

## Supporting document 1

### Risk assessment – Proposal P1029

### Maximum Level for Tutin in Honey

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## Executive summary

Tutin is a plant-derived neurotoxin which is sometimes detected in New Zealand honey. Tutin contamination of honey occurs when bees gather honeydew from an insect that feeds on sap of the shrub *Coriaria arborea* (“tutu”). Consumption of tutu honeydew honey can result in serious acute adverse health effects. Temporary maximum levels (MLs) for tutin in honey and comb honey of 2 mg/kg and 0.1 mg/kg, respectively, currently exist in the *Australia New Zealand Food Standards Code* (Standard 1.4.1 – Contaminants and Natural Toxicants).

For acute neurotoxins, such as tutin, severe symptoms of poisoning (e.g. tonic-clonic convulsions) usually correspond to the time taken to achieve their maximal concentration in serum (typically less than 3-4 hours after ingestion). However, the large variability in the onset time of clinical signs and symptoms of toxicity (0.5 to 17 h with a median of 7.5 h) following tutu honeydew honey ingestion in the 2008 human poisoning incident was difficult to explain. To investigate this variability, which was considered to have potential implications for the existing MLs for tutin, a human pharmacokinetic study was conducted in which 6 healthy adult males each received a single oral dose of tutu honeydew honey. The tutin dose received by the volunteers was equivalent to that received by a high consumer of honey (97.5<sup>th</sup> percentile) that contains tutin at the ML of 2 mg/kg.

The serum tutin concentration profile for all volunteers exhibited two discrete peaks, with the first at 0.5 to 1.5 hours post-dose, and the second and higher serum level occurring at 8 to 16 hours post-dose. Transient mild light-headedness was reported by two out of 6 subjects at a time post-dose corresponding to peak 1, while transient mild headache was reported by the same two subjects at approximately peak 2. No other adverse effects were observed in the study.

It was speculated that peak 1 was due to rapid systemic absorption of tutin while peak 2 was due to tutin released in a delayed and sustained manner from a hitherto unidentified source of additional tutin in honey, such as conjugates of tutin. Subsequent chemical analysis revealed the presence of substantial amounts of various tutin glycosides in the administered honey sample. Further analysis of this honey sample and 14 other honey samples indicated that (i) four tutin glycosides account for the majority of tutin glycosides in honey, (ii) the ratio of the sum of these four tutin glycosides to tutin varied over an approximately 5-fold range, and (iii) honey samples that contained no detectable tutin also contained no detectable tutin glycosides.

Based on the results of the human pharmacokinetic study, it is considered possible that adverse effects such as mild light-headedness and headache may be experienced following the consumption of honey containing tutin at the current ML of 2 mg/kg.

Such adverse effects are more likely if a large amount of honey ( $\geq 0.9$  g of honey per kg bodyweight) is consumed in one sitting, as was the case in the pharmacokinetic study. The risk of adverse effects is increased if the ingested honey has a ratio of tutin glycosides to tutin at the high end of the observed range.

As no method is currently available for the quantification of tutin glycosides in honey, the continued use of an ML based on the level of tutin is necessary. In order to protect consumers from minor adverse effects such as those reported in the pharmacokinetic study, a reduction in the ML by a factor of 3 is proposed, resulting in a revised ML of 0.7 mg/kg for extracted/blended honey.

Assessing the risk for comb honey is not possible because there are insufficient data on the degree of variability of tutin levels across combs. It is conceivable that the tutin level in honey sampled from a specific portion of comb could differ markedly from the tutin level in another part of the comb. Similarly, the tutin level determined for a hive "drip" sample may differ markedly from that in a portion of comb taken from that hive. An ML equivalent to the analytical limit of detection is considered appropriate to minimise the health risk posed by comb honey.

