alata* CYP82E genes act semi-dominantly and individually to increase conversion of nicotine to nornicotine, the *N. langsdorffii* CYP82E genes are both non-functional. Also CYP82E genes encoding nicotine N-demethylase are duplicated at closely linked chromosomal regions in the two diploid *Nicotiana* species of the *Alatae* section, and that the two CYP82E genes in *N. langsdorffii* are rendered non-functional by either transcriptional inactivation or premature translational termination, resulting in the absence of nornicotine in this species (Pakdeechanuan et al., 2012).

1.4.2.3 Spatio-temporal distribution of nicotine to nornicotine conversion

Using grafting techniques in *N. tabacum* and *N. sylvestris*, Hall et al. (1965) studied time and site of nicotine to nornicotine conversion. They reported nicotine to nornicotine conversion happens in converter scions grown on nonconverter stocks, but nonconverter scions grown on converter stocks do not display conversion ability, suggesting that nicotine to nornicotine conversion happens in the leaf (Hall et al., 1965).

In another similar study, Wernsman et al. (1968) carried out further experiments and used lines that harboured converter genes from *N. tabacum*, *N. sylvestris* and *N. tomentosiformis,* to investigate the location and time of conversion. They also employed reciprocal grafting experiments. According to their results, the type of the scions used (i.e. whether it is converter or nonconverter) determines conversion ability and it doesn't depend on the type of stock. Also, considerable amounts of nornicotine are detected in both green and cured leaves (Wernsman and Matzinger, 1968). Hence, all these experiments indicate that conversion of nicotine to nornicotine is restricted to the leaves.

1.4.3 Alkaloid levels and composition in different species

Alkaloid levels and composition can vary greatly among the *Nicotiana* species (Lisko et al., 2013). Leaf tissues of most wild *Nicotiana* species contain predominantly either nicotine or its derivative nornicotine, with anabasine being a major constituent of the alkaloid fraction in a limited number of species (Zhang et al., 2012). In *N. glauca* for example, the level of nicotine is low, while anabasine is high (Lisko et al., 2013). Anatabine is a minor component of the alkaloid fraction in most species and is not found at high levels in leaves or roots of any *Nicotiana* species that has been analysed in detail (Chintapakorn and Hamill, 2003). In a study that investigated the alkaloid composition of 64 different species of the genus *Nicotiana* grown in greenhouse and field, it has been shown that all tested species contained a measurable alkaloid fraction (at least 10 µg. g^{-1}). Both total alkaloid level and the distribution of four major alkaloids nicotine, nornicotine, anabasine and anatabine indicated that these measures were highly correlated between greenhouse and field grown plants, although total alkaloid levels were significantly higher in plants grown in fields (Sisson and Severson, 1990).

A single alkaloid seems to be prominent in each species (Saitoh et al., 1985, Sisson and Severson, 1990). In most of the species, nicotine or nornicotine are the predominant alkaloid (Sisson and Severson, 1990), and in a few species including *N. noctiflora*, *N. petunioides*, (Sisson and Severson, 1990), *N. debneyi* (syn. *N. forsteri*) and *N. glauca* (Saitoh et al., 1985, Sisson and Severson, 1990), anabasine has been reported to be the major alkaloid in fresh leaves. In addition to the major alkaloid, a secondary alkaloid constitutes the remainder of alkaloid composition. Nicotine as major and nornicotine as second abundant alkaloid was the most prevalent distribution, followed by nornicotine as the major and nicotine as the second. In some species the combination of nicotine and anabasine was observed (Saitoh et al., 1985, Sisson and Severson, 1990).

Total alkaloid level and distribution of alkaloids varies in roots and leaves in *Nicotiana* species. The majority of species contain nicotine as the predominant alkaloid in roots (Saitoh et al., 1985), and any conversion to nornicotine that occurs is believed to happen in leaves. Anabasine and anatabine are more concentrated in roots than in leaves in most of species, except for *N. glauca, N. tabacum, N. repanda* and *N. hesperis*, in which anabasine is the major alkaloid in leaves and *N. alata N. corymbosa, N. hesperis* in which anatabine is the major leaf alkaloid (Saitoh et al., 1985).

The composition of alkaloids in air-dried and fresh leaves is different and in some species there is an increased proportion of nornicotine at the expense of nicotine, which reflects their converter trait (Sisson and Severson, 1990). As mentioned before, all of the species of *Nicotiana* endemic to Australia are from the section *Sauveolentes*. A wide range of alkaloid levels is exhibited and alkaloid composition is also heterogeneous in this section. However, there may be an association with the geographic distribution and alkaloid composition and level (Table 1-5). It is hypothesised that different alkaloid profiles is related to the adaption of the species to the climate in which they grow (Sisson and Severson, 1990).

Table 1-5 Subdivision of Australian species of *Nicotiana* in terms of total alkaloid, major alkaloid type and geographic distribution (Sisson and Severson, 1990)

Subdivision (included species)	**Major alkaloid**	**Total alkaloid level**	**Geographic distribution**
Subdivision 1 *N. gossei, N. amplexicaulis, N. benthamiana, N. excelsior, N. ingulba*	Nicotine	Very high	Arid regions of central and north Australia
Subdivision 2 *N. suaveolens , N. maritima, N. velutina, N. exigua, N. megalosiphon, N. goodspeedii, N. debneyi*	Nornicotine	Medium	South-central and east Australia
Subdivision 3 *N. simulans , N. rosulata, N. occidentalis, N. hesperis, N. rotundifolia, N. umbratica, N. cavicola*	Variable Nicotine or Nornicotine	Low	Western Australia

1.4.4 Alkaloid distribution and translocation in *Nicotiana* spp.

According to optimal defense theory, defense metabolites are distributed within plants but are allocated preferentially to tissues with high probability of being attacked and high fitness value to the plant so that their fitness benefits are optimized (Baldwin, 1999). Indeed the spatial and temporal details of nicotine production in *Nicotiana* are consistent with optimizing defence allocation in plants (Baldwin, 2001b).

Concentrations in different plant parts varies up to 10-fold, but the whole-plant amount is remarkably homeostatic and allometrically corrected constant pools maintained via controlling synthesis and biomass accumulation rather than nicotine turnover, regardless of variation in nitrogen supply rates, and even externally supplied nicotine. Variation in amounts of nicotine in different plant parts results from difference in synthesis and transport profile (Baldwin, 2001a).

Nicotine is synthesized exclusively in the roots and then transported to the shoots via xylem. This transport requires a signal from the leaves (Kidd et al., 2006). In general, the highest

concentration of nicotine is found in young leaves, stems, and reproductive parts, while roots and old leaves have the lowest. For example, high concentrations can be found in the trichomes of *N. attenuata,* which cover the calyx of flowers and developing seed capsules. In this case a single trichome can contain between 1.3-3.6 µg of nicotine, while in contrast to the calyx, seeds contain little nicotine (Baldwin, 1999). It is also likely that during elongation and flowering that de novo biosynthesis of nicotine in roots tends to decline; it is redistributed from its location in the central vacuole by symplastic transport routes that are not well known (Baldwin, 2001a).

1.5 Chemical composition of smokeless tobacco products

Smokeless tobacco products such as snuff, chewing tobacco and pituri contain fewer carcinogens than cigarette smoke because most are formed during combustion, but the level of potent carcinogens are still unacceptably high (Hecht, 2003). They mainly contain nicotine and some of them can deliver higher doses of nicotine than cigarettes. Nicotine from smokeless tobacco products is absorbed through the lining of the mouth or the nose (Winstanley, 2012) and the absorption is slower than that from cigarettes, but the level of nicotine in users' blood is similar to its level in smokers (International Agency for Research on Cancer, 2007, Scientific Advisory Committee on Tobacco Products Regulation, 2010). There are also significant quantities of PAHs (polycyclic aromatic hydrocarbons), nitrosamines, aldehydes (like formaldehyde, acetaldehyde and crotonaldehyde) and metals in unburned tobacco which contribute to its carcinogenic potential (Table 1-6). Fire-curing of *Nicotiana* leaves create polycyclic aromatic hydrocarbons (PAHs). Polycyclic aromatic hydrocarbons (PAH), N-nitrosamines and aromatic amines are categorized as strong carcinogens and aldehydes as weaker carcinogens (Hecht, 2003).

Table 1-6 Carcinogens in smokeless tobacco products, and the types of cancer they may cause (Hoffmann et al., 2001, International Agency for Research on Cancer, 2004, Hecht, 2003)

Chemical class	Number of compounds	Representative carcinogens and typical amounts in processed tobacco (ng g^{-1})		Types of cancer they cause
PAH (polycyclic aromatic hydrocarbons)	1	BaP (benzo[*a*]pyrene)	0.4-90	Lung Laryngeal Oral cavity Cervical
Nitrosamines	6	NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone)	1,890	Lung, Nasal, Oral cavity, Pancreatic, Cervical, Liver
		NNN (*N*′-nitrosonornicotine)	8,730	Nasal, Oral cavity, Oesophageal
Aldehydes	2	Formaldehyde	1,600-7,400	Nasal, Lung
		Acetaldehyde	1,400-7,400	
Inorganic compounds	7	Cadmium	1,300-1,600	Lung
Total	16			

1.5.1 Tobacco specific N-nitrosamines (TSNAs)

By far, the most prevalent strong carcinogens in unburned tobacco products like oral snuff, chewing tobacco and other smokeless tobacco products, are nitrosamines (Hecht, 2003). Tobacco specific nitrosamines (TSNAs) are responsible for increased risk of the upper digestive tract and known to cause oral cavity cancer, respiratory tract and pancreatic cancer (Brunnemann et al., 1996, Hecht, 2003). Their formation is via nitrosation of tobacco alkaloids (Fig. 1-5) (Brunnemann et al., 1996) which mostly happens during curing and there have been reports of their presence in smokeless tobacco (Hoffmann et al., 1994). Two of them, 4-(methylnitrosamino)-1-(3-pyridyl)-1 butanone (NNK) and *N*′-nitrosonornicotine (NNN) are the most potent ones and carcinogenic to laboratory animals (Hecht, 2003). Among the list of cancers caused by NNN and NNK are nasal, oral cavity, oesophageal, lung, pancreatic, cervical and liver cancer (Hecht, 1998, Hecht, 2003). *N*′ nitrosoanabasine (NAB) is a weak carcinogen and *N*′-nitrosoanatabine (NAT) apparently lacks activity (Stepanov et al., 2006, Brown et al., 2003, Hoffmann and Hecht, 1985). Each alkaloid forms one primary nitrosamine by N-nitrosation; nornicotine is believed to be the main precursor of NNN. Nicotine forms NNN via demethylation to nornicotine and then N-nitrosation.

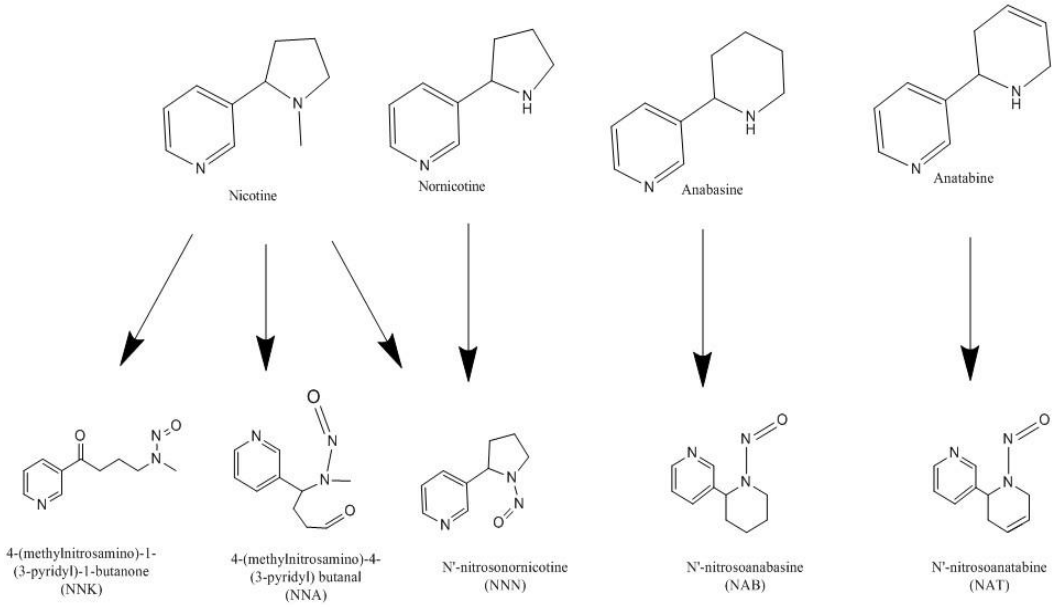


Figure 1-5 Tobacco alkaloids, and the nitrosamines that can be formed from them. With the exception of NNA, all of these compounds are present in tobacco and tobacco smoke (Hoffmann et al., 1995).

Concentration ranges of some TSNAs in fresh/dry leaves and in different smokeless tobacco products from countries such as India, Canada, US, some European countries, Uzbakistan, Sudan, South Africa and Thailand have been compared in the monograph on smokeless tobacco products by the International Agency for Research on Cancer (International Agency for Research on Cancer, 2007). The levels of TSNA present were up to 0.39 µg/g NNN and up to 0.42 µg/g NNK in the fresh top leaves of *N. tabacum* (flue-cured type) grown in the USA. In *N. tabacum* grown in India the levels were up to 0.035 µg/g NNN and 0.0115 µg/g NNK (Djordjevic et al., 1989a, Hoffmann et al., 1994). In smokeless tobacco they are mainly produced after harvest and during processes like drying, curing, ageing and especially fermentation. Storage in elevated temperatures for longer than four weeks results in higher amounts of TSNA (Brunnemann et al., 1996). Curing specifically has a huge impact on increasing the levels and the concentration ranges of NNN and NNK in cured leaves of commercial *Nicotiana* species (*N. tabacum* and *N. rustica*) have been reported to be increased by 6 fold compared to concentration in fresh green leaves (Djordjevic et al., 1989b, International Agency for Research on Cancer, 2007). Up to 3 mg of NNN and NNK could be present in each gram of a smokeless tobacco from Sudan which is called toombak (Idris et al., 1991). The concentration range in saliva of the users has also been compared in different smokeless tobacco products from around the world (International Agency for Research on Cancer, 2007) with the highest levels reported for toombak users (Idris et al., 1992).

1.6 Nicotine delivery in smokeless tobacco products

Tobacco dependence results from nicotine, which through binding to nicotinic cholinergic receptors, increases their expression in the nervous system and results in releasing neurotransmitters and hormones that have effects on mood, cognition, appetite and cause dependence (Albuquerque et al., 2009, Henningfield et al., 1995a). Difficulty in quitting tobacco and withdrawal symptoms are directly linked to the quantity of nicotine intake (Fagerstrom et al., 1990, Hughes and Hatsukami, 1986, Walsh et al., 1994). The rate of absorption has also been shown to be an important drug addictive quality (de Wit et al., 1992, Henningfield and Keenan, 1993).

The addictive and toxic effects of nicotine are maximised when it is delivered with a vehicle like tobacco (Henningfield et al., 1995a). In smokeless tobacco products use, nicotine is extracted from the tobacco and passes through mouth mucosa to enter the blood and finally the brain to have the pharmacological effects that result in addiction. In different tobacco products, the pH level varies and it affects the quantity of nicotine received by the user (Hoffmann et al., 1995, Tomar and Henningfield, 1995). The physiological and psychoactive effect of a few mg of nicotine delivered rapidly via smokeless tobacco products with high pH will be stronger than using 20 mg via nicotine transdermal patch (Henningfield et al., 1995a).

The fact that pH is the main determinant of nicotine delivery through biological membranes has been shown by animal studies (Travell, 1940, Travell, 1960), using a dialysis membrane model (Nair et al., 1997), and studies on humans (Beckett et al., 1972, Ivey and Triggs, 1978). Based on studies on buccal absorption of alkaloids, no absorption of nicotine was observed at pH 5.5, about 10% at pH 7, and more than 30% at pH 9 (Beckett et al., 1972). A similar pattern has been reported for nicotine absorption from the stomach; no absorption at pH 1, increased absorption at pH 7.4 and even a higher absorption at pH 9.8 (Ivey and Triggs, 1978). In order to simulate nicotine absorption through mouth mucosa, the rate of nicotine absorption through a dialysis membrane has been studied, also suggesting that increasing pH results in increased absorption of nicotine. The difference in transferred amount of nicotine across the membrane between high and low pH products was maximum in the first two minutes of applying them (Tomar and Henningfield, 1997). The nicotine release rate from smokeless tobacco product into saliva has also been reported to be increased by pH of the product (Nasr et al., 1998), which in turn can lead to a higher absorption of nicotine.

Transferring nicotine through a biological membrane, depends on its concentration in free-base form. The pKa (dissociation constant) of nicotine is 8.02 (Haynes et al., 2012), which means in an aqueous solution with pH of 8.02, half of it would be in un-ionised and the other half in ionised form. When the pH is higher than pKa, nicotine is mainly in its un-ionised and free-base form; hence its transport through a biological membrane is easier than as the ionised form. Therefore the rate of nicotine absorption and its psychoactive and addictive effects will increase if pH is raised (Tomar and Henningfield, 1997). Considering this, it has been mentioned in the literature that knowing the total amount of nicotine in a smokeless tobacco product without knowing its pH is of no value for estimating nicotine absorption rate or quantity (Djordjevic et al., 1995, Henningfield et al., 1995b, Connolly, 1995).

The pH of the oral environment controls nicotine absorption (Henningfield et al., 1990), especially in smokeless tobacco products which are left in the mouth for long periods. Naturally the tobacco leaf by itself is acidic, but when placed in the mouth due to the buffering action of the bicarbonate in saliva, even in the neutral pH of the oral environment a proportion of the nicotine will be un-ionised, however this process is very slow. Therefore, buffering tobacco products to alkaline levels speeds up this process and improves the rate of release (Tomar and Henningfield, 1997).

Depending on conditions of storage, i.e. temperature, humidity and duration, the pH level of smokeless tobacco products may increase (Andersen et al., 1993, Djordjevic et al., 1993). Manufacturers can also alter the pH through addition of alkalisers such as sodium carbonate, sodium bicarbonate, ammonium carbonate, and calcium carbonate in order to control the dose of nicotine (Connolly, 1995).

1.6.1 Nicotine release from smokeless tobacco products

The extent of nicotine release from any given smokeless tobacco product and its absorption is important in determining its psychoactive and addictive quality (Hatsukami and Severson, 1999). The commercially available smokeless tobacco products undergo different processes such as fermentation and moisture level adjustment and all these processes help in their pH adjustments. Sometimes buffering agents are also added to help pH adjustment. The particle size of the tobacco fragments and the added chemicals for coating or binding the fragments together are very important in determining the nicotine release rate from them. Binding agents are added in products such as long cut smokeless tobacco products that have larger fragments of tobacco leaves to help packing the products tighter and as a result they tend to show slower

nicotine release rates than products with finer tobacco fragments (Connolly, 1995). The *in vitro* release rate has also been shown to be higher in the products with higher pH (Nasr et al., 1998). Some products such as snus, come in tea bag like sachets that could be rate limiting for nicotine release since they need to be moistened and let the saliva to diffuse inside and release the nicotine into the oral environment for absorption through the mucosa (Connolly, 1995). The sachets have been shown to control the nicotine release rate in Skoal Bandit Classic, which is a commercially manufactured American moist snuff (Nasr et al., 1998).

1.7 Toxicity of smokeless tobacco products

Users of smokeless tobacco products are exposed to a spectrum of chemicals with some having carcinogenic potential, although it is less than what is reported for smoked tobacco. Recently, measures have been put in place by the Food and Drug Administration (FDA) to make sure that the manufactured smokeless tobacco products have reduced health risks. Consequently, emerging potential reduced exposure products (PREP) need to be evaluated in terms of their impact on health. Epidemiologic and clinical trials would be the best approach for risk evaluation of these products, however, laboratory models such as *in vitro* cell assays could also be used for the evaluation of their relative toxicity (Johnson et al., 2009). These assays are inexpensive and fast, which allows faster screening of toxic levels and helps extrapolation to humans. Viability of the cells exposed to the studied matrix is evaluated using these *in vitro* cytotoxicity assays and, since cytotoxicity is an important step in disease development mechanisms, it could be used as an indicator for the potential of any given product to cause harm. These assays can also help in defining the concentration range and more importantly the dose resulting in 50% of cell death (IC_{50}) that is essential in comparing the effects of identical compounds delivered in different matrixes or of multiple compounds in one system. Also, the basal cytotoxicity tests are the stepping stone for other *in vitro* assays that determine the genotoxicity, mutagenicity and induction of programmed cell death caused by these products (Eisenbrand et al., 2002). Different biological endpoints are monitored for determining the cytotoxicity. These include the assays that measure plasma membrane permeability, mitochondrial function, cell morphology and reproduction changes or a combination of these effects (Borenfreund and Puerner, 1985, Eisenbrand et al., 2002, Johnson et al., 2009).

There are numerous reports on *in vitro* cytotoxic effects of commercially available smokeless tobacco products such as Swedish snus (Coggins et al., 2012, Costea et al., 2010), American moist snuff (Misra et al., 2014), commercial chewing tobacco (Coppe et al., 2008) and

Kentucky reference smokeless tobacco product (Lombard et al., 2010). There have been reports of cytotoxicity caused by Indian manufactured smokeless tobacco such as gutka (Avti et al., 2010) and khaini (Das et al., 2013). Limited data is available on the toxicity of homemade traditional or indigenous smokeless tobacco products used around the world. Generally, the manufactured products are monitored to contain less TSNAs. It's not the case with traditional and custom-made products; an example is the Sudanese smokeless tobacco product toombak that has been shown to contain at least 100 times higher levels of tobacco specific N-nitrosamines than the commercially available Swedish and American snuff (Idris et al., 1998). Toombak is the only indigenous smokeless tobacco that has been studied for its cytotoxicity and the results indicated its higher toxic potential compared to commercially available Swedish snuff (Costea et al., 2010). There is very little information available on the extent of toxicity caused by indigenous or local smokeless tobacco products similar to pituri.

1.8 Rationale

As reviewed in the literature so far, research on the chemistry of tobacco plants and products helps us gaining a better understanding of the diversity and levels of psychoactive chemicals in them as well as the risks attributed to their consumption. While some scientists suggest substitution of smoking with smokeless tobacco as a harm reduction measure, there is little support for such claims based on the available evidence. Smokeless tobacco products have been reviewed extensively for their ingredients, their chemistry including carcinogenic components and the potential toxicity in the International Agency for Research on Cancer's monograph. Other than commercially available smokeless tobacco products, this monograph reports on the use of indigenous smokeless tobacco products among some native populations around the world. However, pituri, a smokeless tobacco product used by some Aboriginal Australians in Central Australia that is prepared from Australian native *Nicotiana* spp. was not included in the monograph and no data on chewing tobacco use is collected by the Australian Bureau of Statistics and so official acknowledgement of the existence of pituri is lacking.

The research proposed here will help to increase knowledge about the chemistry of Australian *Nicotiana* spp., as the tobacco component used in pituri preparation, which will provide some insight in terms of potential for harm to users.