# Abbreviations

<u>Time</u>		<u>Weight</u>	
sec	Second	bw	Bodyweight
min	Minute	ng	Nanogram
h	Hour	µg	Microgram
d	Day	mg	Milligram
wk	Week	kg	Kilogram
mo	Month		
y	Year		
		<u>Dosing</u>	
<u>Length</u>		IP	Intraperitoneal
cm	Centimetre	IV	Intravenous
		SC	Subcutaneous
<u>Volume</u>		mg/kg bw/day	mg/kg bodyweight/day
µL	Microlitre		
mL	Millilitre	<u>Concentration</u>	
L	Litre	M	Molar
		w/w	Weight per weight

<u>Clinical chemistry &amp; haematology</u>	
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CRP	C-reactive protein
Hb	Haemoglobin
Hct	Haematocrit
LDH	Lactate dehydrogenase
RBC	Red Blood Cells (erythrocyte) (count)
WBC	White Blood Cells (leucocyte) (count)
<u>Clinical terminology</u>	
BP	Blood pressure
ECG	Electrocardiogram
bpm	Beats per minute
<u>Other abbreviations</u>	
ARfD	Acute Reference Dose
AUC	Area under the serum/plasma concentration versus time curve
C <sub>max</sub>	Maximum serum concentration
DEA	Dietary Exposure Assessment
FSANZ	Food Standards Australia New Zealand
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LOAEL	Lowest Observed Adverse Effect Level
ML	Maximum Level
NOAEL	No Observed Adverse Effect Level
NZFSA	New Zealand Food Safety Authority
SD	Standard deviation
SEM	Standard error of the mean
T <sub>max</sub>	Time to maximum serum concentration

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# 1. INTRODUCTION

## 1.1 Background

Tutin is a plant-derived neurotoxin which is sometimes found as a natural toxicant in New Zealand honey. Tutin contamination of honey occurs when bees gather honeydew from an insect that feeds on sap of the shrub *Coriaria arborea* (“tutu”). Consumption of tutu honeydew honey can result in serious acute adverse health effects. In 2009, temporary maximum levels (MLs) for tutin in honey and comb honey of 2 mg/kg and 0.1 mg/kg, respectively, were included in the *Australia New Zealand Food Standards Code* (the Code) (Standard 1.4.1 – Contaminants and Natural Toxicants). These MLs are identical to those brought into force in New Zealand in January 2009. They were introduced as a temporary risk management measure in response to an incident in Coromandel, New Zealand when at least 20 people were poisoned following the consumption of comb honey containing tutin.

## 1.2 Risk Assessment Context

FSANZ has not previously conducted a risk assessment of tutin or related substances found as natural toxicants in honey. Therefore the aims of the current assessment are to:

- (i) Evaluate data relevant to the risk assessment of tutin as a natural toxicant in honey,
- (ii) Evaluate relevant data on substances related to tutin that are also present in toxic honey.

## 1.3 Risk Assessment Questions

For this Proposal, the risk assessment questions were developed in the context of the Section 18 Objectives of the *Food Standards Australia New Zealand Act 1991*.

The following risk assessment questions are addressed in this report:

1. Why do some consumers of toxic tutu honeydew honey experience delayed onset of clinical signs and symptoms?
2. Does a delayed onset of clinical signs and symptoms following consumption of tutu honeydew honey have implications for the current tutin ML of 2 mg/kg?
3. Does comb honey containing tutin at the current ML of 0.1 mg/kg pose a health risk?
4. If so, what magnitude of reduction in the ML for tutin in comb honey would be considered effective to minimise the health risk?

# 2. HAZARD ASSESSMENT

## 2.1 Background

Tutin, a plant-derived neurotoxin, is a potential honey contaminant associated with human poisonings in New Zealand since the late 19<sup>th</sup> century (NZFSA 2008; Goodwin 2013). Toxic effects have been reported from the consumption of as little as 5 g of contaminated honey (Palmer-Jones 1947b). A number of deaths have been reported, however fatalities are rare with the most recent occurring in 1917.

The most recent poisoning incident occurred in early 2008, in which there were 11 confirmed,

9 probable and 2 possible cases of intoxication attributed to tutin with the main clinical signs and symptoms being nausea, headache, vomiting and convulsions (NZFSA unpublished data; Beasley 2008; Chancellor 2013). Confirmed cases were defined as those who experienced vomiting or any neurological symptom within 24 hours of eating comb honey, and for which analysis of left over honey confirmed the presence of tutin. Probable cases were those which experienced vomiting and any neurological symptom within 24 hours of eating comb honey, but for which no honey remained for analysis or tutin was not detected in remaining honey. Possible cases experienced vomiting or any neurological symptoms within 24 hours of eating comb honey, but for which no honey remained for analysis or tutin was not detected in remaining honey. Analysis of honey samples associated with the 11 confirmed cases revealed the presence of tutin at levels ranging from approximately 30 to 50 mg per kg honey. Hyenanchin was present at levels approximately 6-times those of tutin (i.e. 180 to 300 mg/kg).

Observations and experiments in the 1940s indicated that the tutu shrub (*Coriaria arborea*) is the source of tutin in honey (Sutherland and Palmer-Jones 1947a; Palmer-Jones 1965), and that tutin contamination of honey occurs when bees gather honeydew from an insect that feeds on tutu sap (Paterson 1947). The insect, a vine hopper (*Scolytopa australis*) native to Australia, is thought to have been introduced into New Zealand before 1870 (Palmer-Jones 1965). A large fraction of the tutin ingested by *S. australis* is metabolised to 4-hydroxy-tutin (“hyenanchin”) which is found in the resulting honey along with tutin (Sutherland and Palmer-Jones, 1947b; Hodges and White 1966; Perry et al 2001). Studies in mice have indicated that hyenanchin is significantly less acutely toxic than tutin (McNaughton and Goodwin 2008).

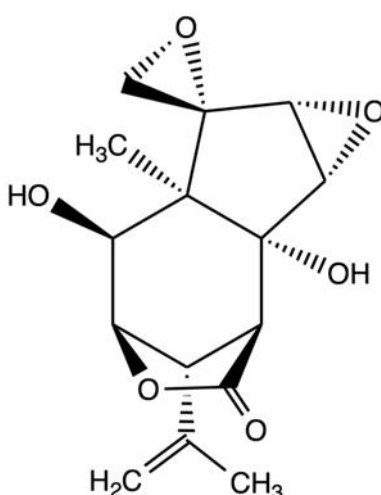
In the late 1970s, analysis of a sample of toxic honey showed the presence of dihydro-derivatives of tutin and hyenanchin, at levels 8-fold lower than those of tutin and hyenanchin (Blunt et al 1979); however, no toxicity information is available on these dihydro-derivatives. Since that publication, no other compounds related to tutin and hyenanchin, or other chemically unrelated potential toxins in tutu honeydew honey have been reported.

There are 8 species of *Coriaria* endemic to New Zealand with *Coriaria arborea* the largest (up to 8 m) and most widespread. It is not known whether the other *Coriaria* species that grow in New Zealand contain tutin and may therefore contribute to tutin contamination of honey. Tutin is present in *Coriaria* species that grow in other parts of the world, for example the Asian *Coriaria nepalensis* (Wei et al 1998) and *Coriaria japonica* (Kinoshita et al 2005), and the South American *Coriaria ruscifolia* (Fuentealba et al 2007). The root of *Coriaria nepalensis*, also known as *Coriaria sinica* Maxim, is used as a Chinese herbal medicine and contains tutin along with the related compounds apotutin, coriamyrtin, coriatin, hydroxycoriatin, coriatone and corianlactone (Wei et al 1998; Shen et al 2004).

## 2.1.1 Assessments by Other Agencies

In 2008, the New Zealand Food Safety Authority (NZFSA) conducted a hazard assessment of tutin and established an Acute Reference Dose (ARfD) of 2.5 µg per kg bodyweight (bw). The ARfD was derived from a mouse toxicity study in which no adverse effects were observed at a dose of 0.25 mg/kg bw (Munday 2008a; see Section 2.2.3 below). A 100-fold uncertainty factor was applied to this no observed adverse effect level (NOAEL) to derive the ARfD (NZFSA 2008).

## 2.1.2 Chemistry

Structural formula:	
Common name:	Tutin
Systematic name:	(7R,8R)-1aβ,1b,5,6,6a,7aβ-Hexahydro-1bα,6β-dihydroxy-6aα-methyl-8-(1-methylethenyl)spiro[2α,5α-methano-7H-oxireno[3,4]cyclopent[1,2-d]oxepine-7,2'-oxiran]-3(2H)-one
Chemical formula:	C <sub>15</sub> H <sub>18</sub> O <sub>6</sub>
Molecular mass:	294.30
CAS number:	2571-22-4

## 2.1.3 Scope of the Hazard Assessment

FSANZ has not previously conducted a hazard assessment of tutin. Therefore, the aims of the current assessment were to:

- Evaluate data relevant to the hazard assessment of tutin;
- Evaluate data relevant to the hazard assessment of substances related to tutin that have been detected in honey.