1.9 Aims and hypotheses to be tested

The overall aim of this thesis is to investigate the *Nicotiana* component of pituri in terms of pyridine alkaloids and tobacco-specific nitrosamines, their production, release and toxicity. Therefore, firstly, this study will establish validated methodologies for routine quantitative analysis of important chemicals such as alkaloids and nitrosamines in tobacco plants and products. Secondly, it will report on the alkaloid composition of Australian *Nicotiana* spp. and search for the presence of an underlying molecular mechanism in nicotine to nornicotine conversion that leads to production of nornicotine. Thirdly, this study will explore the release of nicotine and other alkaloids and nitrosamines from the tobacco constituent of pituri and compare these with other commercially available products. Finally, the potential toxicity of the tobacco constituent of pituri and a reference moist snuff will be compared to that of pure nicotine in an *in vitro* setting. Outcomes of the research described in this dissertation will report alkaloid chemistry of Australian *Nicotiana* species, provide insight into nornicotine production and warn about the potential toxicity of the released chemicals to users.

To help in producing more knowledge and relieve the ambiguity surrounding pituri, which is amongst the least studied and acknowledged traditional smokeless tobacco products, this study aimed to:

1. Establish efficient quantification methods for determination of the alkaloids and tobacco specific nitrosamines in *Nicotiana* spp used in pituri. Based on methods described in the literature, HPLC-UV and LC-MS/MS techniques are hypothesised to be suitable for quantitative analysis of pyridine alkaloids and TSNAs, respectively. Methods will be developed and validated to test their efficiency.

2. Quantify the alkaloids present in all 26 of the currently recognised Australian *Nicotiana* species. Based on alkaloid measurements published 25-30 years ago (Saitoh et al., 1985, Sisson and Severson, 1990) that included 19 of the Australian species, it is hypothesised that Australian *Nicotiana* species vary in terms of abundant alkaloids and are categorised into nicotine dominant versus nornicotine dominant species. This will be addressed by germinating seeds and growing plants in a single common environment, quantifying the alkaloids present in their leaves, and assessing the relative levels of nicotine and nornicotine in order to identify those species that are low and high converters.

Nornicotine production in *Nicotiana tabacum* is mainly through N-demethylation of nicotine and is regulated by a group of cytochrome P450 related subfamily of genes which are triggered

by curing and senescence (Chakrabarti et al., 2007, Gavilano and Siminszky, 2007, Siminszky et al., 2005). The conversion locus, mainly CYP82E4, has been found to be present in all *Nicotiana* investigated, but its transcription is strongly upregulated by curing and during leaf senescence only in high converters (Chakrabarti et al., 2008). Therefore, it was hypothesised that the nicotine to nornicotine conversion locus is present in all Australian *Nicotiana* species and is triggered by curing only in high converter species. This will be addressed by assessing whether the locus is present in all species, and then further investigating its regulation associated with curing for a small number of low and high converter species.

3. Smokeless tobacco use can be associated with fast release of high levels of nicotine, but there is currently no knowledge of nicotine release from pituri. Pituri is sucked rather than chewed so it is hypothesised that the release of nicotine from the tobacco constituent of pituri is more similar to that of commercial smokeless tobacco products such as Swedish snus than from nicotine gum. Additionally, pituri is prepared by mixing alkaline wood ash with *Nicotiana* leaves so it is hypothesised that release is faster at higher pH induced by adding the ash. These hypotheses will be addressed by studying nicotine release with and without chewing, and under both normal and alkaline pH condition, for comparison with nicotine release from Swedish snus and a nicotine replacement therapy product, Nicabate gum.

4. The major reason for the use of tobacco products is the psychoactive alkaloid nicotine. However, based on analyses of other indigenous smokeless tobacco products, pituri users are likely to be exposed to a range of other potentially toxic chemicals. Therefore, the aim of this section is to investigate the toxicity of the *Nicotiana* constituents of pituri, with the hypothesis that the *Nicotiana* constituents are associated with higher cytotoxicity than nicotine alone. This will be addressed by testing *in vitro* cytotoxicity of an extract from *Nicotiana* leaves used in pituri on human lung epithelial cells, and comparison to a similar reference product and to cytotoxicity caused by only nicotine.

Chapter 2. HPLC-UV and LC-MS/MS method development and validation for quantification of six alkaloids and two nitrosamines in tobacco plants and products

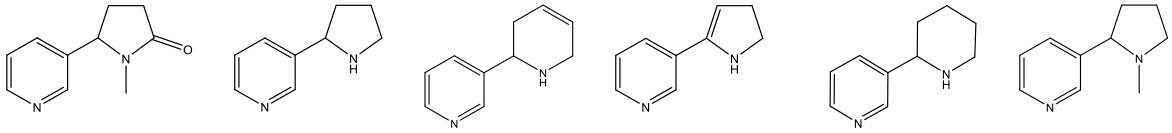
2.1 Introduction

Nicotiana species (*Solanaceae*), known as tobacco plants, are the most widely used drug plants in the world (Chase et al., 2003, Knapp et al., 2004). Tobacco products are responsible for nearly 6 million deaths around the world each year, and it is expected to reach to 8 million by 2030 (World Health Organization, 2011). All members of the *Nicotiana* genus produce nicotine, which accounts for the widespread human use of tobacco plants and products. They also produce many other pharmacologically active pyridine alkaloids, however, these are less potent than nicotine (Clark et al., 1965, Keinanen et al., 2001). Structurally related minor alkaloids present in several wild *Nicotiana* species and cultivars of commercial tobacco (*Nicotiana tabacum*) are nornicotine, anabasine, anatabine and myosmine (Huang and Hsieh, 2007, Lisko et al., 2013) (Fig. 2-1 A). Cotinine has been reported to be present at low levels (Luanratana and Griffin, 1982) although it is primarily recognised as the major metabolite of nicotine in the human body (Benowitz et al., 2009). The number and abundance of the different alkaloids are highly variable within the genus (Sisson and Severson, 1990).

Apart from alkaloids, tobacco specific nitrosamines (TSNAs) are also present in tobacco plants and products (Fig. 2-1 B). They are produced through nitrosation of tobacco alkaloids during curing and post-harvest processing of tobacco leaves and some of them are strong carcinogens. Among the reported TSNAs, N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are produced from nicotine and nornicotine and have been classified as potent carcinogenic compounds to humans. Corresponding TSNA compounds derived from anatabine and anabasine are N'- nitrosoanatabine (NAT), which hasn’t reported to have carcinogenic activity, and N-nitrosoanabasine (NAB) that is considered to be a weak carcinogen (International Agency for Research on Cancer, 2007, Hecht et al., 1983).

The concomitant presence of multiple alkaloids, which are responsible for the formation of carcinogenic TSNAs, drives the need for their quantitation in tobacco plants and products (Lisko et al., 2013). It is also of high importance in the tobacco industry for quality control purposes (Lu and Ralapati, 1998) and is necessary for the understanding of plant secondary and defence metabolism (Gaquerel et al., 2009).

A



Cotinine	Nornicotine	Anatabine	Myosmine	Anabasine	Nicotine
MW=176.22	MW=148.20	MW=160.22	MW=146.19	MW=162.23	MW=162.23
pKa=4.88	pKa=9.83	pKa=8.76	pKa=6.66	pKa=9.86	pKa=8.02

B

N'-nitrosonornicotine	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
(NNN)	(NNK)
MW=177.20314	MW=207.22912
pKa=5.25	pKa=3.79

Figure 2-1 Structure, molecular weight (MW, g/mol) and pKa values of A) the common tobacco alkaloids shown in order of HPLC elution and B) the two major carcinogenic TSNAs, NNN and NNK

Many reports have focused on the quantitative analysis of nicotine and other minor alkaloids in tobacco plants by employing gas chromatography (GC) with a variety of detection methods such as flame ionization detection (FID) (Sheng et al., 2005), nitrogen-phosphorus detection (NPD) (Sisson and Severson, 1990, Yang et al., 2002), nitrogen chemiluminescence detection (NCD) (Cai et al., 2012b) and mass spectrometry (MS) (Hossain and Salehuddin, 2013). Less frequently used in tobacco alkaloid analysis are techniques such as micellar electrokinetic capillary chromatography with UV detection (MEEC-UV) (Lu and Ralapati, 1998), microemulsion electrokinetic chromatography with UV detection (MEEKC-UV) (Huang and Hsieh, 2007) and capillary electrophoresis (CE) with UV (Lochmann et al., 2001) or electrochemical (ED) detection (Matysik, 1999). High performance liquid chromatographic (HPLC) methods for alkaloid determination in tobacco plants mainly involve the use of UV detection (Keinanen et al., 2001, Tambwekar et al., 2003, Ciolino et al., 1999a, Sudan et al., 1984, Švob Troje et al., 1997, Manceau et al., 1992, Saunders and Blume, 1981), and HPLC-MS has been employed for the determination of only nicotine from tobacco (Vlase et al., 2005). HPLC-UV is generally accepted to be a cost effective and convenient method for separation

and accurate quantification of chemicals with a chromophore, as is present in alkaloids, but so far the methods in the literature are restricted to the quantification of nicotine, as the main alkaloid (Tambwekar et al., 2003, Ciolino et al., 1999b), or only two (Sudan et al., 1984, Švob Troje et al., 1997) three (Manceau et al., 1992) or maximum of four (Saunders and Blume, 1981) major alkaloids in tobacco plants and products.

Similar to alkaloids, there have also been numerous reports of the conventional methods for quantification of TSNAs mainly in cigarette smoke and some other tobacco products. Until recently the majority of these reports used GC coupled with thermal energy analyser (TEA) (Carmella et al., 2000, Stepanov et al., 2002) or mass spectrometry (MS) (Song and Ashley, 1999, Zhou et al., 2007, Sleiman et al., 2009), or HPLC with various detectors such as UV (Hecht et al., 1975), or TEA (Hoffmann et al., 1979). It is now more common to see LC-MS/MS being used extensively for quantitative analysis of TSNAs (Kim and Shin, 2013, Wagner et al., 2005, Wu et al., 2008, Ding et al., 2008, Xiong et al., 2010, Jansson et al., 2003) due to its high sensitivity.

In order to study the alkaloids of wide range of wild *Nicotiana* species and tobacco products, this study aimed to develop and validate two methods. Firstly, a new fast, convenient and efficient HPLC-UV method for the simultaneous separation and quantification of six common tobacco alkaloids was developed. This method used a reverse-phased column with bidentate C18-C18 bonding technology and basic pH mobile phase, and achieved high sensitivity, reproducibility, precision and linear response with elution over 13 minutes. Secondly, an LC-MS/MS method was performed, adapted from methods in the literature (Kim and Shin, 2013), to quantify the two main carcinogenic TSNAs, NNN and NNK in some smokeless tobacco products. This analysis took less than 2 minutes using a HILIC column with a triple quadrupole tandem MS and was validated for linearity, detection limits, accuracy, recovery and repeatability.

2.2 Experimental

2.2.1 Chemicals

Acetonitrile and methanol of HPLC grade and hydrochloric acid (HCl) were from Merck (Darmstadt, Germany), triethylamine (TEA; pro-analysis) was from Ajax Finechem (Sydney, Australia) and ammonium formate (for HPLC, ≥99.0%) was from Sigma-Aldrich (St. Louis, MO). The water was from a Milli-Q system (Millipore, Billerica, MA). The analytical

standards used for HPLC were nornicotine, myosmine and cotinine from Sigma, nicotine from Fluka (Milwaukee, WI), anabasine from Sigma-Aldrich, anatabine from Cayman Chemical Company (AnnArbor, MI), and the internal standard used was caffeine from Ajax Finechem (Sydney, Australia). The standards used in LC-MS/MS analysis were N'-nitrosonornicotine (NNN) and 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) from sigma and their corresponding internal standards were N'- nitrosonornicotine-d4 (NNN-d4) and 4-(methylnitrosoamino)-1-(3-pyridyl-d4)-1-butanone (NNK-d4) from Toronto Research Chemicals (Ontario, Canada).

2.2.2 Instrumentation and chromatographic conditions

2.2.2.1 HPLC-UV

An Agilent Technologies 1200 series HPLC system (Agilent Technologies, Boeblingen, Germany) with a binary pump, degasser, an auto sampler, a thermostat column compartment, and a diode array detector was used. ChemStation for LC 3D software version B.04.02 (Agilent Technologies, Wilmington, DE) was used for data acquisition and handling. Chromatography was performed on a reversed-phase Zorbax Extend C-18 column (Agilent Technologies) of 150 × 3 mm i.d. dimensions and 3.5 µm particle size. The mobile phase consisted of 15 mM ammonium formate in water, pH adjusted to 10.5 with TEA (solvent A) and acetonitrile (solvent B). Gradient elution used was: 0-5 min from 9 to 10% solvent B; 5-13 min from 10 to 13% solvent B; the flow rate was 0.8 mL/min. The column temperature was set at 25ºC and the injection volume was 40 µL. Detection was performed at 260 nm.

2.2.2.2 LC-MS/MS

The LC-MS/MS analysis was conducted on a 2.1 × 150 mm Poroshell 120 HILIC column with 2.7 µm particle size (Agilent Technologies). The MS-MS detection was performed in positive ion mode on an Agilent 6460 Triple Quadrupole tandem mass spectrometer with a Jet Stream source and the workstation operated with MassHunter software (Agilent Technologies). Multiple reaction monitoring (MRM) mode was used for detection. Mass spectrometry parameters such as the m/z of precursor and product ions, fragment voltage and collision energy were as shown in Table 2-1. The nozzle and capillary voltages were 2000 and 5000 V, respectively. The mobile phase was composed of 10 mM ammonium formate in methanol (70%) and 10 mM ammonium formate in water (30%) with a flow rate of 0.35 mL/min. The total run time was 3 min and the injection volume was 1 µL.

Table 2-1 Mass spectrometry parameters used in MRM mode for two TSNAs

Compound	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
NNN	178.1	148.1	60	11
NNN-d4	182.1	152.1	60	11
NNK	208.1	122.1	60	11
NNK-d4	212.1	126.1	60	11

2.2.3 Sample preparation

2.2.3.1 HPLC-UV

For alkaloid extraction, samples of fresh/dried tobacco leaves or smokeless tobacco products were freeze dried at -80ºC, and 50 mg were ground to a fine powder. These then were extracted by shaking vigorously for 2 h with 1 mL of extraction solution (40% aqueous methanol containing 0.1% 1 N hydrochloric acid). After centrifugation the supernatant was removed and the remaining pellet subjected to two further extractions with 0.5 mL of extraction solution each time and the supernatants combined; no alkaloids were detected in any further extracts from the pellet. The extracts then were evaporated to dryness with a vacuum speed evaporative concentrator (Savant Speed Vac SC-110 with cooling unit RVT-100, Savant instruments, Holbrook, NY) and reconstituted in 10% aqueous acetonitrile. All standards and extracts were filtered through a 0.45 µm nylon filter (Grace Davison Discovery Sciences).

2.2.3.2 LC-MS/MS

Aqueous extract of smokeless tobacco products (100 µl) were prepared for analysis of NNN and NNK by adding 50 ng/ml of both internal standards (NNN-d4 and NNK-d4), evaporating to dryness and reconstituting in the mobile phase (10 mM ammonium formate in methanol and 10 mM ammonium formate in water) before analysis.

2.2.4 Method validation

2.2.4.1 HPLC-UV

To validate the method, system suitability and sensitivity, linearity, precision and reproducibility were assessed. System suitability was evaluated by taking data from six replicate analyses of a mixture of alkaloid standards at a concentration of 5 µg/mL for all alkaloids and IS. Parameters used for monitoring system suitability were tailing factor (Tf), plate number (N), peak area (Pa), resolution (R_s) and capacity factor (K′), all calculated using

the United States Pharmacopeia (USP) equations (United States Pharmacopoeial Convention, 2011). System sensitivity was assessed using limits of detection (LOD) and quantification (LOQ) (Shabir, 2003, United States Pharmacopoeial Convention, 2011). The linearity of the method was assessed by linear regression analysis. Eight concentrations of each alkaloid standard, over an expected concentration range of each alkaloid in tobacco plants, were used to construct the calibration curves. A fixed concentration of IS was added to all of the concentrations and the peak area ratio of each alkaloid to that of IS was used to build the calibration plots. To assess the precision of the method, repeatability within intra and inter-day runs was checked by calculating the relative standard deviation (RSD) for the retention time and peak area ratio of six replicate injections. This was performed for each alkaloid standard in a mixture of alkaloids and IS all at a concentration of 5 µg/mL. Method reproducibility was evaluated by performing the analytical method on two other HPLC systems within the lab: Instrument 2 was an Agilent technologies 1100 series HPLC system with a capillary pump, degasser, auto sampler and diode array detector operated with ChemStation for LC 3D software version B.04.02, and Instrument 3 was a Shimadzu HPLC system with a dual piston pump, degasser, auto sampler, and diode array detector connected by the controller module and operated with LabSolutions software (Shimadzu Corporation, Koyoto, Japan). The RSD of six replicate injections of the same mixture of alkaloids (5 µg/mL for all alkaloids and IS) for both retention time and peak area ratio to IS was calculated for each alkaloid standard.

2.2.4.2 LC-MS/MS

For validation of the LC-MS/MS method, sensitivity, linearity, precision and accuracy were assessed. Sensitivity was assessed by determining the limit of detection (LOD) and limit of quantitation (LOQ). For linearity, regression statistics were calculated from peak area ratios for NNN and NNK to their corresponding internal standard (NNN-d4 and NNK-d4) for at least eight concentrations in an expected range. Precision of the method was estimated by intra-day repeatability in analysing 10 individual aliquots of samples, performed for a mixture of NNN and NNK and their corresponding internal standards at a concentration of 10 ng/mL. Accuracy was evaluated for recovery of added amounts in six samples at concentrations of 10 ng/ mL for NNN and NNK.

2.3 Results and discussion

2.3.1 Method development

2.3.1.1 HPLC-UV

HPLC-UV can be difficult due to lack of resolution between the multiple compounds. Obtaining acceptable peak parameters for the analytes is essential in quantitative studies. In chromatographic analysis, the addition of salts and ion-pairing agents to the mobile phase can improve resolution and peak parameters (Mallet et al., 2004). It is also important to consider the ionization constants (pKa) for each of the ionisable groups on the analytes when separating them by HPLC because the mobile phase pH can play a pivotal role in retention, peak shape, resolution, and reproducibility (Claessens, 2001, Kele and Guiochon, 1999, Neue et al., 1999, Heyrman, 1999). Most of the common tobacco alkaloids contain a tertiary amine group with a pKa value of more than 8 (Fig. 2-1A), so at acidic and neutral pH they are mainly in a ionised form with associated shorter retention under reversed-phase HPLC conditions; this can make it difficult to quantify them simultaneously using HPLC-UV due to lack of peak separation. Increasing the pH ensures the alkaloids are unionised, increasing retention times (Heyrman, 1999, Chen and Xu, 2011).

All six alkaloids were fully retained and eluted within less than 13 min with a flow rate of 0.8 mL/min and pH 10.5 (Fig. 2-2 A); previously it took 24 min to elute and separate just four alkaloids using HPLC-UV (Saunders and Blume, 1981). The short run time consumes less mobile phase and will prove cost effective for large sample population analysis. The developed method was successfully applied to the quantification of alkaloids from fresh leaves of *Nicotiana benthamiana*. No interference with the matrix was observed (Fig. 2-2 B). The quantitation shows that the fresh leaves of *Nicotiana benthamiana* contained 0.2, 0.3, 0.3, and 2.6 mg/g DW of anatabine, nornicotine, anabasine and nicotine, respectively.

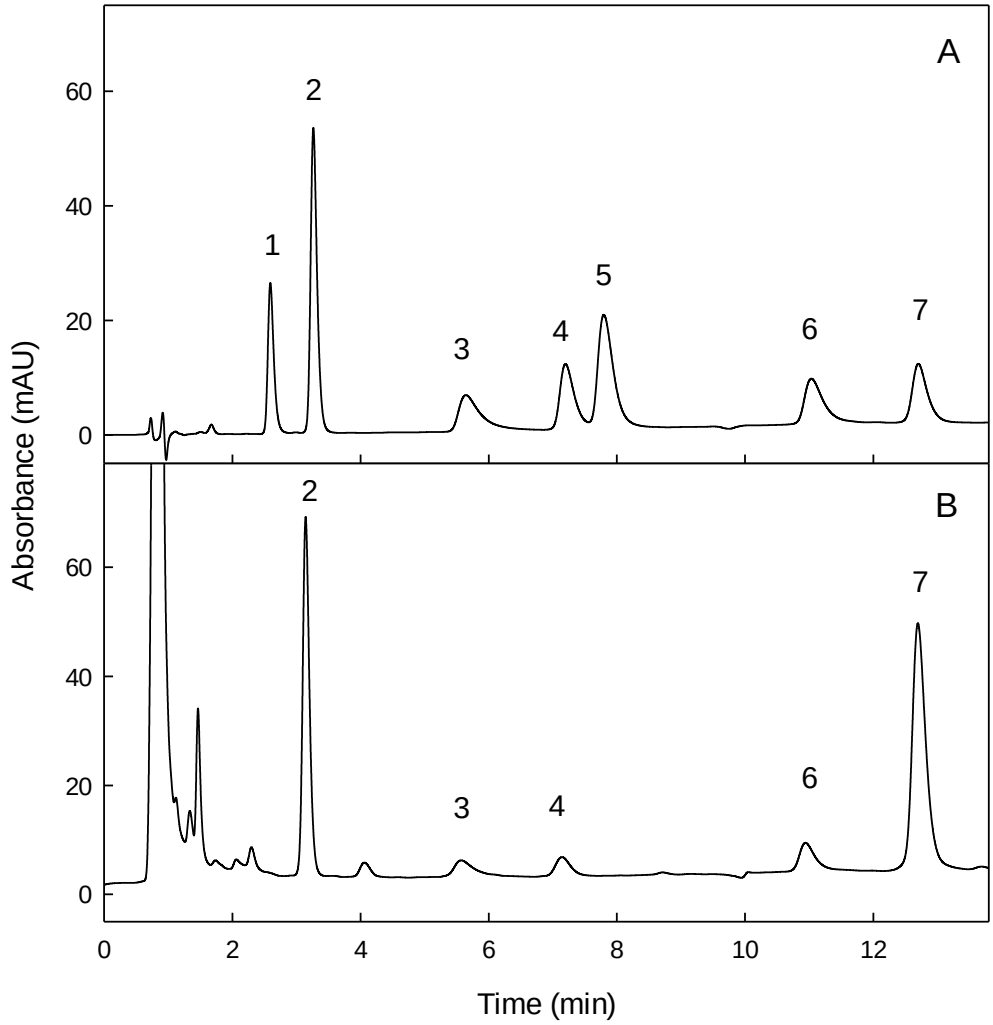


Figure 2-2 Chromatograms depicting separation of alkaloids on a 150 × 3 mm, 3.5 µm particle size Zorbax Extend C-18 column and detection at 260 nm. The mobile phase (flow rate 0.8 mL/min) consisted of a gradient of acetonitrile and 15 mM ammonium formate in water (pH adjusted to 10.5 with triethylamine); the gradient elution was 9 to 10% acetonitrile from 0-5 min and then 10 to 13% acetonitrile from 5 to 13 min. (A) Standard solution of alkaloids and IS, (B) Extraction from *Nicotiana benthamiana* fresh leaves. 1. Cotinine; 2. Caffeine (IS); 3. Nornicotine; 4. Anatabine; 5. Myosmine; 6. Anabasine; 7. Nicotine.