## 2.2 Evaluation of Data

There are limited published toxicity data for tutin (reviewed by McNaughton and Goodwin, 2008). Several additional studies have been conducted since the above review was conducted and these studies are evaluated here, along with other relevant published information. A human pharmacokinetic study was conducted in 2012 to investigate the basis of the large variability in the onset time of clinical signs and symptoms of toxicity following honey consumption.

### 2.2.1 Biochemistry and Pharmacology

Tutin is a non-competitive antagonist of the GABA<sub>A</sub> receptor (Curtis et al 1973; Hosie et al 1996; Ozoe et al 1998; Olsen 2006). γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS). GABA activation of the GABA<sub>A</sub> receptor, a member of the cysteine-loop ligand-gated ion channels (LGICs), results in inhibitory CNS effects. Inhibition of the GABA<sub>A</sub> receptor by tutin can result in excessive CNS stimulatory effects sometimes resulting in convulsions and death. Tutin and related

compounds such as picrotoxin (Appendix 1) do not compete directly with GABA in binding to the GABA<sub>A</sub> receptor and are therefore termed non-competitive antagonists (Chen et al 2006). Based on the extensive data on picrotoxin, these molecules probably inhibit LGICs by an allosteric mechanism (Hawthorne and Lynch 2005).

In an unpublished study, Lees and Khanh (2009) quantified the activities of tutin, and a mixture of hyenanchin and dihydrohyenanchin, in inhibiting the GABA<sub>A</sub> channel in cultured nerve cells from rat brain. Tutin was the most potent (IC<sub>50</sub> 5.9 μM) followed by hyenanchin<sup>1</sup> (IC<sub>50</sub> 33 μM) and dihydrohyenanchin (IC<sub>50</sub> 41 μM). Each substance was able to completely occlude the GABA-ergic currents. The lowest concentration for complete inhibition for tutin was 32 μM, with values for hyenanchin and dihydrohyenanchin of 64 μM and 100 μM, respectively. At these high concentrations all three compounds evoked epileptiform activity in the electrophysiology experiments. An approximate IC<sub>50</sub> for picrotoxin of 3.4 μM was also determined, indicating approximately 2-fold greater potency than tutin under the conditions of this experiment.

Tutin has also been shown to inhibit glycine receptors in spinal neurons *in vitro* (Fuentealba et al 2007 and 2011). This is not surprising considering that glycine receptors, the main inhibitory receptors in the spinal cord and the brain stem, are closely related to the GABA<sub>A</sub> receptor. Moreover, picrotoxin has been shown to inhibit several members of the cys-loop LGIC superfamily of receptors including GABA, glycine, serotonin type 3 and the nicotinic acetylcholine receptor (Erkkila et al 2008). Structural characterisation of the binding of picrotoxin to a glutamate-gated chloride channel revealed in detail how picrotoxin functions as a channel blocker (Hibbs & Gouaux 2011). It is possible that tutin possesses antagonist activity at other LGICs in addition to the GABA<sub>A</sub> receptor and glycine receptors.

## 2.2.2 Absorption, Distribution, Metabolism and Excretion

No laboratory animal studies are available on the absorption, distribution, metabolism and excretion of tutin. However, based on the time of onset of clinical signs consistent with neurotoxicity (e.g. <15 min in mice, ~1 h in dogs), systemic absorption after oral ingestion of purified tutin appears to be rapid in animals (Fitchett and Malcolm 1909; McNaughton and Goodwin 2008). Also, animals recover rapidly from non-lethal doses of tutin suggesting rapid elimination (McNaughton and Goodwin 2008).

In contrast to the apparent rapid absorption observed following oral administration of purified tutin, the onset time of toxicity following consumption of honey containing tutin as a natural toxicant is highly variable. For example, in guinea pigs fed honey containing tutin, the onset of signs of toxicity was between one and seven hours after ingestion (Palmer-Jones 1947a). In humans, a noteworthy feature of honey poisoning cases is the large variability in the onset time of the first clinical sign or symptom of toxicity. For the 11 confirmed cases in 2008, this onset time ranged from 0.5 h to 17 h post-ingestion, with a median of 7.5 h (NZFSA, unpublished data).