2.3.1.2 LC-MS/MS

A variety of mobile phases were tested for LC-MS/MS analysis of the NNN and NNK (data not shown) following the different combinations reported in the literature (Kim and Shin, 2013). The conditions selected for use, due to excellent sensitivity achieved, were 10 mM ammonium formate in methanol and 10 mM ammonium formate in water with a ratio of 70% to 30%. This was programmed to be an isocratic 2 minutes long run with flow rate of 0.35 mL/min.

2.3.2 Method validation

2.3.2.1 HPLC-UV

The system suitability test is used to verify that resolution and reproducibility are adequate (Shabir, 2003). The RSD of peak area and retention time for all alkaloid standards and IS were within 2% (Table 2-1), which indicates the suitability of the system. Also the capacity factor value (K′), the efficiency of the column as expressed by plate number (N), USP tailing factor (Tf) and the baseline separation expressed as resolution (R_s) were all within the acceptable range (K′>2.0, N>2000, 0.5≤Tf≤2 and R_s>2) (Shabir, 2003, United States Pharmacopoeial Convention, 2011) for all of the six alkaloids and IS, for the six replicate injections (Table 2-2).

Table 2-2 System suitability data obtained for validation of the developed method with mobile phase pH 10.5. The values are the average or relative standard deviation (RSD) of six consecutive injections of a mixture of alkaloids and IS (caffeine) all at a concentration of 5 µg/mL using the developed method.

	Tobacco alkaloids						
Parameter	Cotinine	Caffeine	Nornicotine	Anatabine	Myosmine	Anabasine	Nicotine
Rt	2.5	3.1	5.4	6.9	7.5	10.8	12.6
Pa	175.2	391.6	146.4	181.7	345.1	158.7	172.0
K´	2.4	3.2	6.5	8.5	9.2	13.7	16.2
Tf	0.7	0.7	0.5	0.6	0.6	0.6	0.6
N	11113	14393	5207	15847	16524	22918	43020
R_s		5.8	11.7	5.8	2.3	12.8	6.8
RSD (Rt)	0.1	0.2	0.1	0.1	0.1	0.0	0.0
RSD (Pa)	0.1	0.3	0.3	0.2	0.3	0.7	0.3

Rt=retention time; Pa=Peak area; K´=capacity factor; Tf=tailing factor; N=plate number; R_s=resolution

The sensitivity of the method was expressed as limit of detection (LOD) and limit of quantification (LOQ) (Table 2-3), using the residual standard deviation of the regression line (σ) and slope (S) of the calibration curve for each alkaloid standard with LOD being 3.3 σ/S and LOQ 10 σ/S (Shabir, 2003). The method proved to be sensitive for the detection of less than 1 µg/mL of all except for nicotine, which had a detection limit of 1.6 µg/mL. The method was also capable of quantifying as low as 2 µg/mL of cotinine, anatabine, anabasine and myosmine; 3 µg/ml of nornicotine and 5 µg/ml of nicotine in unknown tobacco samples (Table 2-3).

Table 2-3 Regression statistics, limit of detection (LOD) and limit of quantification (LOQ) for alkaloid standards.

	Tobacco alkaloids					
Parameter	Cotinine	Nornicotine	Anatabine	Myosmine	Anabasine	Nicotine
Conc. Range, µg/mL	2 to 20	2 to 50	2 to 20	2 to 20	2 to 20	5 to 250
Regression equation	y =0.01x-0.0030	y =0.010 - 0.012	y =0.009x +0.003	y =0.019x - 0.002	y =0.009x +0.003	y =0.010x - 0.012
R^2	0.9991	0.9997	0.9993	0.9997	0.9999	1.0000
LOD, µg/mL	0.6	0.9	0.6	0.4	0.2	1.6
LOQ, µg/mL	1.8	2.8	1.7	1.2	0.8	4.8

R^2=correlation coefficient; LOD=lower limit of detection; LOQ=lower limit of quantification

The regression statistics were calculated from the calibration curves constructed for all of the studied alkaloids (Table 2-4) and showed satisfactory linearity with the correlation coefficient (r^2) greater than 0.999 for all. For both intra and inter-day analysis of multiple injections, the variation of the RSD for peak area ratio of alkaloids to internal standard and retention time (Table 2-4) were within the acceptable range of 2% (Shabir, 2003).

Table 2-4 Method accuracy results obtained by calculating the relative standard deviation (RSD) value for the retention time (Rt) and average peak area ratio (Pa) of alkaloids to internal standard from six replicate injections within intra and inter-day runs in a mixture of alkaloids and IS (caffeine) all at a concentration of 5 µg/mL.

	Tobacco alkaloids						
Parameter	Cotinine	Caffeine	Nornicotine	Anatabine	Myosmine	Anabasine	Nicotine
Precision (RSD)							
Pa							
Day 1	0.107	0.118	0.316	0.002	0.227	0.273	0.211
Day 2	0.039	0.023	0.504	0.002	0.309	0.119	0.024
Day 3	0.029	0.045	0.666	0.000	0.113	0.222	0.058
Rt							
Day1	0.128	0.171	0.073	0.048	0.041	0.017	0.029
Day2	0.700	0.338	0.193	0.178	0.178	0.014	0.019
Day 3	0.024	0.070	0.105	0.153	0.153	0.058	0.075

The obtained values for the RSD (n=6) of the peak area ratio to IS for each alkaloid standard and RSD of their retention time in two other HPLC systems in the lab (Table 2-5) were within the acceptable criteria of less than 2% (Shabir, 2003) confirming that the method is reproducible.

Table 2-5 Within-laboratory reproducibility results. Relative standard deviation (RSD) for the retention time (Rt) and peak area ratio (Pa) of six replicate injections of a mixture of alkaloids and IS at a concentration of 5 µg/mL for each, obtained from performing the developed method on two different HPLC systems within the lab.

	Tobacco alkaloids					
Parameter	Cotinine	Nornicotine	Anatabine	Myosmine	Anabasine	Nicotine
Reproducibility (RSD)						
Instrument 2 (n=6)						
Pa ratio (n=6)	0.4	0.9	0.9	1.9	0.6	0.5
Rt (n=6)	0.5	0.6	0.6	0.7	0.6	0.7
Instrument 3 (n=6)						
Pa ratio (n=6)	0.2	1.0	1.6	0.9	1.1	0.5
Rt (n=6)	1.6	0.9	0.7	0.6	0.4	0.4

2.3.2.2 LC-MS/MS

The LOD and LOQ were calculated as 3.14 and 10 times the standard deviation of peak area ratio to internal standard obtained by 6 replicate injections of samples spiked at a concentration of 10 ng/ml for each compound, and these were satisfactory for both NNN and NNK. Regression statistics calculated from peak area ratio for concentrations (0.1, 0.2, 1, 2, 4, 5, 10, 20, 100, 500 and 1000 ng/ml) in the range 0.1-1000 ng/ml for NNN and NNK proved sufficient linearity with correlation coefficients greater than 0.995 for both compounds (Table 2-6). The method was demonstrated to be repeatable with percentage standard deviations of less than 15% (FOOD AND DRUG ADMINISTRATION, 2007) for both compounds in the studied concentration range. Accuracy of the method, expressed as the recovery of the actual amount added from six samples with concentration of 10 ng/ml was within the range of 91-109% (Table 2-6).

Table 2-6 The LC-MS/MS validation parameters for the two major TSNAs, NNN and NNK.

	Tobacco Specific Nitrosamines (TSNAs)	
Parameters	NNN	NNK
Concentration range (ng/ml)	0.1 to 1000	0.1 to 1000
Regression equation	y = 0.0265x – 0.0386	y = 0.0245x – 0.0299
Correlation coefficient (R^2)	0.9996	0.9989
Detection limit (ng/ml)	0.83	1.4
Quantitation limit (ng/ml)	2.6	4.3
Accuracy (n=6)	91.5 ± 10.4	108.7 ± 2.7
Repeatability RSD (n=10)	11.30%	2.40%

2.4 Conclusion

The new reversed-phase HPLC-UV method developed and validated herein is simple, does not require expensive instrumentation and can therefore be easily applied for simultaneous determination and quantification of nicotine, nornicotine, anabasine, anatabine, myosmine and cotinine from tobacco plants and products. This approach offers the advantage of using a short run time of 13 minutes for separation of six structurally related alkaloids on a Zorbax Extend C-18 column with a mobile phase consisting of acetonitrile (gradient 9 to 13%) and 15 mM ammonium formate in water adjusted to pH 10.5 with TEA, and detection at a single UV wavelength of 260 nm. Results from validation of the method proved satisfactory with respect to sensitivity, linearity, accuracy and reproducibility that make it suitable for routine quantification of the target alkaloids in tobacco plants and products. The LC-MS/MS method developed and validated here for quantification of NNN and NNK is a simple method adapted from already published methodologies for fast and efficient quantification of these two carcinogenic compounds in smokeless tobacco products.

Chapter 3. Chemical and molecular characterization of nicotine to nornicotine conversion in Australian *Nicotiana* species used as chewing tobacco

3.1 Introduction

All species of the genus *Nicotiana* (L) have the characteristic feature of producing pyridine alkaloids; but the number and abundance of the different alkaloids are highly variable within the genus (Sisson and Severson, 1990) (Saitoh et al., 1985). While nicotine is considered to be the most abundant alkaloid (~95%) in *Nicotiana* species, there are many other structurally related pyridine alkaloids in various *Nicotiana* plants and their cured leaves, primarily nornicotine, anabasine, anatabine, myosmine and cotinine. Nornicotine, the major secondary alkaloid in most species, is a demethylated derivative of nicotine. N'-demethylation of nicotine to nornicotine mainly occurs in the senescing leaves of *Nicotiana* plants. *Nicotiana* species that convert a large portion of their nicotine content to nornicotine during senescence and curing are called “converters”, whereas the species that mainly accumulate nicotine in their leaves are called “nonconverters” (Chakrabarti et al., 2007, Gavilano and Siminszky, 2007, Siminszky et al., 2005).

In tobacco research, nicotine to nornicotine conversion has a vital importance. This is because nornicotine affects tobacco quality by causing unwanted flavour and decreasing smoking quality (Roberts, 1988). Similar to nicotine, nornicotine accumulates in the brain, evokes dopamine release and contributes to the pharmacologic profile of nicotine (Crooks et al., 1997). However, nornicotine is believed to be a source of many undesirable health effects. The most important health implications attributed to nornicotine are due to its being the main precursor of N’-nitrosonornicotine (NNN) which is a tobacco specific nitrosamine (TSNA) with high potential in inducing cancer in laboratory animals (Hecht, 1998, Hecht, 2003, Hoffmann et al., 1994). Nitrosation of nornicotine may also take place in the stomach, which can also lead to formation of NNN (Porubin et al., 2007). Other than that, nornicotine induces the aberrant glycation of proteins within the cells and alters the pharmacological properties of the commonly prescribed steroid medication, prednisone (Dickerson and Janda, 2002). It also catalyses retinal isomerization that is responsible for age-related macular degeneration, birth defects associated with smoking, and other smoking-associated abnormalities that stem from disruption of retinoid metabolism (Brogan et al., 2005). Nornicotine is also believed to be responsible for periodontal disease associated with smoking (Katz et al., 2005). Research efforts have focussed on understanding the conversion phenomenon, and in breeding low nornicotine-producing species (Mann et al., 1964). Nornicotine constitutes about 3-5% of the total alkaloid content in major commercial varieties of *Nicotiana* (mainly *N. tabacum*), but this

could be up to 97% in high converter wild species or varieties of commercial tobacco (Gavilano et al., 2006, Pakdeechanuan et al., 2012).

The molecular identity of the nicotine to nornicotine conversion factor in *Nicotiana* species has been of great importance, since the encoding of nicotine demethylase enzymes is the major determinant in formation and accumulation of nornicotine in these species. A group of cytochrome P450 genes, belonging to the 82E subfamily have been reported to be involved in encoding of these functional N-demethylases in *Nicotiana* spp. (Siminszky et al., 2005). This group of P450 genes are differentially regulated between high converter and low or nonconverter *Nicotiana* (Siminszky et al., 2005). In human liver microsomes, nicotine N-demethylation is catalysed by the P450 subfamilies CYP2A and CYP2B (Yamanaka et al., 2005). There are at least five CYP82E related genes in *N. tabacum*: CYP82E4, CYP82E5 and CYP82E10 encode functional nicotine N-demethylases (Siminszky et al., 2005, Gavilano and Siminszky, 2007, Lewis et al., 2010) whereas two others CYP82E2 and CYP82E3 encode inactive enzymes (Chakrabarti et al., 2007, Gavilano and Siminszky, 2007). Investigations conducted on conversion locus analysis in *Nicotiana* spp. are limited to the varieties of the important commercial tobacco, mainly *N. tabacum* (Cai et al., 2012a, Siminszky et al., 2005, Chakrabarti et al., 2008, Gavilano et al., 2006, Gavilano and Siminszky, 2007, Lewis et al., 2010) and its progenitor species *N. tomentosiformis* (Mann et al., 1964), and *N. sylvestris* (Chakrabarti et al., 2007). There is also a report of the loci and their functionality in a few South American wild species including *N. langsdorffii* and *N. alata* (Pakdeechanuan et al., 2012).

In Australia, there are 26 wild species and subspecies of *Nicotiana*, all belonging to *Suaveolentes* section (Ladiges et al., 2011, Marks et al., 2011). Australian species of *Nicotiana* are mostly annuals, or in exceptionally good seasons they may survive for a second year. In southern parts there are a few short-lived perennial species (Latz et al., 1995, Peterson, 1979). A range of these species have been used for preparing a chewing tobacco products by the Aboriginal population of central Australia for hundreds of years (Latz et al., 1995). This chewing tobacco product is usually known as ‘pituri’ (Latz et al., 1995, Peterson, 1979, Young, 2005, Ratsch et al., 2010), though other names may be used, such as ‘mingkulpa’, within some Aboriginal communities (Ratsch et al., 2010, Young, 2005). Pituri is a mix of fresh or dry leaves of wild *Nicotiana* with a burnt alkaline wood ash that is chewed to form a ‘quid’ (Latz et al., 1995, Ratsch et al., 2010, Watson et al., 1983). A range of species have been reported to be used for chewing, depending on their availability in the different geographic locations. The

most preferred one is *N. gossei* (Latz et al., 1995, Peterson, 1979, Symon, 2005), but when not available other species such as *N. excelsior*, *N. rosulata* subsp. *ingulba*, *N. goodspeedii*, *N. benthamiana* and *N. cavicola* might be used (Latz et al., 1995, Peterson, 1979, Symon, 2005).

The alkaloid profile of *Nicotiana* species has been reported previously for 19 out of 26 of the recognised Australian taxa (Saitoh et al., 1985, Sisson and Severson, 1990), but so far there is no information on nicotine to nornicotine conversion and the molecular characteristics of the responsible locus in Australian *Nicotiana* species. This study aims to quantify the pyridine alkaloids in all Australian *Nicotiana* spp. and investigate the presence and functionality of CYP82E related genes.

3.2 Materials and methods

3.2.1 Plant Material

Seeds of Australian *Nicotiana* species and subspecies were obtained from seedbanks, botanic gardens and herbaria across Australia. Seed viability for each seedlot was determined with tetrazolium staining. Seeds were imbibed in distilled water for 24 hours and then incubated on filter paper soaked with 1% 2,3,5-triphenyltetrazolium chloride (Sigma) solution for 24 hours at 35°C in dark. The % viability was determined by scoring the embryos stained red or pink as viable. For germination, 10 seeds for each seedlot were incubated in 55 mm plastic Petri dishes on a double layer of filter paper (Advantec NO.1 55 mm) soaked with 5 mL distilled water containing 200 ppm GA3 (Sigma) at 20°C under 16 hours photoperiod with cool white light. After 24 hours seeds were transferred to Petri dishes with filter papers soaked in only distilled water. Germination % was monitored: a seed was considered to have germinated once the radicle emerged. After 10-14 days, germinated seeds were transferred into seedling trays filled with Osmocote seed raising and cutting mix (Scotts, Australia). After 2 weeks seedlings were moved to 100×50 mm black tube pots containing Osmocote native potting & planting mix (Scotts, Australia). Plants were grown in pots and under controlled environment (Thermoline, Climatron 520 & 1100-DL/SL growth cabinet) with 16 h light provided by cool white fluorescent lamps, 23°C day and 21ºC night temperature, constant 70–90% relative humidity and watered daily as needed. For seedlots with low seed viability, this process was repeated until 3 healthy plants were obtained.

Green leaves were harvested from 8-10 week old non-flowering adult plants. Three plants were sampled for each taxon. Fresh leaf material was used for DNA extraction for CYP82E gene

analysis. Fresh leaves were freeze-dried and stored at -80ºC for alkaloid analysis. To further investigate the conversion locus, fresh leaves of six selected species were cured by incubating at 35ºC for 6 hours in the dark and used for RNA extraction and RT-PCR analysis, and cured leaves were freeze-dried and stored at -80ºC for alkaloid analysis.

Herbarium vouchers were prepared and submitted using the laboratory-grown plants unless a record for the specific seedlot already existed; the confirmation of identity for six taxa was performed by Neville Walsh (Senior Conservation Botanist, Royal Botanic Gardens Victoria).

3.2.2 Chemical analysis

3.2.2.1 Chemicals and reagents

The solvents used for alkaloids extraction and HPLC analysis were methanol and acetonitrile, from Merck (Darmstadt, Germany), and a 15 mM ammonium formate buffer prepared using ammonium formate from Sigma-Aldrich (St. Louis, MO). The water was deionised and filtered using a Milli-Q system (Millipore, Billerica, MA). Hydrochloric acid and sodium hydroxide used for adjusting pH were from Merck (Darmstadt, Germany). The analytical standards used were nornicotine, myosmine and cotinine from Sigma, nicotine from Fluka (Milwaukee, WI), anabasine from Sigma-Aldrich, anatabine from Cayman Chemical Company (AnnArbor, MI), and the internal standards used were caffeine from Ajax Finechem (Sydney, Australia).

3.2.2.2 Alkaloid analysis

Both fresh and cured freeze-dried leaves (50 mg) were exhaustively extracted for pyridine alkaloids using 40% aqueous methanol containing 0.1% 1N hydrochloric acid following our previously published method (Moghbel et al., 2015) and were quantified for nicotine, nornicotine, anatabine, anabasine, myosmine and cotinine. Quantitative analysis of the extracts was carried out on an Agilent 1100 series high performance liquid chromatography (HPLC) system equipped with a UV detector and on a Zorbax Extend C18 column (Agilent Technologies, Mulgrave, Vic, Australia) with a gradient mobile phase consisting of 15 mM ammonium formate buffer and acetonitrile as described in chapter 2 (Moghbel et al., 2015). Percent conversion was calculated as [nornicotine content / (nornicotine content + nicotine content)] x 100 (Pakdeechanuan et al., 2012). Plants with less than 10% were scored as low or nonconverters, plants with 10-50% and 50-100% conversion were assigned as medium and high converters, respectively.

3.2.3 Molecular analysis

3.2.3.1 PCR amplification of CYP82E related genes

Genomic DNA was extracted from fresh leaves using the ZR Plant/Seed DNA MiniPrep™ (Zymo Research, CA, USA) following the manufacturer's instructions. Genomic fragments containing CYP82E related genes were obtained by amplifying genomic DNA (around 20 ng) with PCR primer set that was designed based on the conserved sequences of the published tobacco CYP82E subfamily genes (Table 3-1). Gel electrophoresis was then performed by loading 5 µl of the PCR products to investigate the presence of the bands representing the amplified targets in studied samples. A 1kb DNA ladder (New England biolabs Inc, MA, U.S.A) was used as marker.

Table 3-1 Sequences of the primers used for PCR amplification of CYP82E related genes designed from *N. tabacum* CYP82E4 mRNA (Accession: KC120817.1) and internal gene GAPDH designed from *N. benthamiana* glyderaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Accession: JQ256517.1).

Primers	Sequence (5'->3')	Product length	Comment
82E-F	CTGCGGACACAGTTGCTCTT	536	For amplifying CYP82E subfamily genes from genomic DNA and cDNA
CYP82E-R	AGTTATGCCTGCACCTTCCT		
GAPDH-F	AACCGGTGTCTTCACTGACAAGGA	562	For amplifying internal gene GAPDH from cDNA
GAPDH-R	GCTTGACCTGCTGTCACCAACAAA		

3.2.3.2 Sequencing the amplified CYP82E regions for selected species

The amplified genomic DNA from three low or nonconverter species (*N. benthamiana, N. excelsior, N. gossei*) and three high converter species (*N. cavicola, N. goodspeedii, N. velutina*) were purified using Rapid PCR Cleanup Enzyme Set (New England biolabs Inc, MA, U.S.A) according to the protocol of the manufacturer. Each purified PCR product was sent for BDT labelling, purification and sequencing to the Australian Genome Research Facility and sequenced following high throughput Sanger sequencing using Applied Biosystems 3730 and 3730xl capillary sequencers. These automated platforms use ABI Prism BigDye Terminator kit (BDT) chemistry version 3.1 (Applied Biosystems) under standardised cycling PCR conditions. cDNA sequences of CYP82E subunits were retrieved from GenBank and BLASTn was used for confirmation of the identity of CYP82E subunits.

3.2.3.3 Agarose gel electrophoretic analysis of CYP82E related genes transcript levels in fresh versus cured leaves of selected species

Total RNA was extracted from fresh and cured leaves of three low or nonconverter species (*N. benthamiana*, *N. excelsior*, *N. gossei*) and three high converter species (*N. cavicola*, *N. goodspeedii*, *N. velutina*) using the ZR Plant RNA MiniPrep™ (Zymo Research, CA, USA) following the instructions provided by the manufacturer. First strand cDNA was then synthesized by ProtoScript II First Strand cDNA Synthesis Kit (New England biolabs Inc, MA, U.S.A) according to the protocol of the manufacturer. The CYP82E related cDNA fragments were amplified by PCR amplification of isolated cDNA from the fresh and cured leaves of the studied species. A set of primers was also optimised to amplify 562 bp GAPDH fragments from the obtained cDNA to be used as the internal gene (Table 3-1). Optical densitometric analysis from the picture of the obtained agarose gel electrophoresis bands was then performed using ImageJ software (version 1.48b, https://imagej.nih.gov/ij/) to determine the relative gene expression of the CYP82E related genes in fresh versus cured leaves. The optical density of the CYP82E bands obtained from loading 5 µl of the PCR product was normalised to that of GAPDH bands obtained from loading similar amount of 5 µl of the PCR product used as internal gene.

3.2.4 Statistical analysis

Statistical analysis was performed using Prism (GraphPad, San Diego, CA) software. One-way analysis of variance (ANOVA) was applied with Bonferroni multiple comparisons and $P < 0.05$ marked as significant. ImageJ Software version 1.48b (https://imagej.nih.gov/ij/) was used for the optical densitometric analysis of expression levels in fresh versus cured leaves.

3.3 Results

3.3.1 Plant growth

Plants were successfully grown for 24 out of the 26 recognised taxa, with two seedlots grown for five of the taxa (Table 3-2). In total, 73 seed lots were obtained for the 26 Australian species and subspecies (Appendix 1). Not all of the received seed lots were viable and of high enough quality to result in successful germination. Germination and growth of *N. wuttkei* J.R.Clarkson & Symon and *N. umbratica* N.T.Burb. Seeds were not achieved because seeds were either inviable or grew into plants that were identified to be other species; *N. tabacum* for seeds

labelled as *N. wuttkei*, and *N. amplexicaulis* instead of *N. umbratica*. Records showing the presence of *N. wuttkei* in a small remote area of northern Queensland are over 20 years old and *N. umbratica* has a limited distribution in northern Western Australia.

Table 3-2 Sources of seeds used for growing the *Nicotiana* species for this study, with their corresponding herbarium voucher number, collection date, tetrazolium (V) and germination (G) results.

Taxon	Seed Lot	Voucher	Collection	% V	% G
N. amplexicaulis N.T.Burb	NSW4157044	NSW 234670	21/10/1990	60	80
	TS 298 (AusTRCF317546)	MEL 2396301	-	50	50
N. benthamiana Domin	A109412	DNA A0109412	18/07/2001	90	100
N. burbidgae Symon	DJD3167	Voucher confirmed	09/2015	20	20
N. cavicola N.Burb.	NSW877057	NSW 951497	3/08/1995	100	90
N. excelsior (J.Black) J.Black	D194512	AD 246203	21/09/2009	50	30
N. forsteri Roem. & Schult.	BGQLD.0727	BRI AQ0840316	27/05/2010	50	50
N. goodspeedii H.Wheeler	DJD213	AD 192908	22/11/2005	100	100
N. gossei Domin	D204093	DNA D0204093	25/04/2011	100	100
	NSW4022326	Identity confirmed	09/1997	90	80
N. heterantha Symon & Kenneally	AusTRCF313551	MEL 2396300	-	60	30
N. maritima H. Wheeler	MKJ140	AD 187318	15/11/2005	80	80
	DJD3341	Voucher confirmed	7/01/2013	50	30
N. megalosiphon Van Heurck & Mull. Arg. ssp. *megalosiphon*	NSW870550	NSW 2010147	8/11/2010	100	90
N. megalosiphon Van Heurck & Mull. Arg. ssp. *sessifolia* P.Horton	AusTRCF303829	MEL 2396303	-	30	20
*N. monoschizocarpa (*P.Horton) Symon & Lepschi	AusTRCF303666	Voucher confirmed	09/1986	50	50
N. occidentalis H.Wheeler ssp.*hesperis* (N.Burb.) P.Horton	TS 341 (AusTRCF303767)	MEL 2396304	1960	82	70
N. occidentalis H.Wheeler ssp. *obliqua* N. Burb.	SL17 (AusTRCF303779)	MELU D106540	1/08/1956	70	50
N. occidentalis H.Wheeler ssp. o*ccidentalis*	L 3569 (AusTRCF303738)	MELU D106542, D106543)	1959	30	20
N. rosulata (S.Moore) Domin ssp. *ingulba* (J.Black) P. Horton	TS 75 (AusTRCF303907)	MEL 2396302	-	84	50
N. rosulata (S.Moore) Domin *ssp. rosulata*	20070392	PERTH 7821336	20/07/2006	100	100
N. rotundifolia Lindley		MEL 2396305	08/2015	100	100
N. simulans N.Burb.	RJB70944	AD 206232	12/03/2007	80	70
	NSW 872310	ASBP 20101508	15/11/2010	40	20
N. sp. *'Corunna'* Symon17088	SL23 (MELU D106460, D106461, D106462, D106463, D106464)	AD 239243	14/09/2005	50	50
N. suaveolens H. Wheeler	DJD1980	AD 239956	6/10/2010	100	100
N. truncata Symon	TST1056	AD 250303	7/10/2010	50	40
N. velutina H.Wheeler	DJD233	AD 192706	24/11/2005	70	60
	NSW4139564	NSW 213331	24/01/1989	60	40

3.3.2 Alkaloid profile

Fresh leaves of all 24 taxa contained the major alkaloids nicotine and nornicotine (Table 3-3). Anatabine and anabasine were also found in the majority of the taxa but myosmine and cotinine were not detected. Nicotine constitutes the major portion of total alkaloids in 15 taxa including *N. gossei*, which tends to be the most important chewed species amongst Aboriginal pituri chewers. This was also the case for *N. excelsior* and *N. benthamiana*, which are amongst the preferred species for pituri chewing (Latz et al., 1995, Symon, 2005). Hence, these three species are examples of low or nonconverter phenotypes. In contrast, nornicotine is the predominant alkaloid in 9 taxa including *N. goodspeedii*, *N. velutina* and *N. cavicola* and so these are classified as high converter species. For four of the five species for which two different seed sources were available for comparison (*N. amplexicaulis*, *N. gossei*, *N. maritima* and *N. velutina*) the dominant alkaloid was the same and absolute concentrations were not significantly different (Table 3-3).

Table 3-3 Alkaloid content (mean ± se for 3 replicates) in freeze-dried fresh leaves of Australian *Nicotiana* spp. grown in a plant growth incubator under 16 h photoperiod provided with cool white fluorescent lamps, 23°C day and 21ºC night temperature, constant 70–90% relative humidity. Plants were watered daily as needed. Conversion rate was defined using the formula: [nornicotine content / (nornicotine content + nicotine content)] x 100. Species with less than 10% conversion rate are regarded to be low or nonconverters while those with 10-50% and more than 50% conversion rate are assigned as medium and high converters, respectively.

Species	**Alkaloid Concentrations (mg/g D.W.)**						**Conversion Status**
	Nicotine	**Nornicotine**	**Anatabine**	**Myosmine**	**Anabasine**	**Cotinine**	
N. amplexicaulis	1.61 ± 0.44	0.23 ± 0.12	0.17 ± 0.15	N.D.	0.28 ± 0.29	N.D.	Medium
	5.09 ± 0.15	0.15 ± 0.05	0.05 ± 0.02	N.D.	0.05 ± 0.02	N.D.	Non-Low
N. benthamiana	2.29 ± 1.61	0.25 ± 0.05	0.14 ± 0.10	N.D.	0.41 ± 0.32	N.D.	Non-Low
N. burbidgae	4.05 ± 1.12	0.20 ± 0.09	0.01 ± 0.00	N.D.	0.66 ± 0.20	N.D.	Non-Low
N. cavicola	0.06 ± 0.02	0.10 ± 0.02	BLQ	N.D.	0.01 ± 0.02	N.D.	High
N. excelsior	5.39 ± 1.09	0.17 ± 0.14	0.21 ± 0.05	N.D.	0.17 ± 0.10	N.D.	Non-Low
N. forsteri	1.39 ± 0.35	0.66 ± 0.47	0.24 ± 0.04	N.D.	0.74 ± 0.5	N.D.	Medium
N. goodspeedii	0.24 ± 0.18	1.92 ± 0.08	0.03 ± 0.01	N.D.	0.29 ± 0.11	N.D.	High
N. gossei	5.95 ± 1.33	0.05 ± 0.07	0.07 ± 0.02	N.D.	N.D.	N.D.	Non-Low
	8.27 ± 3.00	0.05 ± 0.02	0.11 ± 0.06	N.D.	0.10 ± 0.04	N.D.	Non-Low
N. heterantha	0.89 ± 0.12	0.19 ± 0.11	BLQ	N.D.	0.48 ± 0.11	N.D.	Medium
N. maritima	3.94 ± 1.39	0.12 ± 0.05	0.05 ± 0.02	N.D.	0.11 ± 0.05	N.D.	Non-Low
	3.17 ± 1.93	0.10 ± 0.06	0.04 ± 0.02	N.D.	0.11 ± 0.07	N.D.	Non-Low
N. megalosiphon* subsp. *megalosiphon	0.11 ± 0.07	0.63 ± 0.2	0.03 ± 0.01	N.D.	0.23 ± 0.12	N.D.	High
N. megalosiphon* subsp. *sessifolia	0.14 ± 0.08	0.76 ± 0.30	0.11 ± 0.06	N.D.	0.03 ± 0.01	N.D.	High
N. monoschizocarpa	2.35 ± 0.38	2.82 ± 0.66	0.14 ± 0.12	N.D.	2.67 ± 0.83	N.D.	High
N. occidentalis* subsp. *hesperis	0.41 ± 0.01	0.14 ± 0.10	0.03 ± 0.01	N.D.	0.17 ± 0.02	N.D.	Medium
N. occidentalis* subsp. *obliqua	0.65 ± 0.32	0.29 ± 0.04	0.10 ± 0.07	N.D.	0.47 ± 0.45	N.D.	Medium
N. occidentalis* subsp. *occidentalis	0.15 ± 0.06	0.47 ± 0.12	BLQ	N.D.	0.10 ± 0.00	N.D.	High
N. rosulata* subsp. *rosulata	0.60 ± 0.04	0.10 ± 0.03	0.19 ± 0.11	N.D.	0.13 ± 0.04	N.D.	Medium
N. rosulata* var. *ingulba	0.81 ± 0.06	0.18 ± 0.08	0.02 ± 0.01	N.D.	0.01 ± 0.02	N.D.	Medium
N. rotundifolia	3.54 ± 1.58	0.15 ± 0.17	0.02 ± 0.03	N.D.	0.24 ± 0.04	N.D.	Non-Low
N. simulans	0.04 ± 0.04	0.44 ± 0.04	0.03 ± 0.00	N.D.	0.10 ± 0.02	N.D.	High
	0.16 ± 0.03	0.22 ± 0.06	BLQ	N.D.	0.23 ± 0.03	N.D.	High
N. sp. 'Corunna'	1.61 ± 1.10	0.07 ± 0.03	0.04 ± 0.02	N.D.	0.25 ± 0.12	N.D.	Non-Low
N. suaveolens	0.15 ± 0.21	0.72 ± 0.35	0.01 ± 0.01	N.D.	0.12 ± 0.06	N.D.	High
N. truncata	3.21 ± 0.46	0.11 ± 0.07	0.23 ± 0.12	N.D.	0.11 ± 0.03	N.D.	Non-Low
N. velutina	0.11 ± 0.06	0.92 ± 0.53	0.05 ± 0.06	N.D.	0.31 ± 0.25	N.D.	High
	0.03 ± 0.03	1.18 ± 0.28	BLQ	N.D.	0.31 ± 0.03	N.D.	High

N.D.: Not Detected

BLQ: Below Limit of Quantification

The two seedlots of *N. simulans*, one collected in NSW and the other in SA, differed significantly in their absolute levels of nicotine, nornicotine and anabasine. Treatment with heat to mimic curing elevated the proportion of nornicotine to nicotine in the leaves of both high converter and low or nonconverter species, however this increase did not change the dominant alkaloid in either group (Table 3-4).

Table 3-4 Alkaloids (mean ± se for 3 replicates) of fresh versus cured (35°C for 6 hours in the dark) leaves for selected species of Australian *Nicotiana* spp. Significant differences ($p<0.05$) between fresh and cured leaves are indicated (*).

	% Nornicotine content / (Nicotine content + Nornicotine content)	
Species	**Fresh leaves**	**Cured leaves**
N. benthamiana	9.6 ± 2.0	21.4 ± 3.1*
N. excelsior	2.9 ± 0.9	6.0 ± 2.6
N. gossei	0.4 ± 0.1	0.7 ± 0.2
N. goodspeedii	83.2 ± 1.1	90.5 ± 0.8*
N. cavicola	69.7 ± 1.6	81.4 ± 2.6*
N. velutina	73.3 ± 3.8	92.6 ± 1.7*

3.3.3 CYP82E genes in Australian Nicotiana spp.

Since nicotine N-demethylase (NND), the main source for conversion of nicotine to nornicotine, is encoded by cytochrome P450 monooxygenase genes of the CYP82E subfamily in *N. tabacum* (Lewis et al., 2010, Siminszky et al., 2005), PCR using CYP82E specific primers based on the sequences of the conserved regions of the subfamily in *N. tabacum* (Table 3-1) was optimised to amplify the potential loci in Australian *Nicotiana* species. Based on the agarose gel electrophoresis results, genomic DNA corresponding to the conserved region of CYP82E related genes have been amplified in all of the studied Australian *Nicotiana* species (Figure 3-1). To confirm the identity, the amplified regions from three low or nonconverter and three high converter species were purified and subjected to Sanger sequencing. The DNA sequences of the CYP82E related genes from *N. benthamiana*, *N. gossei*, *N. goodspeedii*, *N. velutina*, *N. cavicola* and *N. excelsior* have been deposited in NCBI GenBank with the accession numbers KU234094, KU234095, KU234096, KU234097, KU234098 and KU504631, respectively. Blasting the obtained sequences in NCBI BLASTN tool demonstrated 94-97% resemblance and identity to the published sequences of conversion locus CYP82E in other *Nicotiana* species, confirming their proposed identity (DQ131887.2 and EF472002.1).

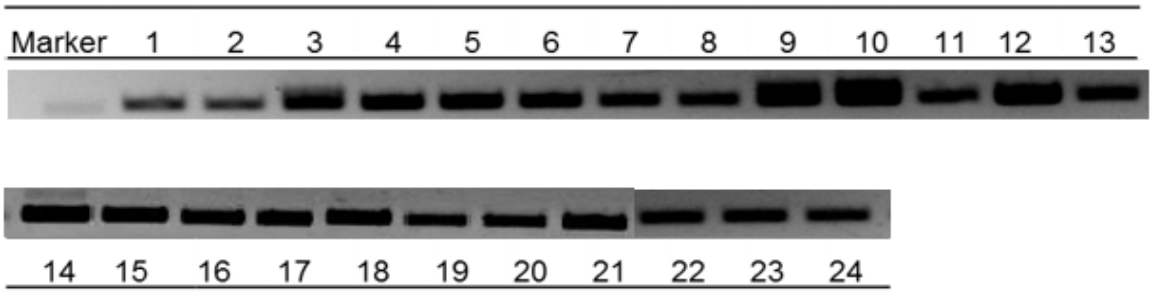


Figure 3-1 Agarose gel electrophoretic photo of the CYP82E related fragment produced by 35 cycles of PCR amplification of DNA isolated from 1. *N. forsteri, 2. N. benthamiana, 3. N. excelsior, 4. N. gossei, 5. N. suaveolens (exigua), 6. N. goodspeedii, 7. N. simulans, 8. N. velutina, 9. N. cavicola, 10. N. amplexicaulis, 11. N. megalosiphon subsp. megalosiphon, 12. N. megalosiphon subsp. sessifolia, 13. N. truncate, 14. N. maritima, 15. N. occidentalis subsp. obliqua, 16. N. occidentalis subsp. Occidentalis, 17. N. occidentalis subsp. hesperis, 18. N. sp. 'Corunna', 19. N. heterantha, 20. N. rosulata subsp. rosulata, 21. N. rosulata var. ingulba, 22. N. rotundifolia, 23. N. burbidgae, 24. N. monoschizocarpa.*

3.3.3.1 Agarose gel electrophoretic analysis of CYP82E related genes transcript levels in fresh versus cured leaves of the selected high converter versus low or nonconverter species

The relationship between transcript levels of CYP82E related genes and observed levels of nornicotine production in fresh and cured leaves of high converter and low or nonconverter species was investigated by analysing relative transcript accumulation levels in fresh versus cured leaves. The ratio between the optical density of the CYP82E band to that from the GAPDH band on the agarose gel photo for each sample was used as an indicator of expression and calculated in fresh and cured leaves of both high converter and low or nonconverter groups of species. The normalised optical density of the bands resulting from transcription of the CYP82E related genes in fresh leaves showed no significant difference across high converter and low or nonconverter species. Curing was associated with a significantly higher (almost 3 fold) optical density in the leaves of the high converter species, but only a small increase or no change was measured in the cured leaves for the low or nonconverter species (Figure 3-2).

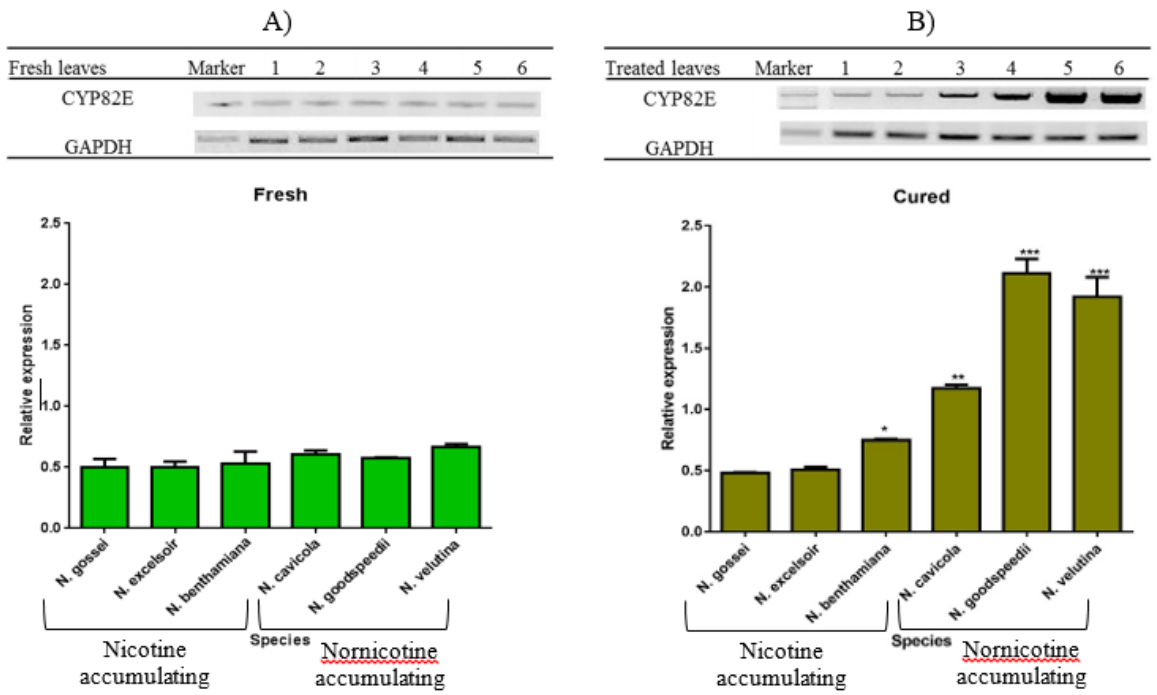


Figure 3-2 Agarose gel electrophoretic analysis and normalised band densitometry values of the CYP82E related fragment produced by 35 cycles of PCR amplification of cDNA isolated from A) fresh leaves B) Cured leaves (at 35° C for 6 hours) of 1. *N. gossei* 2. *N. excelsior* 3. *N. benthamiana,* 4. *N. cavicola,* 5. *N. goodspeedii,* 6. *N. velutina.* A 562 bp GAPDH fragment obtained by 35 cycles of PCR amplification of the cDNA for each sample is shown as an internal gene and for normalising the densitometric values that all were quantified using ImageJ software. The data points in graphs show mean ± std error of 3 independent experiments *: $p<0.05$

3.4 Discussion

This study has expanded the range of Australian wild *Nicotiana* species and subspecies assessed for alkaloid composition to 24 out of the 26 that are currently recognised (Table 3-3). The taxa missing from analysis are *N. wuttkei* and *N. umbratica* for which we were unable to obtain a reliable seed source. Alkaloid composition exhibited large differences between species, with a maximum of 8.3 and minimum of 0.3 mg/g nicotine recorded, which were for *N. gossei* and *N. velutina,* respectively. Alkaloid production in *Nicotiana* is controlled genetically and levels can vary dramatically throughout growth in response to environmental conditions (Keinanen et al., 2001) so it may be expected that different seedlots of the same species vary in alkaloid quantities even when grown in a single environment. However, of the five species for which two seed sources from different geographic locations within Australia were grown, very little difference in alkaloid concentrations was found for four of them (*N. amplexicaulis, N. gossei, N. maritima,* and *N. velutina*). The only significant difference between the two seedlots was for *N. simulans,* which showed statistically different levels of alkaloids for the seedlots from South Australia and New South Wales. However, even

considering the significant difference in absolute alkaloid concentrations for this species, calculations using the nicotine and nornicotine concentrations conclude that both seedlots exhibit the same conversion trait.

The alkaloid composition for 19 Australian *Nicotiana* species were reported almost 30 years ago (Saitoh et al., 1985, Sisson and Severson, 1990). Differences in absolute alkaloid concentrations to those reported here may be associated not only with the different seedlots involved, but also differences in the growth environment and treatment of plants before harvesting leaves. The plants used in previous studies were greenhouse (Saitoh et al., 1985, Sisson and Severson, 1990) or field (Sisson and Severson, 1990) grown plants, whereas the plants in our study were lab grown and usually lab grown plants tend to contain lower alkaloid concentrations than those from field or greenhouse (Dewey and Xie, 2013). Despite differences in reported absolute concentrations for nicotine and nornicotine between this and previous studies, the inferred conversion trait for each species is consistent between studies. For example, using the nicotine and nornicotine quantification results reported by both previous studies (Saitoh et al., 1985, Sisson and Severson, 1990) and here (Table 3-3), the species *N. gossei*, *N. excelsior* and *N. benthamiana* are categorised as low or nonconverter, while *N. goodspeedii*, *N. cavicola* and *N. velutina* are high converter species.

Nornicotine is an undesirable alkaloid in consumed tobacco species, because its production and accumulation has a positive correlation to production of the carcinogenic nitrosamine, NNN. Production of nornicotine via N-demethylation of nicotine in commercial tobacco is mainly governed by CYP82E related genes, specifically E4. Inhibition of the nicotine to nornicotine conversion happens as the result of silencing of the CYP82E gene subfamily. Tetcyclasis is an inhibitor of cytochrome P450 and arrests the nicotine demethylation (Chelvarajan et al., 1993, Donaldson and Luster, 1991). Carbon monoxide also is shown to inhibit nicotine demethylation (Chelvarajan et al., 1993, Donaldson and Luster, 1991), while light of 450 nm reverses this inhibition (Donaldson and Luster, 1991).

Given the vital importance of the CYP82E related genes in nornicotine production and accumulation in tobacco plants and due to considerable nornicotine production in some Australian *Nicotiana* species and the unknown source of conversion in them, in this study, we amplified the conversion loci in the 24 Australian *Nicotiana* species and subspecies. This is the first molecular and biochemical evidence that CYP82E genes are present in Australian *Nicotiana* species.

Although the locus was present in all studied taxa, some of the Australian species are high converters of nicotine to nornicotine and accumulate high levels of nornicotine in their leaves, whereas some others mainly accumulate nicotine. The difference in the observed nicotine to nornicotine conversion phenotype could be due to the presence of the CYP82E related genes with different ability for encoding functional nicotine N-demethylases. It is notable that the main species preferred for chewing are amongst those that mainly accumulate nicotine, i.e. *N. gossei* and *N. excelsior*, both of which are low or nonconverters. *N. benthamiana*, a low converter, has also been mentioned to be used for chewing in Western Australia (Latz et al., 1995, Peterson, 1979, Symon, 2005). In contrast, the high converters *N. megalosiphon* ssp. *megalosiphon, N. simulans, N. occidentalis* ssp. *occidentalis* and *N. velutina* have been listed in the literature as being rarely used or specifically avoided (Latz et al., 1995, Peterson, 1979, Symon, 2005). However, the high converters *N. cavicola* and *N. goodspeedii* have been reported to be used by Aboriginal people (Symon, 2005). *N. rosulata* ssp. *ingulba* is reportedly a species that is preferred for chewing due to its availability in Central Australia (Latz et al., 1995, Peterson, 1979, Symon, 2005), but this is a medium converter.