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<sup>1</sup> The hyenanchin test substance was reported to be only 80% pure and contained an unspecified amount of dihydrohyenanchin. The hyenanchin sample was consistently more active when tested at identical concentrations compared to the dihydrohyenanchin sample. The presence of dihydrohyenanchin in the hyenanchin sample indicates that the potency of hyenanchin is slightly underestimated in these experiments.



### *Human pharmacokinetic study*

In order to investigate the basis of the variability in the onset time of adverse effects following honey consumption, a pharmacokinetic study was conducted in which 6 healthy adult males were administered honey containing tutin as a natural toxicant, and the tutin concentration in serum was measured at multiple time points post-dose (Fields et al, submitted for publication).

The test material was homogenised honey containing tutin and hyenanchin at concentrations of 5.1 and 23 mg per kg, respectively, as determined using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method by a laboratory accredited for the regulatory compliance testing of tutin in honey (ASUREQuality, Wellington, New Zealand).

The tutin dose administered in this study (1.8 µg/kg bw) was equal to that received by a high consumer of honey (97.5<sup>th</sup> percentile = 0.9 g honey/kg bw; see Section 3) containing tutin at the current maximum level (ML) permitted in the Code (2 mg/kg honey) (FSANZ 2014). The mass of honey administered ranged from 25.0 to 32.0 g for subjects with the lowest and highest body weight, respectively (71.4 and 89.6 kg). Subject age range was 21-40 y. Prior to dosing, subjects fasted for 8 h then drank a single dose of honey mixed with approximately 100 mL of warm water (approximately 40 °C). The dose was consumed within approximately 1 min, after which time the dose receptacle was rinsed with approximately 15 mL of warm water and the rinse consumed by the subject. Subjects remained fasted until 4 h post-dose.

Serum concentrations of tutin for the sentinel subject (subject 1) were measured using a validated LC-MS/MS method adapted from the method used for analysis of the honey sample. The serum method had a limit of detection (LOD) of 0.4 ng/mL and a limit of quantification (LOQ) of 1.0 ng/mL. Following analysis of serum samples from the sentinel subject, two additional samples (at 36 and 48 h post-dose) were collected for subjects 2 to 6. Since tutin was not detected in serum at the first two time points post-dose for subject 1, the assay method was modified to improve sensitivity. The resulting LOD and LOQ values were reduced to 0.1 and 0.3 ng/mL, respectively. This modified method was used for the analysis of serum from subjects 2 to 6. Results are shown in Table 1 and graphically in Figures 1 and 2.

Pharmacokinetic parameters were calculated using non-compartmental methods in PKSolver, version 2.0 (Zhang et al 2010). The area under the serum concentration–time curve over the sampling time period ( $AUC_{0-t}$ ) and extrapolated to infinite time ( $AUC_{0-\infty}$ ) were determined using the log-linear trapezoidal method. A two-site absorption model was used to estimate the maximum serum concentration ( $C_{max}$ ) and the time to reach  $C_{max}$  (i.e.  $T_{max}$ ) using the mean serum tutin concentration at each time point as the input data. Terminal half-life ( $t_{1/2}$ ) was calculated by linear regression analysis of the last data points ( $\geq 16$  h) after log-transformation of the data using the best-fit method.

The serum concentration versus time profile for the sentinel subject (Subject 1) exhibited a peak concentration of 2.0 ng/mL at consecutive sampling times of 1.5, 2 and 3 h post dose. The serum tutin concentration then decreased gradually to 1.5 ng/mL by 8 h post-dose before increasing to a maximum concentration of 2.7 ng/mL at the final time point (24 h post-dose). Based on these results, additional blood sampling at 36 h and to 48 h post dose was included for subjects 2 to 6.