Due to the large number of species and the little information on the conversion locus in species other than *N. tabacum*, the varieties and number of different genes have not been identified in detail in this study. Instead, the functionality of the loci has been compared between a few selected high converters and low or nonconverter species. The functional CYP82E loci, especially E4, have been reported to be triggered by curing which leads to a high level of transcript accumulation only in high converter species, while the transcript accumulation happens in very low and negligible levels in low or nonconverters (Lewis et al., 2010). This could be an explanation for the observed significantly elevated levels of transcripts (3 to 4 fold) in the cured leaves of only high converter species *N. goodspeedii*, *N. cavicola* and *N. velutina*, while no significant difference was found between the level of transcript accumulation in fresh leaves with the fresh and cured leaves of the low or nonconverter species *N. gossei* and *N. excelsior*. The level of transcripts in cured leaves of *N. benthamiana* shows a significant increase compared to that observed in fresh leaves, although the increase was not as much as observed for the high converters. This is also consistent with the conversion phenotype of *N. benthamiana*, which tends to accumulate higher nornicotine compared to that observed for the other two low or nonconverter species, specifically in cured leaves, although it accumulates nicotine as the most abundant alkaloid. This might be due to presence of the minor functional NNDs in *N. benthamiana*. The alkaloid phenotypes of the two low or nonconverters, *N. gossei*

and *N. excelsior*, show a lower proportion of nornicotine to nicotine in fresh and cured leaves with a very small increase after curing that is also consistent with the illustrated transcript accumulation levels. This might be due to the loci present in these species being either non-functional or with minor functionality and expressed only at a basic steady level. In contrast, the loci in high converter species, *N. goodspeedii*, *N. cavicola* and *N. velutina*, are functional and tend to be triggered to increase their transcript levels, and this in turn is responsible for their higher levels of nornicotine.

The CYP82E subfamily has several genes in *N. tabacum* and other studied *Nicotiana* species that share a great sequence identity (in some cases more than 90%), however the majority of these are not functional NND genes (Lewis et al., 2010). There are also genes that are functional in some species, but have been mutated and become non-functional in others (Chakrabarti et al., 2007, Gavilano and Siminszky, 2007). This could be due to either transcriptional inactivation or premature translational termination of these genes, resulting in the absence of nornicotine in their carrying species. For example, *N. tabacum* is the result of hybrizidation of diploid *Nicotiana* species closely related to modern *N. tomentosiformis* and *N. sylvestris. N. tomentosiformis* has both active CYP82E3 and CYP82E4 which control conversion in green and senescing leaves, respectively. In contrast in *N. tabacum* a W330C amino acid substitution inactivates CYP82E3; moreover, in nicotine-accumulating nonconverter *N. tabacum* a CYP82E4 is also transcriptionally silenced (Gavilano et al., 2007). Another example is in the two closely related wild species of Alatae section, *N. langsdorffii* and *N. alata*. In *N. langsdorffii* the CYP82E genes are rendered non-functional, whereas CYP82E genes in *N. alata* act semi-dominantly and individually to increase conversion of nicotine to nornicotine (Pakdeechanuan et al., 2012). Furthermore, the tobacco genome contains numerous pseudogenes with CYP82E-like sequences that have been mutated (Pakdeechanuan et al., 2012). CYP82E4 has been reported to be the major functional NND gene, while there are other genes such as CYP8210 and CYP82E5v2 within the *N. tabacum* genome that are also functional, but result in lower level of conversion and hence are considered to be minor functional NNDs. The functionality of the loci has not been studied in Australian *Nicotiana* before, and this study took the first step in investigating the underlying mechanism in nicotine to nornicotine conversion.

In conclusion, the nicotine to nornicotine conversion rate varies between *Nicotiana* species from the *Sauveolentes* section. The conversion locus is present in all 24 studied taxa, but the functional NND enzymes seem to be active only in nornicotine accumulating species. This is

driven mainly by their genetic capability rather than environmental factors (Dewey and Xie, 2013), so even in the species with two different seed sources from different environmental conditions the conversion trait stays the same. The observed high nicotine to nornicotine converter phenotype in some Australian *Nicotiana* species poses a potential risk of toxicity to chewers of these species, especially if the leaves are processed and cured before being used in the preparation of pituri, as nornicotine and its nitrosamine, NNN, can be detrimental to health.

Chapter 4. *In vitro* evaluation of nicotine release from dry leaves of *Nicotiana gossei* used in pituri, the Australian Aboriginal smokeless tobacco

4.1 Introduction

Tobacco consumption contributes to about 6 million deaths a year around the world (World Health Organization, 2011). Global efforts to promote smoking cessation are often centred on its harmful outcomes including premature death and diseases such as lung cancer which are primarily associated with the toxic chemicals inhaled in the burned tobacco smoke (Critchley and Unal, 2003).

Smokeless tobacco products and oral nicotine formulations have been used as measures to aid smoking cessation. The nicotine present in tobacco is rapidly distributed into the brain and provides an instantaneous pleasure with reduced stress and anxiety in the user so it is commonly difficult to cease nicotine intake completely (Benowitz, 2009). Smokeless tobacco products have different health effects than smoking, and not all smokeless tobacco products have been studied for the health outcomes and diseases they may cause (Ebbert et al., 2004, Scientific Advisory Committee on Tobacco Products Regulation, 2010, Winstanley, 2012). Smokeless tobacco products contain tobacco as the main constituent and can be consumed either orally or nasally without combustion (International Agency for Research on Cancer, 2007). The differentiating factor that sets smokeless tobacco products apart from oral Nicotine Replacement Therapy (NRT) formulations is that they contain other plant-derived chemicals such as other alkaloids and the carcinogenic tobacco specific nitrosamines (TSNA) (Gartner and Hall, 2009). In the manufacturing process of smokeless tobacco products, tobacco plant leaves, stems and other parts are blended differently, thus creating products that vary significantly in particle size, pH, flavour, smell, taste and nicotine concentrations. Among these, particle size and pH can affect the release and absorption of nicotine from smokeless tobacco products (Davis and Curvali, 1999). The available content of nitrosamines in these products is influenced by the nitrite content (Burton et al., 1994, Henningfield et al., 1995b). Faster nicotine release from any given product can result in higher nicotine levels in the user's blood, which in turn has been believed to be associated with more subjective drug effects that might lead to abuse (de Wit et al., 1992).

Nicotine from smokeless tobacco products and oral NRT is absorbed by passive diffusion into the systemic circulation through oral mucous membranes, particularly the cheek mucosa. Unionized compounds diffuse rapidly through oral membranes due to their higher solubility in the buccal mucosa that is lipophilic in nature (Ciolino et al., 2001). Therefore, the rate and extent of nicotine absorption depends on the concentrations of unprotonated (unionized)

nicotine available (International Agency for Research on Cancer, 2007). The pH of the environment and the dissociation constant of the alkaloid constituents in the tobacco product influences the yield of unprotonated nicotine ready for absorption (Ciolino et al., 2001). Nicotine is a weak base with a pKa of 8.02 (International Agency for Research on Cancer, 2007). This infers that at a pH of 8.02, half of the nicotine content would be present in the unionized form, and therefore free for absorption into the body, while the remaining quantity is ionized. The mean mucosal pH of the oral cavity is 6.8 while the pH level of saliva ranges from 6.0 to 7.5, which is lower than the pKa of nicotine and promotes a greater proportion of the nicotine as the ionized form (Aframian et al., 2006). As a result, smokeless tobacco products and oral NRT formulations are often buffered to an alkaline pH by adding various agents in order to increase unionized nicotine concentration and enhance the rate of buccal absorption (International Agency for Research on Cancer, 2007, Henningfield et al., 1995b).

There are a range of commercially available smokeless tobacco products, such as loose leaf chewing tobacco, dry snuff, moist snuff (snus), etc. Additionally, indigenous populations of the Americas, Africa, the Indian subcontinent and the Asia-Pacific region have their traditional methods and ingredients for preparing their smokeless tobacco products (International Agency for Research on Cancer, 2007, Latz et al., 1995). Pituri is the most common name for a traditional oral tobacco product produced and used widely in Central Australia by indigenous Australians, using a range of wild Australian tobacco species such as *N. gossei* and *N. excelsior*. There are a variety of other synonyms in the literature for this smokeless tobacco product such as mingkulpa (Latz et al., 1995, Peterson, 1979, Ratsch et al., 2010, Young, 2005). Tobacco leaves are mixed with alkaline wood ash from burned twigs obtained from various plants including species from the *Acacia*, *Grevillea* and *Eucalyptus* genera, and chewed to form a quid. This is then kept in the mouth with some sucking and occasional chewing to further stimulate the release of nicotine (Ratsch et al., 2010). The preparation and consumption method of pituri is similar to other indigenous smokeless tobacco products such as iq'mik used by Indigenous people in Alaska (International Agency for Research on Cancer, 2007, Hearn et al., 2013) and maras from Turkey (International Agency for Research on Cancer, 2007). However, pituri is not included in any of the IARC Monographs and there is little information available in the literature on its formulation, epidemiology and the health outcomes associated with its consumption.

The *in vitro* release of nicotine from General portion (GP, pouched) and Ettan portion (EP, loose) Swedish snus (both Swedish Match brand) has been studied previously, whereby a

model mouth system consisting of modifiable factors influencing buccal conditions such as temperature, saliva composition and the flow rate of simulated saliva was used (Li et al., 2013), however this device wasn't designed to be able to perform mastication. In another study, the rate of nicotine release from four different brands of moist snuff (Copenhagen, Skoal Wintergeen long cut, Skoal Bandit Classic and Skoal Wintergreen Fine Cut) into simulated saliva was compared between a direct release and release through a dialysis bag (Nasr et al., 1998). This is the only study that also investigated the effect of chewing action on *in vitro* nicotine release from smokeless tobacco products; chewing action was simulated manually by pressing with a glass rod (Nasr et al., 1998). There are also reports of devices that have been used for testing NRT gums. They have pistons for striking the gum base (Aslani and Rafiei, 2012, Morjaria et al., 2004, Aslani and Rostami, 2015), according to the description of an appropriate apparatus including stainless steel pistons, operation temperature of 37°C and a chewing rate of 60 strikes per minute that is described in the European Pharmacopeia (European Pharmacopoeia, 2010). However, there has not been sufficient data in support of the suitability of this apparatus (Siewert et al., 2003). These methods have limitations such as expensive instrumentation and the fact that it is not possible to accurately reproduce the mastication process.

In this study the nicotine release rate from dry and ready for mastication leaves of *N. gossei*, as the tobacco constituent of pituri, is compared with that from similar commercially available smokeless tobacco and nicotine replacement therapy products that share similar consumption route, i.e. via putting in the mouth and sucking or chewing. These are Swedish moist snuff or snus, which is a popular smokeless tobacco that only contains tobacco, a similar CORESTA reference smokeless tobacco product containing only tobacco (CRP2, Moist Snuff) and an NRT chewing gum. In addition, the effects of chewing action on the *in vitro* release of nicotine from these products and effect of alkaline pH on nicotine release from pituri is evaluated. A simple *in vitro* model was developed using a dissolution apparatus and different chewing conditions were performed manually to simulate the buccal conditions in a controlled and reproducible manner. Different chewing actions were designed to better simulate the conditions in smokeless tobacco products consumption, since these products are not chewed as much as the NRT gum and are mainly sucked in the mouth with occasional chewing. To get an idea of the potential for exposure to other chemicals that might be present in these products, the final release media in the vessels with most frequent chewing was also quantified for other alkaloids (nornicotine, anatabine, anabasine, myosmine and cotinine) and the carcinogenic TSNAs, N-

nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

4.2 Materials and methods

4.2.1 Materials

Acetonitrile and methanol from Merck (Darmstadt, Germany), and ammonium formate from Sigma-Aldrich (St. Louis, MO) were used for preparing the mobile phase for HPLC-UV and LC-MS/MS analysis. The deionised water was from a Milli-Q system (Millipore, Billerica, MA). Sodium hydroxide and hydrochloric acid from Merck (Darmstadt, Germany) were used for adjusting pH. The analytical standards used for LC-MS/MS analysis of carcinogenic nitrosamines and HPLC-UV analysis of tobacco alkaloids were N'- nitrosonornicotine (NNN), 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), nornicotine, myosmine and cotinine from Sigma, nicotine from Fluka (Milwaukee, WI), anabasine from Sigma-Aldrich, anatabine from Cayman Chemical Company (AnnArbor, MI). Caffeine from Ajax Finechem (Sydney, Australia) was used as the internal standard for HPLC-UV analysis, and N'- nitrosonornicotine-d4 (NNN-d4) and 4-(methylnitrosoamino)-1-(3-pyridyl-d4)-1-butanone (NNK-d4) from Toronto Research Chemicals (Ontario, Canada) were internal standards for LC-MS analysis. Simulated saliva used in the study was formulated according to a standard formula consisting of KH_2PO_4 (2.5 mmol/L), Na_2HPO_4 (2.4 mmol/L), $KHCO_3$ (15 mmol/L), NaCl 10mmol/L, $MgCl_2$ (1.5 mmol/L), $CaCl_2$ (1.5 mmol/L) and citric acid (0.15 mmol/L) (Morjaria et al., 2004), adjusted to pH 6.7 with sodium hydroxide or hydrochloric acid accordingly. This solution also consisted of 0.025 mg/ml of caffeine as an internal standard to be used in the chromatography analysis. To mimic the effect of the ash in increasing the pH of the mouth, the experiment for *N. gossei* dry leaves was repeated with simulated saliva adjusted to pH 9.0 with 1 N NaOH.

Four products were studied: Air dried leaves of *N. gossei* ready for mastication (collected and dried in Alice Springs by an experienced botanist who is very well informed of Australian *Nicotiana* and pituri preparation and use), moist snuff (snus) portions (General Classic Extra Strong, made by Swedish Match, Sweden), CORESTA reference smokeless tobacco product (CRP2 Moist Snuff) obtained from North Carolina State University (Tobacco Analytical Services Laboratory) (Wagner et al., 2014) and Nicabate gum 4 mg (GlaxoSmithKline, Australia). The tobacco products used in this study were stored at -20°C between receipt and conducting the study. The quantity of each product used was standardized to contain 4 mg of nicotine to allow for consistent comparison.

4.2.2 Simulation of a model buccal cavity

A model buccal cavity system was constructed by using simulated saliva as media in a USP dissolution test apparatus II fitted with small volume (100 ml) vessels (Agilent Technologies, Vic, Australia). Each tobacco product was immersed completely in 50 ml of the media in the dissolution vessel. The dissolution system settings used were 50 rpm paddle speed at bath and vessel temperature of 37°C. Samples (0.4 ml) were collected at 2, 5, 10, 15, 20, 30, 60, 90 and 120 minutes using a 1 ml syringe attached to a stainless steel cannula with full flow filter (10 μm, Agilent Technologies) and replaced immediately with 0.4 ml of fresh media.

Three conditions were tested: no chewing action as a control, chewing for 30 seconds following immersion in simulated saliva and chewing for 30 seconds at 15-minute intervals. This chewing action was manually performed by pressing with a glass rod at a rate specified by the European Pharmacopoeia of one press each second (Siewert et al., 2003). Each experiment was replicated three times. The samples obtained were filtered through a 0.45 μm nylon filter (Grace Davison Discovery Sciences) to remove any impurities that may be present.

4.2.3 Chemical analysis

4.2.3.1 Nicotine and other alkaloids analysis with HPLC-UV

The tested tobacco products, snus, CRP2 and *N. gossei* dry leaves were analysed for nicotine and other pyridine alkaloids in order to standardize the amount used to contain 4 mg of nicotine. Nicotine concentration in the dissolution media was assessed for each sample collection time. The concentrations of other alkaloids were quantified in the media for the simulated chewing every 15 minutes at the end of the 120 minute dissolution test. Analysis of the samples was performed using an Agilent Technologies 1200 series High Performance Liquid Chromatography (HPLC) system attached to a binary pump, degasser, an auto sampler, thermostat column compartment and a diode array detector following our previously validated method as mentioned in chapter 2 (Moghbel et al., 2015).

4.2.3.2 TSNAs analysis with LC-MS/MS

The media in the vessels with maximum chewing action for all tested products was quantified for two major TSNAs, NNN and NNK following the validated LC-MS/MS method reported previously as mentioned in chapter 2 (Moghbel et al., 2016).

4.2.4 Statistical analysis

The four products and the three chewing conditions were compared using two-way ANOVA followed by Tukey multiple comparisons both for amount of nicotine released at each time point and for cumulative dissolution of nicotine. Total alkaloids and TSNA contents in smokeless tobacco products, and these concentrations in the media obtained from the release in the vessels with maximum chewing action for all were analysed for differences using two-way ANOVA with Tukey multiple comparisons. All analysis was performed using GraphPad Prism version 6 (GraphPad software, San Diego, CA, USA).

4.3 Results

The CRP2 was most concentrated in nicotine, containing 12 mg/g, which was more than double that in *N. gossei* dry leaves ($p<0.001$), which in turn was significantly higher than that in snus ($p<0.001$) (Table 4-1). All three contained small quantities of the other alkaloids. Nornicotine was significantly higher in *N. gossei* dry leaves compared to snus ($p<0.05$), and CRP2 ($p<0.01$). The products were not significantly different in their anatabine and anabasine content. Myosmine or cotinine were not detected for any of the products.

Table 4-1 Concentration (mg/g; mean ± se for 3 independent replicates) of nicotine and related alkaloids in smokeless tobacco products (fresh weight basis). For each alkaloid, mean values with the same superscript letter are not significantly different ($p<0.05$).

	Products		
Alkaloid (mg/g)	**Snus**	**CRP2**	***N. gossei* dry leaves**
Nicotine	4.083 ± 0.061[a]	11.97 ± 0.32 [b]	4.89 ± 0.29 [c]
Nornicotine	0.117 ± 0.005 [a]	0.08 ± 0.04 [a]	0.41 ± 0.05[b]
Anatabine	BLQ	0.13 ± 0.02	0.08 ± 0.01
Anabasine	0.094 ± 0.017	N.D.	0.14 ± 0.01
Myosmine	N.D.	N.D.	N.D.
Cotinine	N.D.	N.D.	N.D.

N.D.: Not Detected

BLQ: Below Limit of Quantification (1.7 µg/ml)

The quantity of material equivalent in nicotine content to a 4 mg piece of nicotine chewing gum was used to test for nicotine release in a dissolution test (Table 4-1). CRP2 released the greatest quantity of nicotine within the first 10 minutes in comparison to the other products ($p<0.001$). Nicotine release from CRP2 reached the maximum (95-97%) by 30 minutes irrespective of chewing condition (Figure 4-1 A). Snus, which is a commercially available tobacco-based product, released 59% of total nicotine during the 120 minute test when left in

the vessel without any chewing action and a single press at the start did not affect this. More frequent chewing action by pressing at 15 min intervals resulted in an increase ($p<0.001$) in the maximal cumulative nicotine release to 66% (Figure 4-1 B). Nicotine dissolution from *N. gossei* leaves was similar to that of snus, reaching 63%, but with no effect ($p>0.05$) of chewing condition (Figure 4-1 C). Additionally, changing the pH of the release media to pH 9.0 did not affect the rate of release or final amount released (64%) (Figure 4-1 D).

Nicabate gum showed the greatest effect of chewing, with 42% of the nicotine released when pressing occurred at 15-minute intervals and only 1.8% with no chewing ($p\leq0.0001$). Even a single press at the start of the test resulted in greater nicotine release (5.8%) than no pressing at all, and the importance of chewing for gum was displayed clearly by the cumulative amount of nicotine released increasing each time a press was made (Figure 4-1 E). This consistent increase as a result of chewing was not observed with the other tested products, but in these test conditions the maximum released for gum (42%; 1.7 mg) was significantly lower ($p<0.001$) than that achieved by CRP2 (97%; 3.9 mg), *N. gossei* leaves (64%; 2.5 mg) or snus (66%; 2.6 mg).

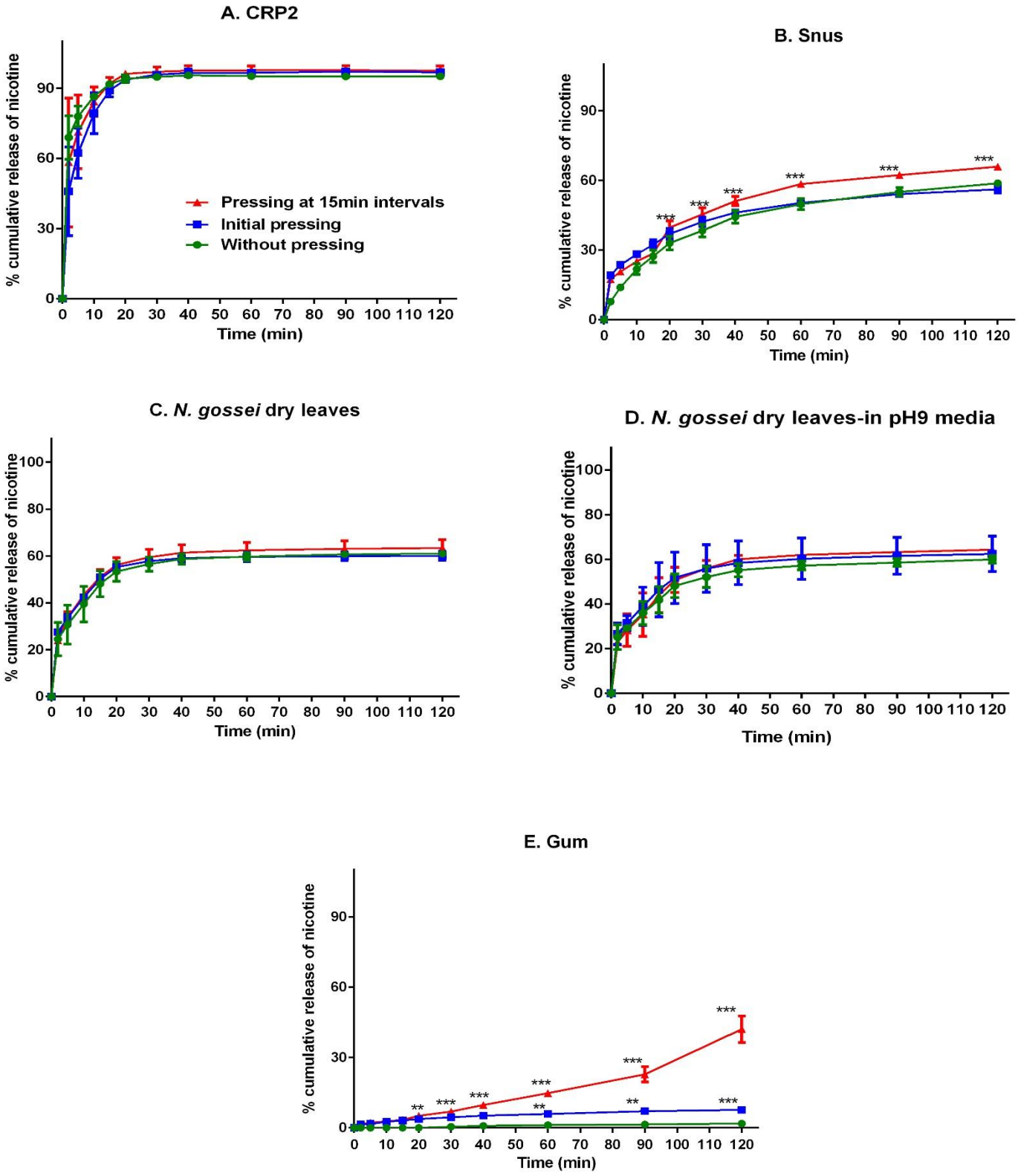


Figure 4-1 Percent cumulative release of nicotine from (A) CRP2 (B) Snus (C) *N. gossei* dry leaves (D) N. gossei dry leaves in alkaline media adjusted to pH 9 (E) and Nicabate gum in 3 conditions of without pressing, initial pressing for 30 seconds and pressing for 30 seconds at every 15 minutes. The data points show mean ± std error of 3 independent experiments.

The tobacco-based products also released other alkaloids into the dissolution media (Table 4-2), with quantities released reflecting the relative concentration within the product (Table 4-1). As seen with the alkaloids, the two TSNAs that were measured in the release media, NNN and NNK, were highest for *N. gossei* dry leaves. Only nicotine was measured in the dissolution media for the chewing gum, with no evidence for the presence of tobacco alkaloids or TSNAs found.

Table 4-2 Concentration of nicotine alkaloids and TSNAs (mean ± se of 3 replicates) released into 50 ml of simulated saliva in vessels with chewing at 15 minute intervals from smokeless tobacco products and Nicabate gum at 120 min.

	Products				
	Gum	**Snus**	**CRP2**	***N. gossei* dry leaves**	***N. gossei* dry leaves-pH9**
Alkaloid (mg)					
Nicotine	1.681 ± 0.321 [a]	2.636 ± 0.057 [b]	3.900 ± 0.067 [c]	2.537 ± 0.20 [b]	2.575 ± 0.008 [b]
Nornicotine	N.D.	0.104 ± 0.005 [ab]	0.067 ± 0.006 [ab]	0.304 ± 0.016 [c]	0.210 ± 0.015 [abc]
Anatabine	N.D.	N.D.	0.003 ± 0.003	0.028 ± 0.002	0.021 ± 0.002
Anabasine	N.D.	0.014 ± 0.065	N.D.	0.019 ± 0.005	0.034 ± 0.005
Myosmine	N.D.	N.D.	N.D.	N.D.	N.D.
Cotinine	N.D.	N.D.	N.D.	N.D.	N.D.
TSNAs (µg)					
NNN	N.D.	0.032 ± 0.095[a]	0.067 ± 0.010 [b]	0.265 ± 0.025 [c]	0.129 ± 0.001 [a]
NNK	N.D.	0.014 ± 0.016 [a]	0.037 ± 0.084 [bc]	0.089 ± 0.032 [a]	0.074 ± 0.065 [a]

N.D.: Not Detected

4.4 Discussion

N. gossei dry leaves are used in preparation of pituri, which is a smokeless tobacco product with a lack of information available on its prevalence of use, chemical composition, nicotine release and absorption pattern. In this study, nicotine release from *N. gossei* dry leaves is compared with CRP2 and Swedish snus, which are used as smokeless tobacco products, and a nicotine replacement therapy chewing gum, Nicabate. A quantity of each product containing 4 mg nicotine was used in the comparisons.

N. gossei dry leaves released nicotine at a similar rate to snus, with the maximum of 63 and 66% released at the end of the study for *N. gossei* dry leaves and snus, respectively. The nicotine content of *N. gossei* dry leaves used in this study (4.9 mg/g) was slightly but significantly higher than snus (4.1 mg/g), though *N. gossei* leaves can contain even higher levels of nicotine, with values between 6.2 and 17.0 mg/g of nicotine reported previously (Saitoh et al., 1985, Sisson and Severson, 1990). This may pose a potential health risk to the user as the exact amount of *N. gossei* dry leaves used in each quid will vary and excessive

nicotine levels lead to high blood pressure and significant cardiac risk (Council on Scientific Affairs, 1986, Gupta et al., 2007). The percentage of cumulative nicotine released from snus at the end of the first hour was 58%, which is less than reported previously for snus in the literature for the same duration using different apparatus (65% (Nasr et al., 1998) and 60%-90% (Li et al., 2013)). The snus in the present study achieved this level of release (66%) by the end of the 2 hour duration tested. The *in vitro* studies performed to evaluate nicotine release in smokeless tobacco products are limited to studies on moist snuff (snus) (Li et al., 2013, Nasr et al., 1998) performed with a maximum measurement time of 60 minutes. In the present study a time of 120 minutes was used because moist snuff may be kept in the mouth for more than 60 minutes; pouched snus is commonly used for 32-115 minutes with up to 124 minutes reported in male users (who tend to keep snus in their mouth for longer than female users), and loose snus was between 36-121 minutes with the longest time of 122 minutes in males (Gupta et al., 2007). The smokeless tobacco standard tested in this study, CRP2, released the highest cumulative amount of nicotine, with almost 97% of the 4 mg being released over the 2 hours.

Nicabate gum released the lowest cumulative amount of nicotine (42%) in this study, and in contrast to the smokeless tobacco products, simulated chewing was clearly the essential component driving nicotine release. Reported *in vitro* measurements for nicotine gum formulations are much higher; 79-83% release for 18 different formulations (Aslani and Rafiei, 2012) and 80-90% release for 3 formulations during a 1 hour period (Morjaria et al., 2004). However, in addition to Nicabate gum not being included in those studies, the experimental designs were quite different as they used a purpose-made mastication device with a piston that struck the gum in various points at a chewing rate of 1 strike per second applied continuously for 60 minutes (Aslani and Rafiei, 2012, Morjaria et al., 2004). This procedure is aligned with the European Pharmacopeia method for chewing gum (European Pharmacopoeia, 2010), for which the Erweka DRT-3 chewing gum dissolution tester has recently become available. To date the only published *in vitro* testing of release using this device is for the medicated chewing gums of dextromethorphan hydrobromide (Swamy et al., 2012) and so far there has not been any report for nicotine gum testing with this device. Nicotine chewing gums such as Nicabate are specifically designed to provide sustained release of small amounts of nicotine to help smokers gradually wean themselves off the addiction to nicotine or smoking (Hurt et al., 1995). The nicotine chewing gums have a fine sugar coating consisting of powdered sugar or a sugar substitute surrounding the gum base. This coating is formulated to prevent the gum from losing its adhesive property and to enhance the taste, and chewing causes the sugar coating to

disintegrate and the gum base to crumble and form a softer and smoother texture, thus promoting a faster release rate of nicotine. Nicotine gum is generally chewed until a tingling sensation is felt (associated with the nicotine absorption) and then placed against the buccal mucosa during periods without chewing. Although continuous application of 1 chew per second may be somewhat faster or constant than consumers use in practice, the strike rate indicated in the Pharmacopoeia (European Pharmacopoeia, 2010) provides a standard for comparison of nicotine chewing gum formulations.

The maximum chew rate used in the present study, with the primary aim of investigating nicotine release from the tobacco component of pituri, was 1 press per second for 30 seconds applied at fifteen minute intervals, because pituri and snus are generally held in the buccal cavity for extended periods of time with occasional sucking and/or chewing (International Agency for Research on Cancer, 2007, Ratsch et al., 2010). Simulated chewing had little or no effect on increasing the release from snus, *N. gossei* dry leaves and CRP2, and they all had significantly faster initial release of nicotine compared to Nicabate gum. Indeed, i*n vivo* studies on comparative release and absorption of nicotine from gum and smokeless tobacco products such as snus have shown the maximum concentration of nicotine in the blood resulting from one 4 mg nicotine chewing gum is achieved slower than Swedish snus and the maximum concentration is also lower (Benowitz et al., 1988, Lunell and Curvall, 2011). Swedish snus is a commercially manufactured product that is in powdered form sealed in a permeable sachet, so the total concentration of nicotine in each sachet available to be absorbed into the body can be known. Sucking the sachet enables saliva to diffuse through the sachet and, while chewing may be expected to speed up the release, in the present study chewing leads to a slightly faster release rate (7%) compared to no chewing. Finer tobacco leaves or the ‘fine-cut’ form with a smaller particle size have greater surface area to be penetrated with saliva and moistened, thus promoting fast diffusion of nicotine from the product and absorption into the mucosal membranes (Lunell and Curvall, 2011, Connolly, 1995). CRP2, which is also a manufactured moist snuff standard in powder form with small particle size and controlled levels of nicotine, pH and humidity (Wagner et al., 2014), showed a very fast release of nicotine without need for chewing. The *N. gossei* dry leaves used here, which are not commercially processed, exhibited large variation between replicates which is likely to be associated with variable nicotine distribution within the leaves and inconsistent particle size.

To simulate the effect of ash in pituri formulation, the simulated saliva was adjusted to pH 9.0. The rate and quantity of nicotine released was unaffected. While there is no evidence that the

higher pH associated with the presence of ash affects nicotine release from the leaves, it may result in faster absorption across mucous membranes. In commercial tobacco products, the total nicotine amount available for uptake into the body can be manipulated by the pH of the surrounding environment, so snus is commonly combined with alkalinizing agents such as sodium carbonate and ammonium carbonate in order to increase the absorption rate (Tomar and Henningfield, 1997).

As tobacco products originate from plant material, they may contain other alkaloids and TSNAs other than only nicotine. The simulated salivary fluid sampled at the end of the 2 hour dissolution test for the maximal chewing actions performed on the tobacco products, snus, *N. gossei* dry leaves and CRP2, contained other alkaloids and TSNAs. In contrast Nicabate gum released only nicotine (Table 4-2). The concentration of nornicotine was greatest in the simulated saliva from *N. gossei* dry leaves, followed by snus and CRP2. Nornicotine is linked to health hazards such as age-related macular degeneration (Brogan et al., 2005) and periodontal disease (Katz et al., 2005), which again is an indication of a higher health risk for pituri chewers. The simulated saliva at the end of testing with *N. gossei* dry leaves contained higher concentrations for both NNN and NNK than CRP2 or snus (Table 4-2). NNN and NNK are identified as the most prevalent strong carcinogens in unburned tobacco products. They are mainly produced via nitrosation of tobacco alkaloids after harvest and during processes like drying, curing, ageing and especially fermentation (Brunnemann et al., 1996).

4.5 Conclusion

Dry leaves of *N. gossei* used in pituri, an indigenous smokeless tobacco product that is held in the mouth with occasional sucking or chewing, contain higher nicotine content than commercially available smokeless tobacco products such as snus and, in the absence of continuous chewing action, higher release rate than Nicabate chewing gum. In addition to high levels of nicotine, higher concentrations of other alkaloids and carcinogenic compounds are also released into the saliva of the users of *N. gossei*. Further studies are recommended in order to specifically investigate the pharmacokinetics of the nicotine and other alkaloids released from pituri in comparison to the similar products. These all help in better understanding how pituri use affects both health outcomes and quality of life as it remains widely used by the Aboriginal population of central Australia.

Chapter 5. The alkaloids chemistry and *in vitro* cytotoxicity of the *Nicotiana gossei* leaves used in Australian Aboriginal smokeless tobacco, ‘pituri’ compared to nicotine

5.1 Introduction

Smokeless tobacco products are considered by many to be less hazardous than cigarette smoking and therefore a suitable substitute to promote harm reduction (Benowitz, 2011). Indeed, smokeless tobacco can deliver similar quantities of nicotine as smoking and avoids exposure to an extensive suite of deleterious chemicals present in smoke (International Agency for Research on Cancer, 2007). However, there is global concern about increasing the consumption of smokeless tobacco due to its adverse effects on human health. Smokeless tobacco products have different health effects than smoked tobacco, but few smokeless tobacco products have been studied for the health outcomes and diseases they may cause (Ebbert et al., 2004, Scollo and Winstanley, 2015). A short-term increase of blood pressure and heart rate is attributed to smokeless tobacco use, and users are more at risk of dying from stroke and heart disease (International Agency for Research on Cancer, 2007). Also, significant effects on the soft and hard tissues of the mouth caused by smokeless tobacco can lead to oral disease such as bad breath, tooth decay, receding gums, leucoplakia and lesions in the mouth (Critchley and Unal, 2003, International Agency for Research on Cancer, 2007). In pregnant women using smokeless tobacco, there is a high risk of low birth weight, premature birth and preeclampsia (Critchley and Unal, 2003, Ebbert et al., 2004, International Agency for Research on Cancer, 2007). In male users of smokeless tobacco, there is an increase in number of abnormal sperm, while sperm count and semen volume decrease (International Agency for Research on Cancer, 2007). Based on animal and epidemiological studies, smokeless tobacco products have been classified as carcinogenic to humans (Hecht, 2003, International Agency for Research on Cancer, 2007), with mouth cancer being the most prevalent cancer type (Ebbert et al., 2004) though cancer of the pancreas and oesophagus have also been reported to be caused by smokeless tobacco products (Secretan et al., 2009). The carcinogenicity of tobacco products is mainly attributed to tobacco-specific N-nitrosamines (TSNAs), which are nitrosated derivatives of tobacco alkaloids. The main two TSNAs are N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); both have been classified as group 1 human carcinogens by the International Agency for Research on Cancer (IARC) (Hecht, 1998, International Agency for Research on Cancer, 2007).

The two main types of smokeless tobacco products are snuff and chewing tobacco (Ebbert et al., 2004) and there are a wide range of products used around the world. Snuff is prepared from cured and ground leaves of *Nicotiana* spp., and can be held in the mouth and sucked or inhaled (in its dry form). Chewing tobacco consists of *Nicotiana* spp. leaves chewed in combination

with substances such as lime, betel leaf or areca nut (Scollo and Winstanley, 2015). Smokeless tobacco products vary in their composition and process of production, so each smokeless tobacco product will vary in terms of chemical composition and consequently pharmacological activity (Ebbert et al., 2004, Scollo and Winstanley, 2015).

Tobacco plants are chewed by indigenous populations of the Americas, Africa, the Indian subcontinent and the Asia-Pacific region (Latz et al., 1995, International Agency for Research on Cancer, 2007). A range of endemic *Nicotiana* species, such as *N. gossei* and *N. excelsior*, are chewed by indigenous Australian people, primarily in the central region of Australia (Latz et al., 1995). Pituri is the most commonly recognised word used for this (Latz et al., 1995, Peterson, 1979, Young, 2005, Ratsch et al., 2010), though there are a variety of synonyms such as mingkulpa (Ratsch et al., 2010, Young, 2005) also in use. Currently there is no national or international acknowledgement of the existence of chewed tobacco use by Australian indigenous people. Australia was not mentioned in the IARC monographs on smokeless tobacco (International Agency For Research On Cancer, 2012, International Agency for Research on Cancer, 2007) published by the World Health Organisation, yet pituri chewing is a common feature in everyday life of contemporary Aboriginal populations in Central Australia and other areas; 41% of Aboriginal people in Central Australia were chewing pituri according to a survey conducted in 1987 (Fleming et al., 1991) and in a recent study over 30% of Aboriginal women giving birth at a large hospital regularly chew pituri throughout pregnancy and lactation (Ratsch, 2011).

To prepare pituri, fresh or dry leaves of *Nicotiana* are broken into pieces, mixed with burnt wood ash and chewed to form a 'quid' (Latz et al., 1995, Ratsch et al., 2010) (Watson et al., 1983). A range of wood is burned to form the ash; some species mentioned in the literature are *Acacia* spp., *Grevillea* spp. and *Eucalyptus* spp. (Peterson, 1979), with *Acacia salicina* being a particular preference because it has been reported to contain high levels (51%) of calcium sulphate and is considered to be a very alkaline plant ash (Higgin, 1903). Pituri is held in the mouth, the lower lip and buccal cavity or the cheek, for extended periods of time for absorption. The oral cavity has a thin epithelium and rich blood supply so absorption of the nicotine is fast and avoids first pass metabolism (Hukkanen et al., 2005). The quid may be passed between chewers and stored behind the ear, under a breast, under an arm-band or a head-band when not being chewed (Peterson, 1979). A quid may be retained in the buccal cavity overnight, enabling continuous exposure (Ratsch et al., 2010). The way in which pituri is prepared and used is similar to iq'mik used by indigenous people in Alaska (International Agency for Research on

Cancer, 2007, Hearn et al., 2013) and maras from Turkey (International Agency for Research on Cancer, 2007).

In vitro studies have been used to elucidate any cell-specific effects of smokeless tobacco consumption that can indicate possible toxicity and carcinogenicity of smokeless tobacco chemicals in humans. There have been reports of cytotoxicity caused by smokeless tobacco products such as gutkha (Avti et al., 2010), khaini (Das et al., 2013), Sudanese toombak (Costea et al., 2010), American moist snuff (Misra et al., 2014), Swedish moist snuff (Coggins et al., 2012, Costea et al., 2010), Kentucky reference moist smokeless tobacco product (Lombard et al., 2010) and commercial chewing tobacco (Coppe et al., 2008). The present study is the first assessment of cytotoxicity induced by tobacco constituents of pituri as a means of investigating its potential carcinogenicity. Direct comparison is made for *N. gossei* leaves which are the main ingredients of pituri and the main source of nicotine for chewers, with pure nicotine and with an official reference chewing tobacco (CRP2) which also contains only tobacco.

5.2 Materials and methods

5.2.1 Chemicals and reagents

The solvents used for HPLC analysis were acetonitrile, from Merck (Darmstadt, Germany), and buffer prepared using ammonium formate from Sigma-Aldrich (St. Louis, MO). The water was deionised and filtered using a Milli-Q system (Millipore, Billerica, MA). Hydrochloric acid and sodium hydroxide used for adjusting pH were from Merck (Darmstadt, Germany). The analytical standards used were N'-nitrosonornicotine (NNN), 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), nornicotine, myosmine and cotinine from Sigma, nicotine from Fluka (Milwaukee, WI), anabasine from Sigma-Aldrich, anatabine from Cayman Chemical Company (AnnArbor, MI), and the internal standards used were caffeine from Ajax Finechem (Sydney, Australia) for HPLC-UV analysis and N'-nitrosonornicotine-d4 (NNN-d4) and 4-(methylnitrosoamino)-1-(3-pyridyl-d4)-1-butanone (NNK-d4) from Toronto Research Chemicals (Ontario, Canada) for LC-MS analysis. Reagents and culture medium used for cell culture including DMEM/F-12 (Dulbecco's Modified Eagle Medium, nutrient mixture F-12), FBS (Fetal Bovine Serum) and Trypsin-EDTA (0.25%) were from Gibco (Thermo Fisher Scientific, Vic, Australia). Triton X-100 solution used as positive control was from Sigma-Aldrich (St. Louis, MO). MTS assay reagent, CellTiter 96 AQueous One Solution Assay was from Promega (Madison, WI).

5.2.2 Preparation of extracts

Aqueous extracts of air dried ready for mastication leaves of *N. gossei* (pituri) collected in Alice Springs NT, Australia and Coresta reference smokeless tobacco product (CRP2 Moist Snuff) obtained from North Carolina State University (Tobacco Analytical Services Laboratory) stored at -20°C (Wagner et al., 2014) were prepared according to the method described by Coggins et al. (Coggins et al., 2012) with some modifications. Water (20 ml) was added to 1 g of material in a 50 ml tube and homogenised gently before incubating at 37°C with shaking for 24 hours. The mixture was centrifuged for 20 min at approximately 5000 *g*, the supernatant was decanted into an empty tube and centrifuged again at 5000 *g* for 20 minutes. The supernatant was decanted again and the pH was adjusted to 7.4 ± 0.2 with 1 M hydrochloric acid or 1 M sodium hydroxide. The final extracts were pre-filtered through 0.45 μm nylon filter (Grace Davison, Discovery Sciences) to remove fine particulates and finally filter-sterilized using a nylon filter with 0.2 μm pore size (Grace Davison, Discovery Sciences). The sterile filtrate was then lyophilized and stored at -80°C to be used for chemical analysis and preparing stock concentrations in sterile media as required for experiments (Das et al., 2013). All containers and solutions used in the extraction steps were sterilised prior to use in order to prevent contamination.

5.2.3 Quantification of nicotine and other alkaloids

The freeze-dried extracts from pituri leaves and CRP2 were quantified for nicotine and other alkaloids in order to determine the concentrations for treatments. Chemical analysis of the extracts was carried out on an Agilent 1100 series high performance liquid chromatography (HPLC) system equipped with a UV detector and Zorbax Extend C18 column (Agilent Technologies, Mulgrave, Vic, Australia) with a mobile phase consisting of 15 mM ammonium formate buffer and acetonitrile according to our previously validated method as mentioned in chapter 2 (Moghbel et al., 2015). The powdered extracts were dissolved in 10% aqueous acetonitrile containing 25 mg/ml caffeine as internal standard and quantification was performed using a constructed calibration curve of nicotine, nornicotine, anatabine, anabasine, myosmine and cotinine over a range of concentrations in 10% aqueous acetonitrile.

5.2.4 Quantification of NNN and NNK

SLT products, pituri leaves, CRP2, and their freeze-dried extracts, were quantified for two major TSNAs, NNN and NNK using LC-MS/MS. The analysis was performed on a 2.1 × 150

mm Poroshell 120 HILIC column with 2.7 µm particle size (Agilent Technologies). The MS-MS detection and quantification was performed following the validated LC-MS/MS method described in chapter 2.

5.2.5 Cell culture, treatment and viability assay

Human Lung Carcinoma Epithelial Cells (A549), provided by Professor Tom Gonda (School of Pharmacy, The University of Queensland), were seeded onto T25 plastic tissue culture flasks in DMEM-F12 medium containing 10% FBS, and incubated at 37°C in a 5% CO_2-air humidified incubator. Passaging of the cells was carried out at 3-day intervals when cell growth in culture flasks reached about 80% confluency. For experimental cultures, cells were detached by trypsinisation using trypsin-EDTA solution (0.25% trypsin, 0.53 mM MEDTA-4Na). The detached cells were suspended in culture medium and then centrifuged at 1500 rpm for 2 min. The resulting cell pellet was resuspended in media, the number of cells determined using a hemocytometer, cells plated at a density of 1×10^4 cells per well in 96-well culture plates and incubated overnight to allow adequate attachment of cells. Triplicate sets of cells were treated with 2, 10, 20, 50 mg/ml of the aqueous extracts from pituri leaves or CRP2 dissolved in DMEM-F12, or nicotine standard in DMEM-F12 at a concentration range covering the corresponding concentrations present in the extracts (0.1, 0.2, 0.5, 1 and 1.5 mg/ml), and incubated for 1, 3, 6 and 24 hours at 37°C. Triplicate sets of cells were treated with DMEM-F12 medium as a negative control, or 1% Triton X-100 in DMEM-F12 medium as a positive control.

Cytotoxicity testing was carried out using the Promega CellTiter 96 AQueous One Solution Assay (MTS) to estimate the viability of cells in culture according to the manufacturer's instructions and Malich et al. (Malich et al., 1997). The AQueous One Solution reagent (20 µL) was added to each well and the culture plates were incubated for 3 h before measuring the absorbance at 490 nm with iMark microplate reader (Bio-Rad, NSW, Australia). The absorbance at 490 nm, which corresponds to the quantity of formazan product, is proportional to the number of living cells in the culture. To correct for the potential for the extracts to contribute to the absorbance, triplicate sets of blank wells without cells were treated and tested in exactly the same way and their absorbance at 490 nm measured. The cell viability (%) was calculated as (abs-t – abs-b)/abs-c × 100, where abs-t is the average absorbance of treated wells, abs-b is the average absorbance of blank wells, and abs-c is the average absorbance of control wells containing only cell culture medium. Each experiment was performed in triplicate.