**Table 1. Serum tutin concentration versus blood sampling time.**

Sampling time (hours post-dose)	Tutin concentration in serum (ng/mL)						
	Subject 1 <sup>†</sup>	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Mean ( $\pm$ SEM)
0	ND	ND	ND	ND	ND	ND	-
0.25	ND	0.7	0.8	0.8	2.0	2.0	1.26 (0.30)
0.5	ND	1.9	2.0	1.7	3.2	2.5	2.26 (0.27)
0.75	1.4	1.8	1.8	2.3	2.8	2.0	2.12 (0.19)
1	1.6	1.9	1.6	2.1	2.1	2.0	1.94 (0.09)
1.5	2.0	1.7	1.7 <sup>^</sup>	2.6	3.0	2.1	2.22 (0.26)
2	2.0	‡	1.3	2.0	2.3	1.8	1.84 (0.21)
3	2.0	1.3	1.4	1.6	2.1	1.4	1.56 (0.14)
4	1.7	1.1	1.5	1.6	2.0	1.4	1.52 (0.15)
6	1.9	1.0	1.9	3.2	3.0	1.4	2.10 (0.43)
8	1.5	0.9	3.0	3.9	5.3	1.7	2.96 (0.78)
12	1.9	1.7	3.9	5.3	4.4	3.3	3.72 (0.60)
16	2.6	3.0	3.5	4.6	3.7	3.3	3.62 (0.27)
24	2.7	2.8	2.3	3.7	2.9	2.7	2.88 (0.23)
36	NA	2.0	1.7	1.5	1.5	0.9	1.52 (0.18)
48	NA	0.7	0.5	0.6	0.5	0.4	0.54 (0.05)

<sup>†</sup> Not included in calculation of the mean.

<sup>^</sup> Actual time of collection for this sample was 103 min post-dose. All other sampling times were within 1 min of the scheduled time.

D Not Detected NA Not Applicable (blood samples not collected)ng/mL.ND Not Detected

<sup>‡</sup> Probable outlier (concentration reported as 17.9 ng/mL). Data point not used in calculations.

NA Not Applicable (36 h and 48 h blood samples were not collected for subject 1, the sentinel subject)

The concentration-time profiles for each of the subjects 2 to 6 indicated two major peaks, with the second, much broader, peak corresponding to the maximum serum concentration ( $C_{max}$ ) determined for each subject (Figure 1).

Figure 1. Plot of serum tutin concentration versus time post-dose for subjects 2 to 6