5.2.6 Data analysis

The cell viability values obtained after 24 hours treatment of the cells with 2, 10, 20 and 50 mg/ml of the aqueous extracts of both pituri leaves and CRP2 were used for calculating IC_{50}. For nicotine, in addition to 0.1, 0.2, 0.5, 1 and 1.5 mg/ml, which were used as comparable concentrations of the nicotine present in the extracts, treatments with 5 and 10 mg/ml were included for calculating the IC_{50}.

Prism (GraphPad, San Diego, CA) was used to conduct statistical analysis. One-way analysis of variance (ANOVA) with Bonferroni multiple comparisons test were applied. Values of $p < 0.05$ were considered to be significant.

5.3 Results

5.3.1 Chemical analysis of the extracts

Concentrations of nicotine and other alkaloids and two major carcinogenic TSNAs, NNN and NNK, were assessed in the aqueous extracts from pituri leaf and CRP2 snuff and the results were used to calculate the concentrations present in the original dry leaves and snuff product, respectively. Nicotine was the main alkaloid constituent in pituri leaf (4.8 mg/g) and CRP2 (11.97 mg/g) (Table 5-1). A much smaller quantity of nornicotine was extracted from both products, but at a greater concentration from the pituri leaves (0.4 mg/g) than CRP2 (0.08 mg/g). Low levels of anatabine and anabasine were also quantified in both extracts (Table 5-1).

Table 5-1 Alkaloid and TSNA composition (mean ± se for 3 independent replicates) of the Coresta reference smokeless tobacco product (CRP2 Moist Snuff) and dry leaves of *Nicotiana gossei*, along with freeze-dried aqueous extracts of CRP2 and dry leaves of *N. gossei*. ND: not detected.

	Concentration in original product (fresh weight)		Concentration in freeze-dried aqueous extract (dry weight)	
	N. gossei dry leaves	CRP2	*N. gossei* dry leaves	CRP2
Alkaloid (mg/g)				
Nicotine	4.89 ± 0.29	11.97 ± 0.32	25.54 ± 2.10	26.37 ± 0.70
Nornicotine	0.41 ± 0.05	0.077 ± 0.04	2.15 ± 0.20	0.17 ± 0.09
Anatabine	0.08 ± 0.01	0.13 ± 0.02	0.41 ± 0.04	0.30 ± 0.01
Anabasine	0.14 ± 0.01	ND	0.14 ± 0.02	ND
Myosmine	ND	ND	ND	ND
Cotinine	ND	ND	ND	ND
TSNAs (µg/g)				
NNN	8.37 ± 2.22	2.39 ± 1.42	43.70 ± 14.49	10.52 ± 6.24
NNK	6.91 ± 0.07	0.36 ± 0.01	36.07 ± 0.35	3.21 ± 0.02

Neither of the extracts contained myosmine and cotinine, both of which would have been detected and quantified by the HPLC-UV method if they were present at levels above 1.2 and 1.8 µg/ml, respectively. The concentration of nicotine in CRP2 moist snuff measured here was the same as that reported during official annual testing (12 mg/g) (Wagner et al., 2014). The alkaloid composition of *N. gossei* was lower than that reported previously for this species (6.2 – 16.8 mg/g dw) (Saitoh et al., 1985, Sisson and Severson, 1990), which is a reflection of the very different seed source, growth environment and chemical extraction method used between studies.

This study is the first to report the concentration of NNN and NNK in pituri leaves; NNN was present at 8.37 µg/g and NNK was 6.91 µg/g. The concentration of NNN and NNK have been analysed in CRP2 in different years (2010, 2011) and different laboratories, and ranged from 1.439 to 2.157 µg/g for NNN and 0.370 to 0.537 µg/g for NNK (Wagner et al., 2014). The concentration quantified in this study was 2.39 ± 1.42 and 0.36 ± 0.01 µg/g of original CRP2 product for NNN and NNK, respectively (Table 5-1).

5.3.2 Effect of nicotine and smokeless tobacco extracts on cell viability

Four concentrations of the aqueous extract from *N. gossei* leaves and CRP2 were tested for cytotoxicity: 2, 10, 20 and 50 mg/ml dissolved in DMEM-F12 medium. Based on the values in Table 5-1, the concentration of nicotine in these four quantities of aqueous extract from pituri leaves was calculated to be 0.051, 0.255, 0.511 and 1.270 mg/ml respectively, and for the aqueous extract from CRP2 the values were 0.053, 0.264, 0528 and 1.320, respectively. A corresponding concentration range of the nicotine standard was selected for testing, i.e. 0.05, 0.2, 0.5, 1 and 1.5 mg/ml. The growth of the cells was negatively affected by increasing concentrations and duration of treatment for both extracts (Fig. 5-1 A, B). For the 10 mg/ml concentration of extract from pituri leaves only 20% of the cells survived at 24 hours of cultivation while the inhibition was almost three-fold lower for CRP2 with about 60% of survival for the same duration of exposure to 10 mg/ml of the extract ($p < 0.05$). Both extracts resulted in significant reduction of viability after 24 hours of treatment at concentration of 20 mg/ml with viability reaching 5 and 24% for the extracts from pituri leaves and CRP2, respectively ($P < 0.01$). The maximum inhibition for both products was caused by treating the cells with 50 mg/ml of extracts for 24 hours, resulting in only 1.5 and 6% viability for extracts from pituri leaves and CRP2, respectively ($p < 0.001$) (Fig. 5-1 A, B).

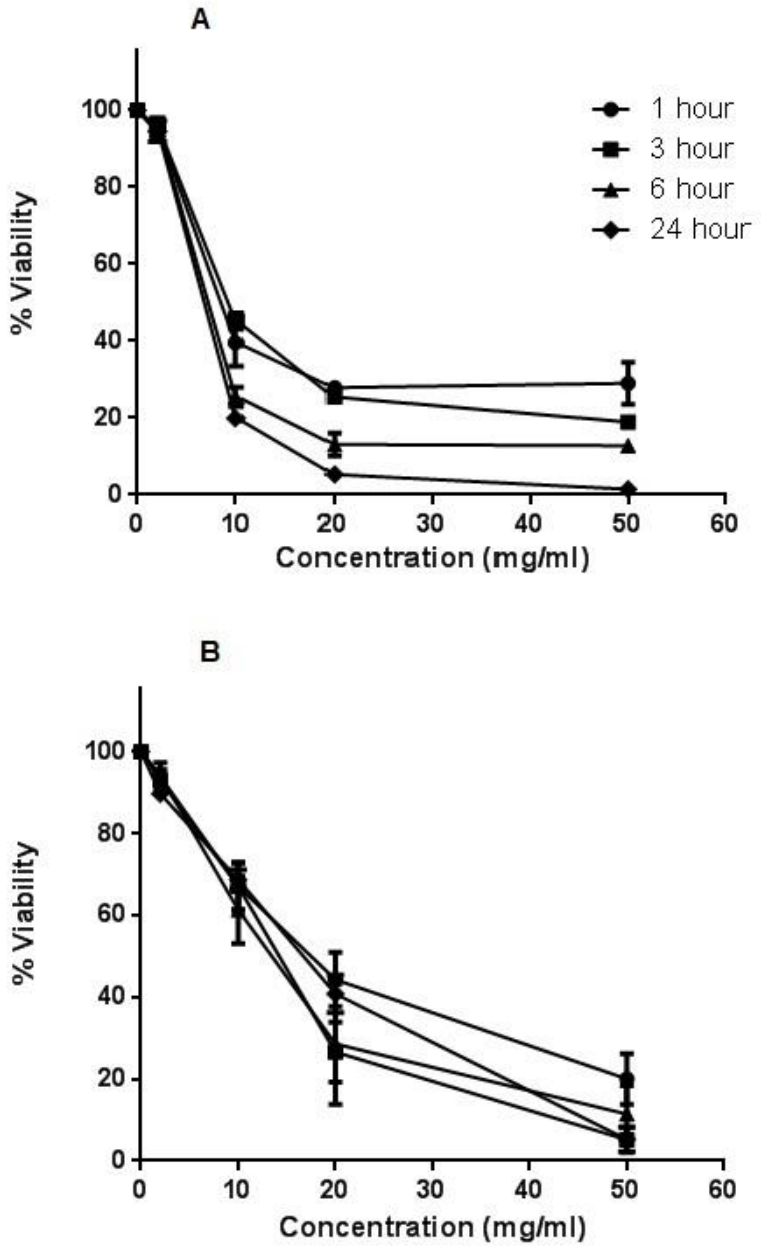


Figure 5-1 MTS assay results showing the effects of A) aqueous extract of *N. gossei* leaves used in pituri and B) aqueous extract of CRP2 on cell viability of human lung epithelial cells (A549). Applied concentrations of both extracts were 2, 10, 20, 50 mg/ml that were equivalent to approximately 0.05, 0.2, 0.5, 1-1.5 mg/ml of nicotine respectively. The data show the mean ± SEM of the 3 replicate experiments.

Exposing cells to nicotine alone also resulted in a decrease in cell viability. This was remarkably lower than observed for pituri leaf and CRP2 extracts. The nicotine concentrations of 1 and 1.5 mg/ml, between which lies the concentration of nicotine found in 50 mg/ml of

extracts from pituri leaves and CRP2, resulted in cell viability of 86.4 and 76.8%, respectively, for 24 hour treatments of the cells (Fig. 5-2).

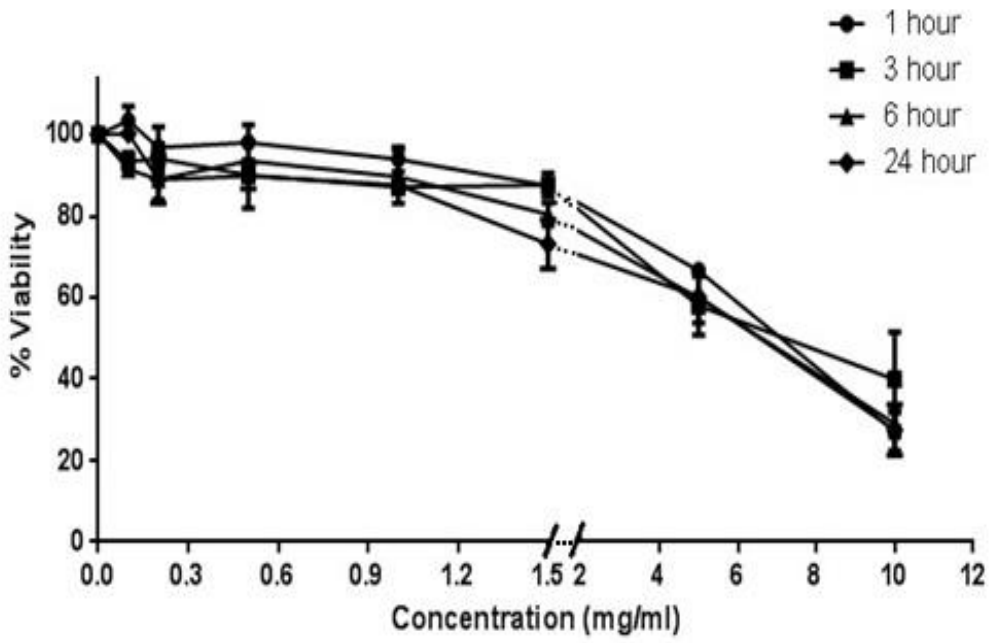


Figure 5-2 MTS assay results showing the effects of a concentration range of nicotine standard on cell viability of human lung epithelial cells (A549). Concentrations of 0.05 to 1.5 mg/ml covers the concentration range found in the 2-50 mg/ml of the pituri leaf and CRP2 extracts. Higher concentrations of 5 and 10 mg/ml were applied for calculation of IC50. The data show the mean ± SEM of the 3 replicate experiments. .

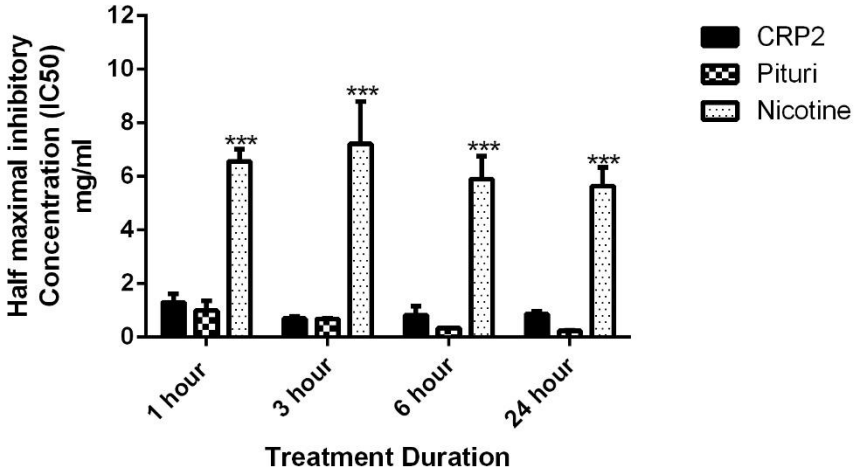


Figure 5-3 Nicotine equivalent concentrations of extracts from pituri leaves and CRP2, and concentration of nicotine standard that resulted in 50% inhibition of growth in human lung epithelial cells (A549). The data show the mean ± SEM of the 3 replicate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The concentrations causing 50% inhibition of cell growth (IC_{50}) after 24 hours incubation were 9.1 mg/ml of the aqueous extract from pituri and 32.5 mg/ml of the aqueous extract from CRP2. Calculated in terms of their nicotine equivalent concentrations (Fig. 5-3), the IC50 was 0.23 mg/ml for pituri leaves and 0.85 mg/ml for CRP2. The IC_{50} for nicotine was 5.6 mg/ml for the same treatment duration, which is substantially higher than both pituri and CRP2 ($P<0.01$) (Fig. 5-3).

5.4 Discussion

Nicotine is the most abundant alkaloid (~95%) in tobacco plants and products such as snuff, chewing tobacco and pituri and some of them can deliver higher doses of nicotine than cigarettes. Nicotine from smokeless tobacco products is readily absorbed through the lining of the mouth or the nose (Scollo and Winstanley, 2015) and although absorption is slower than that from cigarettes, blood levels of nicotine in users can be similar to that in smokers (International Agency for Research on Cancer, 2007). Nicotine has been shown to cause cell cycle arrest and growth inhibition (Lee et al., 2005). It also causes selective mutagenesis that relates to an increased risk of squamous cell carcinoma (Waggoner and Wang, 1994) and induces differentiation in immortalized oral keratinocytes (Lee et al., 2005)

According to our results, a large amount of nicotine is required to cause toxicity in the human lung epithelial cells (A549) used here. Toxic effects of nicotine usually require higher concentrations than is physiologically attainable and can vary with cell type (Lee et al., 2005). It has been reported that the concentration of nicotine in the blood of smokers (~100 nM or 16.2 ng/ml) does not affect the growth of several lines of lung cancer cells (Maneckjee and Minna, 1990). In contrast, some studies reported the positive effects of nicotine on cell growth in other cell types, such as primary human endothelial cells at 50 ng/ ml (Boutherin-Falson and Blaes, 1990) and lung cancer line neuroendocrine carcinoma at concentration of 1 M (162 ng/ml) (Schuller, 1989). In our study the growth of lung epithelial cells remained unaffected until 6 and 24 hours treatment with 1.5 mg/ml of nicotine, and all the treatments with 5 and 10 mg/ml nicotine significantly decreased cell viability ($p < 0.05$).

The cytotoxic effect on mitochondrial function of aqueous extracts from dry leaves of *N. gossei*, as the main ingredient of pituri, and reference smokeless tobacco products (CRP2) was measured. The IC_{50} calculated for 24 hours of treatment were 0.23 and 0.86 mg/ml for pituri leaves and CRP2, respectively, which is substantially lower than the IC_{50} for nicotine (5.6 mg/ml). In fact, the quantity of extract giving rise to this concentration of nicotine would be

220 and 212 mg/ml for pituri leaves and CRP2, respectively, indicating that the smokeless tobacco extracts exhibited greater toxicity than nicotine is responsible for. Nicotine can inhibit cell growth and proliferation (Chang et al., 2002)and can cause chromosome aberrations, but only in higher dose (Trivedi et al., 1990). Nicotine has been reported to increase the intracellular thiol level (Chang et al., 2002), but the levels of glutathione (GSH) and malondialdehyde (MDA) is higher in cells exposed to smokeless tobacco extracts than those exposed to only nicotine. This suggests that nicotine is less effective in induction of oxidative stress than the extracts of smokeless tobacco which contain other active compounds (Yildiz et al., 1999). These compounds trigger the formation of harmful free radicals. Pituri is prepared from a range of wild Australian species of *Nicotiana*, the major species being *N. gossei*. Other biologically active compounds are present in smokeless tobacco, such as the other alkaloids measured here (Table 5-1). More importantly, during curing and fermentation, tobacco alkaloids can give rise to tobacco specific N-nitrosamines via nitrosation which are by far the most prevalent strong carcinogens in unburned tobacco products (Hecht, 2003). Their levels per unit dose are reported to be even higher in SLTs compared with the levels in the mainstream cigarette smoke. TSNAs have been detected and measured in fresh leaves of tobacco plants, but drying is the main driver to increase levels by about 6 fold. Levels of TNSAs vary considerably between different smokeless tobacco products due to differences in type of product (manufactured, indigenous), type of tobacco plants used, climatic conditions, post-harvest processing and curing methods, storage conditions and the applied analytical methods (International Agency for Research on Cancer, 2007). Two specific TSNAs, NNK and NNN, are believed to be involved in the induction of oral cancer in SLT users (Hecht, 2003), while N-nitrosoanabasine (NAB) is reported to be a weak carcinogen and N-nitrosoanatabine (NAT) apparently lacks activity (Hecht et al., 1983). Levels range from 3 mg of NNN and 8 mg of NNK in each gram of Toombak, which is an SLT used in Sudan, to 0.5 and 0.1 µg/g of NNN and NNK, respectively in Naswar from Uzbekistan (International Agency for Research on Cancer, 2007). Smokeless tobacco from Australia was not included in this international comparison of TSNA levels. The present study has measured dry *Nicotiana* leaf used in the preparation of pituri rather than the prepared smokeless tobacco product, but the levels of NNN (8.37 µg/g) and NNK (6.91 µg/g) are consistent with smokeless tobacco products in use around the world. NNN and NNK have been extensively studied for their mechanism of action in different model tissues (Hecht et al., 1983). Their metabolic transformation through α-hydroxylation is believed to be important in making them electrophilic and leading to their

binding to nucleophilic centres in DNA. This binding results in point mutations that can eventually activate oncogenes (Barbacid, 1986). Other than nitrosamines, PAHs such as benzo(a)pyrene that are present in smokeless tobacco products, have been reported to form covalent bonds with DNA, triggering carcinogenesis (Hoffmann and Hecht, 1990).

5.5 Conclusion

In conclusion, this study has taken the first steps in considering the potential for negative consequences of pituri use. We have demonstrated that the induced cell death of human lung epithelial cells by aqueous extracts from pituri leaves and CRP2 is much higher than that by nicotine alone and that carcinogenic TSNAs are present in pituri.

Chapter 6. Summary, conclusions and future research

6.1 Summary and conclusions

Smokeless tobacco products, commercially manufactured or traditional, are relatively similar to smoked tobacco in terms of health outcomes and causing addiction, although they are considered to cause milder harms and in some cases recommended as possible substitute in quit smoking schemes (Arabi, 2008). The urgent need for further evaluation of their chemical composition for risk assessment purposes has resulted in studies mainly on manufactured and commercially available products such as American and Swedish snus (International Agency for Research on Cancer, 2007). *Nicotiana tabacum* is the main species in tobacco products and therefore has been studied extensively for its alkaloid chemistry and biosynthetic pathways (Dewey and Xie, 2013). In addition to nicotine, the production of certain other alkaloids, particularly nornicotine, is more important from a health perspective because of its higher risk to users, and consequently the tobacco industry has been focused on breeding low nornicotine producing varieties (Roberts, 1988).

Australian *Nicotiana* species have received very little attention, largely because so little is known about the extent of their use. Some of these species have a traditional use as pituri, a smokeless tobacco used by Aboriginal Australians in Central Australia (Ratsch et al., 2010). Hence, the overall objective of this thesis was to investigate the *Nicotiana* component of pituri in terms of pyridine alkaloids and tobacco-specific nitrosamines, their production, release and toxicity. This was dealt with by addressing four aims, and each of these will be discussed below before subsequently consider wider implications of the findings. As the focus of the study was on the chemistry of species due to their use in pituri, the nicotine release from the most common species chewed in pituri was investigated in the presence and absence of mastication and compared to the similar smokeless tobacco products and a commercial nicotine chewing gum. Finally the potential of the most common species used in pituri to be cytotoxic was investigated and compared to a standard smokeless tobacco and pure nicotine in the same concentrations as found in pituri.

AIM 1: to establish suitable analytical methods to ensure reliable and efficient chemical analysis that was planned for the alkaloid characterisation of the studied species, and alkaloids and TSNAs analysis of the products used in different phases of the study.

A new method was developed, optimised and validated for quantitation of alkaloids. This is a faster and more efficient HPLC-UV method than any previously available in the literature, and allows quantitative analysis of six alkaloids, nicotine, nornicotine, anatabine, anabasine,

myosmine and cotinine, in tobacco plants and products (Moghbel et al., 2015). Also an efficient LC-MS/MS method was adopted from published methods and validated in our lab for quantifying the two important carcinogenic TSNAs, NNN and NNK that stem from tobacco alkaloids during post-harvest processing of tobacco products. The validation results proved the efficiency of both developed methods to meet the aim to quantify the target alkaloids and TSNAs in studied plants and products (Chapter 2).

AIM 2: To determine the abundance of psychoactive alkaloids in Australian *Nicotiana* species, and to consider the nicotine to nornicotine conversion characteristics in some nicotine dominant versus nornicotine dominant species.

There are 26 *Nicotiana* species and subspecies currently recognised as Australian, all within the section *Suaveolentes*. A range of these species have been reported to be used by Aboriginal Australians for the preparation of smokeless tobacco known as pituri or mingkulpa (Latz et al., 1995, Peterson, 1979, Symon, 2005). The leaves are used either fresh or fire/sun dried and are mixed with ash that is prepared by burning wood such as *Acacia*. The mixture is then chewed to make a 'quid' (Latz et al., 1995, Ratsch et al., 2010, Watson et al., 1983). The particular species used in pituri can be quite variable and depends on availability in any given geographic location (Latz et al., 1995). Therefore the aim was to investigate all 26 Australian taxa of *Nicotiana* to ensure all of those potentially used for chewing were included. One approach for studying the chemistry of all these species was collecting their leaves from the plants grown in their natural habitat for analysis, but the wide distribution of the species and the need for proper identification before collection of their leaves made this option too difficult to achieve within a reasonable timeframe. More importantly, since the chemical composition of the leaves could be affected by their different geographical localization, it was decided to grow them all from seed under the same condition to obtain the needed leaf samples for chemical analysis. Therefore, a total of 73 seedlots (Apendix 1) were obtained from seed banks, botanic gardens and herbaria across Australia to grow all the taxa. However, some of the seedlots were from very old collections, also not all of the obtained seedlots were stored and maintained properly before receipt; hence the majority of them didn't have sufficient viability and failed germination. Out of the 73, only 29 seedlots covering 24 taxa achieved satisfactory germination and resulted in healthy plants that were then used for different analysis in the course of this study. These covered the main species that have been reported or are likely to be chewed due to their distribution across the areas where pituri chewing is reported to be practiced. The

remaining 2 taxa for which no reliable seed source or germination was obtained have very restricted distribution (Ladiges et al., 2011) and most probably aren't used for chewing.

Chemical analysis of the 24 species confirmed the hypothesis that Australian *Nicotiana* spp. have different levels of alkaloids and nicotine to nornicotine conversion phenotypes, although the conversion locus is present in all. The main species preferred to be chewed by Aboriginal pituri users are amongst rather safer, nicotine dominant species. These include *N. gossei* and *N. excelsior*, both of which are low or nonconverters. *N. benthamiana*, also a low converter, has been reported to be chewed mainly by Western Australian Aboriginals (Latz et al., 1995, Symon, 2005). However, there are reports in the literature of more hazardous high converter species consumption in some areas. These include *N. cavicola* in Western Australia (Symon, 2005) and *N. goodspeedii* by Aboriginal people at the western end of the Nullarbor Plain in Western Australia (Symon, 2005). *N. rosulata* ssp. *ingulba*, a medium converter has been also a preferred species for chewing due to its availability in Central Australia (Latz et al., 1995, Peterson, 1979, Symon, 2005). There are also reports mentioning some high converters as rarely used or specifically avoided which include *N. megalosiphon* ssp. *megalosiphon, N. simulans, N. occidentalis* ssp. *occidentalis* and *N. velutina* (Latz et al., 1995, Peterson, 1979, Symon, 2005). Therefore, the nicotine to nornicotine conversion, which is regulated by a group of cytochrome P450 genes that encode active demethylase enzymes (Siminszky et al., 2005), was further studied in six selected species with different conversion and nicotine or nornicotine accumulation capability by investigating the functionality of the locus before and after curing the leaves with heat. The studied species included *N. gossei*, *N. excelsior* and *N. benthamiana* as none or low converters and *N. cavicola, N. goodspeedii* and *N. velutina* as high converters of nicotine to nornicotine. The active genes have been reported to be triggered to express more by drying or curing and also during senescence (Lewis et al., 2010). The result obtained in this study also confirmed the hypothesis on the importance of the curing process in increasing the transcription of the functional locus that are active only in high converter species. Leaves from *Nicotiana* plants used in pituri preparation might undergo a sun or fire drying process that can lead to higher accumulation of nornicotine in high converter chewed species which can in turn, result in higher toxicity with nornicotine and its derivative carcinogenic TSNA, NNN (Chapter 3).

AIM 3: to investigate the nicotine release pattern of the tobacco constituent of pituri in comparison with available similar smokeless tobacco and NRT products.

Oral consumption of smokeless tobacco products implies the release and absorption of the addictive component, nicotine, through the lining of the mouth, which is slower than through the lungs and via smoking (Benowitz et al., 1988). Nicotine replacement therapy gums are also designed to provide nicotine through the same path, however, the release from the gum formulation depends highly on the chewing and mastication process performed by parallel function of the teeth and tongue (Hurt et al., 1995). The result from the *in vitro* release studies conducted here using a model buccal cavity system showed that this is not the case in oral consumption of the smokeless tobacco products, such as the CORESTA reference smokeless tobacco (CRP2), the tobacco component of pituri and Swedish snus, as these showed minimal or insignificant dependence on chewing actions. Similar to snus, the dry and ready for mastication leaves of *N. gossei* used here, which is one of the most common ingredients for pituri users, are generally held in the mouth and sucked rather than being chewed. Both these products tend to release higher concentrations of nicotine faster than the formulated chewing gums under non-chewed conditions, which proved the hypothesis for this section. It is also important to note that extensive nicotine uptake from *N. gossei* leaves can result in cardiovascular implications (Savitz et al., 2006). The hypothesis on the positive effect of ash for increasing the release of nicotine was rejected as the induced alkaline pH for mimicking the ash component in pituri in terms of pH was shown to be insignificant in increasing the release rate; it is likely that the pH is of more relevance to the absorption of nicotine through the mucosa rather than release from the plant tissues. The chemical analysis of the released media indicated that the consumption of *N. gossei* leaves in pituri exposes the users to other toxic chemicals such as nornicotine and carcinogenic TSNAs, NNN and NNK (Chapter 4).

AIM 4: To consider the cytotoxic potential of tobacco constituent of pituri compared to that from nicotine alone.

Nicotine is believed to be the main psychoactive chemical in tobacco plants and products which result in the addiction in users. However, nicotine is not the only chemical received through consumption of smokeless tobacco products. So it was hypothesised that tobacco constituents of *N. gossei* are more toxic than nicotine alone. The cytotoxic effect of the extracts from smokeless tobacco products such as *N. gossei* dry leaves used in pituri and a reference moist snuff, CRP2, on human lung epithelium cells (A549) was compared with that from only nicotine in similar concentrations. The observed viability of the cells treated with nicotine only, in the similar concentration as what has been determined for the extract from *N. gossei* dry leaves and CRP2, was much higher than the viability obtained after the treatment with the two

aforementioned extracts, which proved the hypothesis made for this section. Biologically active compounds such as alkaloids (other than nicotine) and carcinogenic TSNAs, NNN and NNK, are present in both extracts. Levels of NNN and NNK in different smokeless tobacco products reported so far range from 3 mg to 0.5 µg/g for NNN and 8 mg to 0.1 µg/g for NNK (International Agency for Research on Cancer, 2007). The levels measured for NNN (8.37 µg/g) and NNK (6.91 µg/g) for *N. gossei* leaves, which reflects the levels in the final product, pituri, are consistent with smokeless tobacco products in use around the world. The presence of other alkaloids and carcinogenic NNN and NNK is in line with the observed higher cytotoxicity of these products rather that attributed to nicotine alone (Moghbel et al., 2016) (Chapter 5).

In conclusion, pituri as a smokeless tobacco product is considered to be less harmful than cigarette smoke, however possible harm might arise from the lack of knowledge on the different chemistry of the wide variety of species that might be used in its preparation and due to general misconception of its being "safe" rather than "less hazardous". While the main concerns about cigarette smoke are the chemicals such as polycyclic aromatic hydrocarbons that are produced during combustion, there are compounds such as TSNAs which come in the tobacco constituent such as the wild Australian *Nicotiana* species used in pituri.

The *Nicotiana* species studied here vary in their alkaloid composition, with genetics driving some to have higher nornicotine contents than others, which can lead to production of higher levels of carcinogenic TSNAs such as NNN. Although the most preferred species *N. gossei*, is a nicotine dominant and a safer species, there are sporadic mentions in the literature of the use of some high converter species as pituri, due largely to accessibility within specific geographic locations. *N. cavicola, N. goodspeedii, N. megalosiphon* ssp. *megalosiphon, N. simulans, N. occidentalis* ssp. *occidentalis* and *N. velutina* as high-nornicotine species have a more hazardous nature especially due to the chemical changes that occur during the storage and processing practices such as curing (drying). The reports in the literature on which species are chewed is limited and it is possible that some other species such as *N. monoschizocarpa, N. megalosiphon* ssp. *sessilifolia* (high converters) and *N. occidentalis ssp. obliqua* (medium converter) are also chewed since their distribution range includes the central desert area where pituri chewing is believed to be most common. Therefore, the information on nicotine to nornicotine conversion status of these species indicates that chewers should avoid these species.

The *N. gossei* dry leaves tested in this study contain more nicotine than the commercial Swedish snus, but the nicotine release rate is similar for these two products and is not apparently increased by chewing. However, the release of other toxic chemicals such asNNN and NNK makes *N. gossei* dry leaves a more toxic smokeless tobacco than Swedish snus. The nicotine chewing gum is a better alternative for nicotine supply because no other alkaloids or nitrosamines were released from it, however it might not be favoured by Aboriginal chewers since they have to pay for it, whereas pituri is a free source of nicotine. Testing the extract from dry leaves of *N. gossei* against human lung epithelium cells resulted in higher toxicity compared to nicotine at similar concentrations, indicating its higher toxic potential that could be due to other alkaloids and carcinogenic NNN and NNK present in the extract from *N. gossei* leaves. It is likely that nornicotine dominant species have even higher levels of these TSNAs and therefore higher toxic potential if used in preparation of pituri, especially if their leaves undergo drying before consumption. Therefore, avoiding consumption of high converters, and preferably medium converters, will help in harm reduction to pituri chewers.

The work undertaken in this thesis has been conducted with respect for the Convention on Biological Diversity and the Nagoya Protocol. The biological resources necessary to conduct this project were provided by a range of contributors across Australia. The vast majority of these are members of the Australian Seedbank Partnership, which has the Convention on Biological Diversity and the preservation of traditional knowledge, resources and rights as a major focus. Agreements regarding the use of the material for this project and information sharing were signed where available, and the seedbanks, herbaria, botanic gardens and individual collectors have been acknowledged at each stage. The research outcomes from this project are shared with those contributors, and, through publication of the results, with the wider public.

6.2 Future direction

The knowledge of processing and preparing pituri has a sacred ritual significance in Aboriginal life, yet there is a paucity of information on the species and processes used in preparation of pituri (Low, 1987, Watson et al., 1983). To date there are limited and sporadic studies in the literature on pituri, the majority of which are very old and not thoroughly focused on it as a stand-alone product. Furthermore, apart from *Nicotiana* species, *Duboisia hopwoodii* has also been mentioned and known as the plant constituent in pituri. Although the plant material currently used for pituri preparation in central Australia is the wild *Nicotiana* spp. and not *D.*

hopwoodii, there is a great deal of confusion in the scientific and lay media and no clear distinction between the species used in any given geographic locations where pituri chewing is practiced (Ratsch et al., 2010). Apart from pyridine alkaloids like nicotine, *D. hopwoodii* contains tropane alkaloids such as scopolamine (de Rios and Stachalek, 1999). Chemical analysis of *D. hopwoodii* and the possible metabolites of its tropane alkaloids in the users, if any, will be of value for elucidation of possible harms. Therefore, ethnobotanical research in the area where pituri remains very popular with Aboriginal chewers, could help in getting a better picture on the species as well as details on the production process to further expand the investigation on the possible health effects and the scale of chemical exposure to the users.

This thesis took the first step in amplifying the putative loci encoding N-demethylases in 24 out of 26 Australian *Nicotiana*. The loci was found to be triggered by heat and expressed more in the cured leaves of only high converter species. The difference in the observed expression levels between the cured leaves of high converter and low or nonconverter species could be due to the presence of the different members of the CYP82E subfamily and also silencing of the active conversion loci in the low or nonconverter species (Chakrabarti et al., 2007). It would be interesting to study this further by identification of the present members of the CYP82E in studied high converter and low or nonconverter species and investigating if any such transcriptional silencing of the active conversion loci is involved in the observed conversion phenotypes in Australian *Nicotiana* spp.

In terms of nicotine release, the next step would be to consider release from quids. These would have to be prepared in the manner practiced by Aboriginal chewers, which involves mastication to create a unified piece of pituri quid. A closer determination of the size and amount of each ingredient in the quids as well as the extent to which each quid is chewed or sucked can help in better investigating the release rate. A proper chewing device may be more appropriate for *in vitro* testing, such as the Erweka DRT-3 mastication device that has been used for testing the medicated chewing gums of dextromethorphan hydrobromide (Swamy et al., 2012), or a device may need to be designed that simulates sucking to better represent the case in pituri consumption. The need for release testing with proper methods is even more urgent recently with the growing number of tobacco companies which have started producing products such as tobacco gums and lozenges that are similar in design to the nicotine replacement therapy products produced by pharmaceutical companies (Kostygina et al., 2016). The rate of absorption of tobacco chemicals such as nicotine and nornicotine present in pituri through mucosal membranes could also be investigated in *in vitro* setting using an appropriate

membrane such as a dialysis membrane (Nasr et al., 1998), bovine (Ìkinci et al., 2004) or porcine (Nair et al., 1997) buccal mucosa that have been implemented for nicotine absorption and diffusion studies, to get an idea of the rate of nicotine absorption in the presence and absence of different concentrations of the ash.

Ultimately, *in vivo* testing would be the most valuable approach for determining differences in nicotine absorption between pituri quids, commercial tobacco or nicotine products and other traditional smokeless tobacco products. Bioanalytical studies of the nicotine and hazardous TSNAs metabolites in biological matrices of the pituri users with consideration of different methods and duration of chewing/sucking, different ingredients such as high/low nornicotine species or fresh/dry leaves, proportion of tobacco to ash and the quantity of total product can be a starting point. Then comparison of these metabolites and their pharmacokinetics in pituri chewers with the metabolites in the users of other similar smokeless tobacco products, nicotine replacement therapy products, and even smokers as well as a control groups of non-chewer/non-smokers could really broaden the knowledge surrounding pituri risk assessment.

Investigation into the cytotoxicity of pituri could be expanded by checking the cytotoxic potential of the different species that might be used as the tobacco constituent of pituri, the ash and the final quids for effects. Determining the chemistry of the different types of the ash used in pituri especially in regard to the toxic chemicals such as the polycyclic aromatic hydrocarbons (PAHs) or heavy metals that might affect cell survival will help to get a better understanding of the extent of toxicity as well as the pathways involved in cytotoxicity. Also parallel to this would be applying a range of other cell assays such as mutagenicity assays (Wan et al., 2009) to detect genetic damage that leads to gene mutations, genotoxicity (Misra et al., 2014) to investigate DNA damage as an indication of potential carcinogenicity, apoptosis assays (Bagchi et al., 1999) to look for higher than normal apoptosis that can be attributed to pituri chemicals, and assays that evaluate the inflammatory response (Nordskog et al., 2003) to exposure with pituri chemicals.

The information available on the prevalence and frequency of pituri consumption is minimal, except for a mention of pituri as a form of tobacco in major drug use pattern and tobacco epidemiologic studies. The main study reporting on prevalence of pituri dates back to 1987, in which 38% of Aboriginal women and 11% of men in the NT were reported to be chewing pituri. According to this survey chewing has been particularly common among women in the central desert region, with nearly two thirds of them (61%) chewing tobacco (Fleming et al., 1991). The epidemic seems to be unabated and might even be accelerating. According to a

more recent study from 2011, over 30% of Aboriginal women giving birth at a large hospital in Alice Springs regularly chew pituri throughout pregnancy and lactation (Ratsch, 2011). With this notion of the considerable affected population, more systematic epidemiologic studies conducted in the area where it remains popular will make the interpretation of pituri and its pathology more precise. Currently, there is ongoing research to investigate the maternal and neonatal outcomes of pituri consumption among the pregnant Aboriginal women chewing pituri compared to smokers and non-users. Associated with this, it would be valuable to investigate the prevalence of tobacco related disease such as oral disease, heart disease, cancers, etc. not only in pregnant women and neonates, but also in the wider population of chewers.

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Appendix

Appendix 1. Australian *Nicotiana* spp. seedlots received, with their corresponding internal seedlot number, source seedlot code, herbarium voucher number, collection date, the Australian state from which they were collected, and seed viability (V) according to tetrazolium staining. Seed germination and plant growth was attempted for all seedlots; those that resulted in successful germination of sufficient replicate plants to have been used in this study are highlighted. For successful seedlots without a herbarium voucher number, vouchers were identified by Dr Neville Walsh (NW), senior conservation botanist at Royal Botanic Gardens Victoria, and Dr. Daniel Duval (DD) at South Australian Seed Conservation Centre, Botanic Gardens of South Australia. Missing data occurred for some seedlots not tested for viability due to small numbers of seeds being provided, and seedlots for which insufficient germination was achieved were not pursued to complete their collection information and confirm identification.
Seed sources in the table are: **ATGGC**: Australian Tropical Grains Germplasm Centre, Biloela, Qld; **Herbalistics:** purchased from herbalistics.com.au; **NSW:** Australian Botanic Garden, Mount Annan; **NT:** Alice Springs Desert Park; **Qld**: Brisbane Botanic Gardens; **SA:** Botanic Gardens of South Australia; **UniMel:** University of Melbourne; **Vic:** Royal Botanic Gardens Victoria; **WA:** Botanic Garden and Parks Authority, Perth; **WASABC:** WA State Agricultural Biotechnology Centre

Species	**Seedlot**	**Source seedlot code**	**Voucher**	**Source**	**collection date**	**Collection state**	**% V**
N. amplexicaulis N.T.Burb	19	NSW4157044	NSW 234670	NSW	21/10/1990	QLD	60
	65	TS 298 (AusTRCF317546)	MEL 2396301	ATGGC	-	-	50
	49	TS 286 (AusTRCF303897)		ATGGC			50
N. benthamiana Domin	3	A109412	DNA A0109412	NT	18/07/2001	WA	90
	4	D0182096	DNA D0182096	NT	12/07/2007	SA	80
	28	-		Herbalistics	-	NT	90
N. burbidgae Symon	73	DJD3167	Voucher confirmed (DD)	SA	Sep-15	SA	20
	40	SL54		UniMel	11/10/2005	SA	0
N. cavicola N.Burb.	18	NSW877057	NSW951497	NSW	3/08/1995	SA	100
	30	-		Herbalistics	-		0
	33	SL9 (AusTRCF303902)	-	UniMel	4/09/2013	WA	0
	50	TS 205 (AusTRCF303692)	-	ATGGC	1956		100
N. excelsior (J.Black) J.Black	5	D194512	AD 246203	NT	21/09/2009	SA	50
	10	D0221380	DNA D0221380	NT	8/09/2012	NT	50
	27	MJT438	AD 264269	SA	4/09/2013	SA	90
N. forsteri Roem. & Schult.	1	BGQLD.0727	BRI AQ0840316	QLD	27/05/2010	QLD	50

Species	Seedlot	Source seedlot code	Voucher	Source	collection date	Collection state	% V
	2	RJ 3238	NSW 906082	NSW	1/02/2013	NSW	90
N. goodspeedii H.Wheeler	12	DJD213	AD 192908	SA	22/11/2005	SA	100
N. gossei Domin	6	D204093	DNA D0204093	NT	25/04/2011	NSW	100
	21	NSW4022326	Voucher confirmed (NW)	NSW	Sep-97	NT	90
	31	-		Herbalistics	-		80
N. heterantha Symon & Kenneally	64	AusTRCF313551	MEL 2396300	ATGGC	-	-	60
	38	SL33	MELU D106511, D106512, D106513	UniMel	20/03/2005		50
N. maritima H. Wheeler	16	DJD3341	Voucher confirmed (DD)	SA	7/01/2013	SA	50
	69	MKJ140	AD 187318	SA	15/11/2005	SA	80
	51	TS 107 (AusTRCF303768)	-	ATGGC	1954		80
N. megalosiphon Van Heurck & Mull. Arg. ssp. m*egalosiphon*	26	NSW870550	NSW2010147	NSW	8/11/2010	NSW	100
	41	-		Herbalistics	3/07/1905	NSW	100
N. megalosiphon Van Heurck & Mull. Arg. ssp. *sessifolia* P.Horton	52	AusTRCF303829	MEL 2396303	ATGGC	-	-	30
N. monoschizocarpa (P.Horton) Symon & Lepschi	72	AusTRCF303666	Voucher confirmed (NW)	WASABC	1/09/1986	NT	50
N. occidentalis H.Wheeler ssp.*hesperis* (N.Burb.) P.Horton	56	TS 341 (AusTRCF303767)	MEL 2396304	ATGGC	1960	-	82
	55	TS 331 (AusTRCF303764)	-	ATGGC	1970	-	70
N. occidentalis H.Wheeler ssp. *obliqua* N. Burb.	34	SL17 (AusTRCF303779)	MELU D106540	UniMel	1/08/1956	WA	70
	29	-		Herbalistics	-	NT	20
	22	NSW4150998	NSW4150998	NSW	25/11/1980	SA	-
	67	-	-	WA	1992	-	100
	42	DJD3051	voucher submitted	SA	25/09/2014	-	100

Species	Seedlot	Source seedlot code	Voucher	Source	collection date	Collection state	% V
	45	19921000	-	WA	-	-	10
N. occidentalis H.Wheeler ssp. *occidentalis*	54	L 3569 (AusTRCF303738)	MELU D106542, D106543	ATGGC	1959	-	30
	53	TS 182 (AusTRCF303732)	-	ATGGC	1956	-	4
N. rosulata (S.Moore) Domin ssp. *ingulba* (J.Black) P. Horton	58	TS 75 (AusTRCF303907)	MEL 2396302	ATGGC		-	84
	7	98A97266	98A97266	NT	2/09/1998	NT	0
	43	-	-	WA	-	-	30
	57	TS 63 (AusTRCF303906)		ATGGC	-	-	70
	68	-	-	WA	1/01/2015	-	100
N. rosulata (S.Moore) Domin ssp. r*osulata*	44	20070392	PERTH 7821336	WA	20/07/2006	SA	100
	48	AD112312	AD112312	SA	24/12/2000	SA	100
N. rotundifolia Lindley	71	-	MEL 2396305	WASABC	Aug-15	WA	100
	23	NSW4080337	NSW4080337	NSW	17/10/1980	WA	-
	59	73/229 (AusTRCF303745)	-	ATGGC	1953	-	10
	60	TS 192 (AusTRCF317526)	-	ATGGC	-	-	32
	35	SL20 (AusTRCF303626)	MELU D106465, D106466, D106467, D106468	UniMel	-	-	0
N. simulans N.Burb.	13	RJB70944	AD 206232	SA	12/03/2007	SA	80
	25	NSW872310	ASBP 20101508	NSW	15/11/2010	NSW	40
N. sp. 'Corunna' Symon17088	36	SL23 (MELU D106460, D106461, D106462, D106463, D106464)	AD 239243	UniMel	14/09/2005	SA	50
	70	DES17088	AD 169953	SA	17/09/2004	-	80
N. suaveolens H. Wheeler	11	DJD1980	AD 239956	SA	6/10/2010	**SA**	100
	61	TS 261 (AusTRCF303886)	-	ATGGC	-	-	90

Species	Seedlot	Source seedlot code	Voucher	Source	collection date	Collection state	% V
	47	NSW 4084762	NSW 4084762	NSW	20/01/1989	NSW	0
	46	NSW 874839	ASBP 20110237	NSW	30/03/2011	NSW	100
	17	NSW4088842	NSW 197546	NSW	22/10/1986	NSW	0
	37	SL24	MELU D106487 (field); D106488, D106489, D106490, D106491	UniMel	-	VIC	0
N. truncata Symon	14	TST1056	AD 250303	SA	7/10/2010	SA	50
	39	SL44	MELU D106487 (field); D106488, D106489, D106490, D106491	UniMel	10/10/2005	SA	0
N. umbratica N.Burb.	66	TS 303 (AusTRCF317549)	-	ATGGC	-	-	-
	74	-	-	WASABC	2013	SA	-
N. velutina H.Wheeler	15	DJD233	AD 192706	SA	24/11/2005	SA	70
	24	NSW4139564	NSW 213331	NSW	24/01/1989	VIC	60
	8	98A97216	-	WASABC	2/09/1998	NT	0
	9	MEL2338142	MEL2338142	Vic	24/11/2010	VIC	60
N. wuttkei J.Clarkson & Symon	62	ZZ 305 (AusTRCF323099)	-	ATGGC	-	-	-
	63	73/276 (AusTRCF323100)	-	ATGGC	-	-	20
	75	73/280 (AusTRCF323101)	plants identified as *N. tabacum*	ATGGC	-	-	