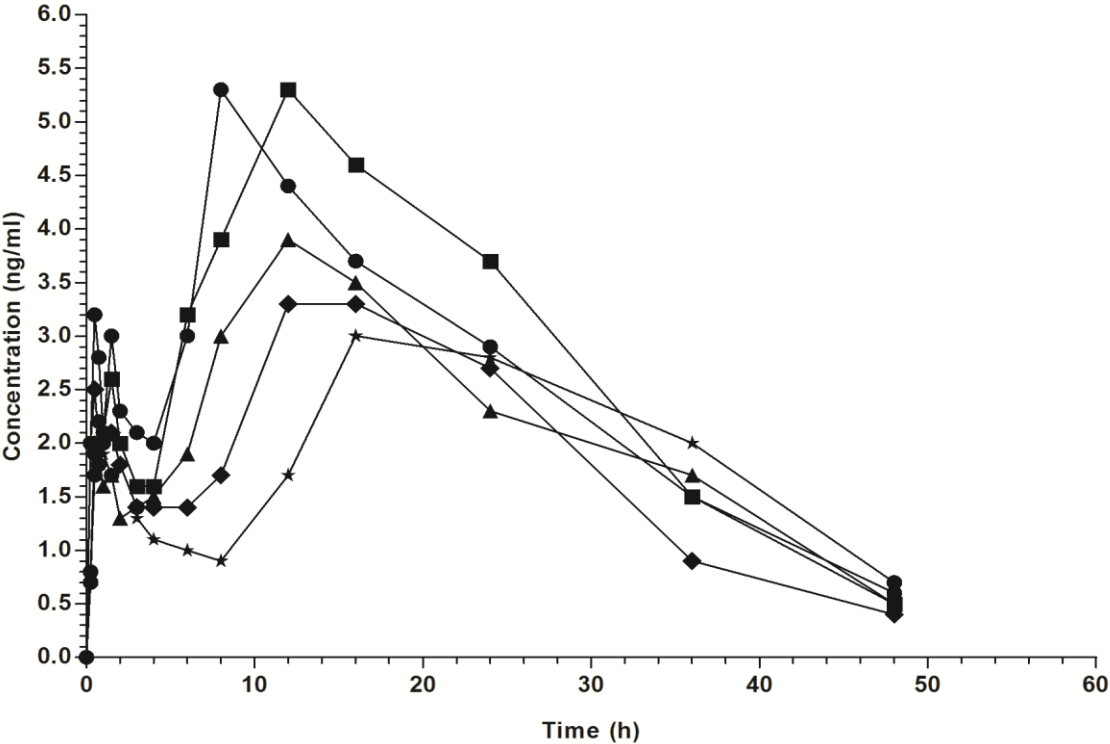
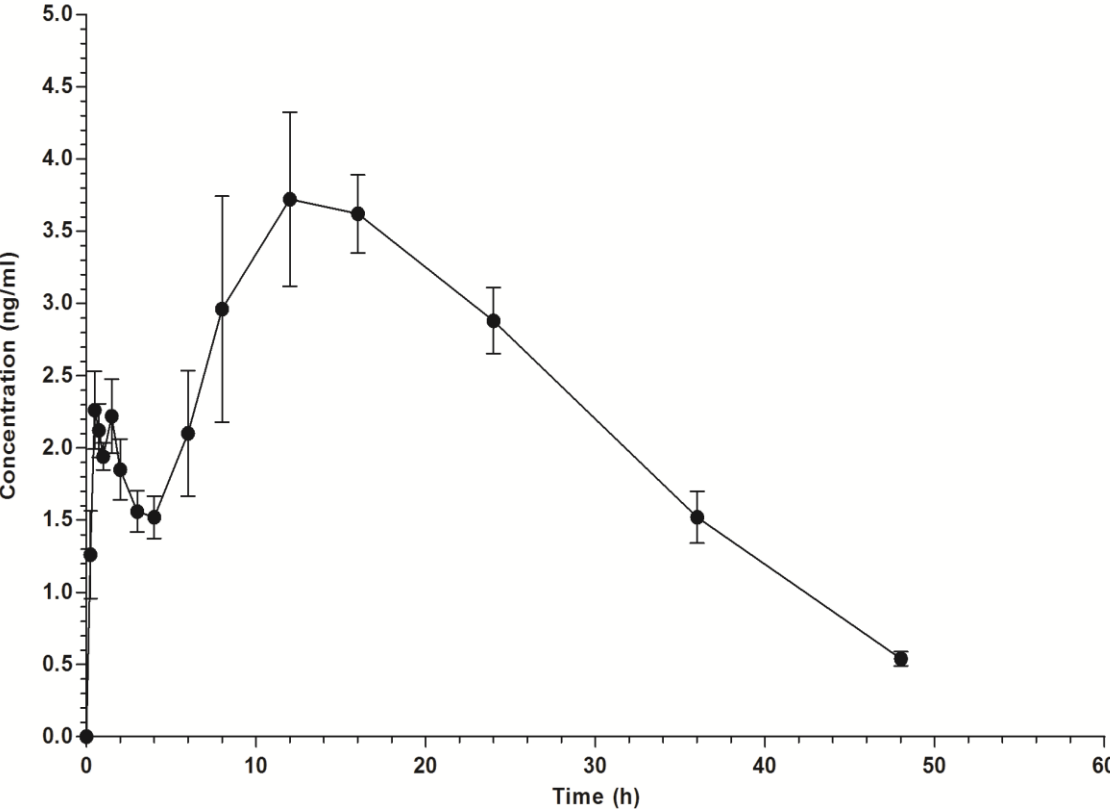
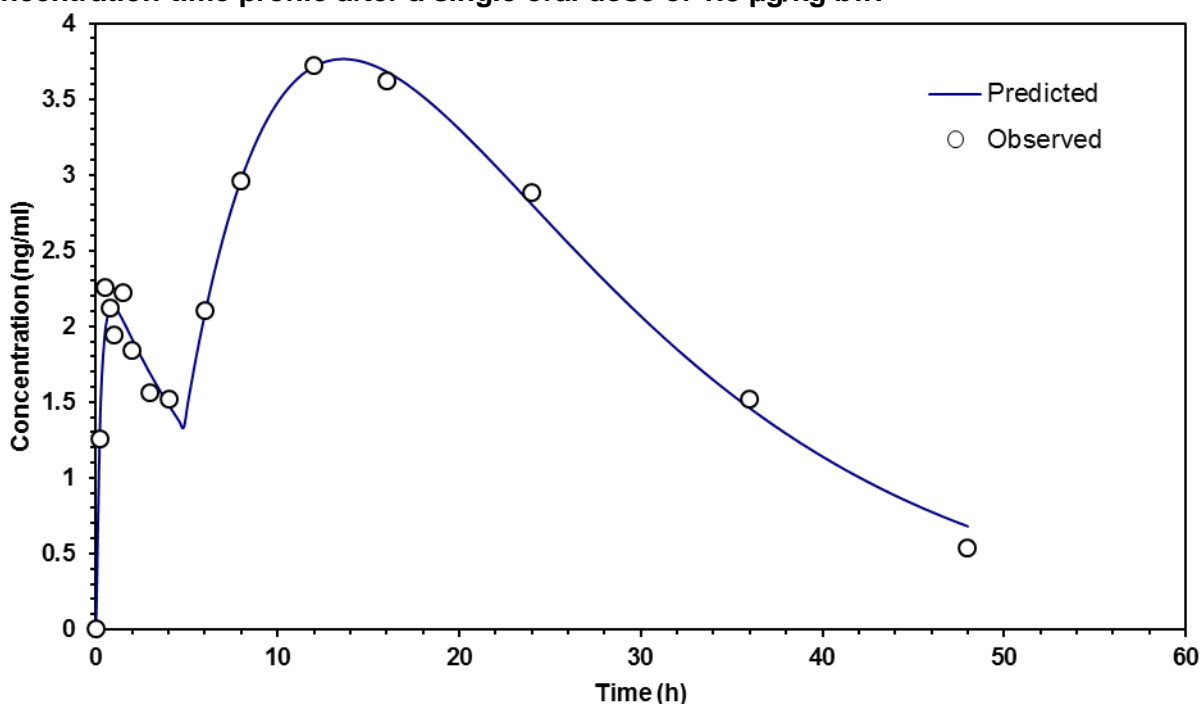


Figure 2. Plot of mean serum tutin concentration ( $\pm$ SEM) versus time



Two-site absorption modelling of the mean tutin serum concentration data resulted in the predicted profile shown in Figure 3 and pharmacokinetic parameters shown in Table 2. As the data for subject 1 contained fewer data points and was generated using an assay of lower sensitivity it was decided to exclude them from the pharmacokinetic calculations. Following a lag time of 4.8 h,  $T_{max}$  for the second peak ( $T_{max2}$ ) occurred approximately 14 hours after  $T_{max}$  for the first peak ( $T_{max1}=0.9$  h). The goodness-of-fit of the two-site absorption model (Figure 3) as visually judged by the concordance of the observed and predicted values is good and the model has a correlation coefficient ( $r^2$ ) of 0.997. The calculated fraction of tutin that experiences delayed absorption ( $f \sim 84\%$ ) estimated by the proposed model is consistent with the observation that the area under the second peak is much larger relative to the first peak.

**Figure 3. Measured mean (open circles) and predicted (solid lines) serum tutin concentration-time profile after a single oral dose of 1.8  $\mu\text{g}/\text{kg}$  bw.**



It was speculated that peak 1 was due to rapid systemic absorption of tutin present in honey while peak 2 was due to tutin released in a delayed and sustained manner from a hitherto unidentified source of additional tutin, such as conjugates of tutin. Subsequent chemical analysis revealed the presence of substantial amounts of various tutin glycosides in the administered honey sample (Appendix 2).

Further analysis of the pharmacokinetic honey sample and 14 other honey samples indicated that (i) four tutin glycosides accounted for the majority of tutin glycosides present in honey, (ii) the ratio of the sum of these four tutin glycosides to tutin aglycone ( $\text{tutin}_{\text{glyc}}:\text{tutin}_{\text{aglyc}}$ ) varied over an approximately 5-fold range, (iii) the ratio showed no apparent association with the tutin aglycone level, (iv) the highest  $\text{tutin}_{\text{glyc}}:\text{tutin}_{\text{aglyc}}$  ratio was for a honey sample associated with poisoning in 2008, (v) this ratio was 1.7-times greater than the ratio for the pharmacokinetic honey sample, and (vi) honey samples that contained no detectable tutin aglycone also contained no detectable tutin glycosides (Appendix 4).

**Table 2. Pharmacokinetic parameters derived from two-site absorption model.**

Parameter	Value	Unit
Frac	0.16	
T <sub>lag</sub>	4.83	h
k <sub>a1</sub>	3.87	1/h
k <sub>a2</sub>	0.07	1/h
k <sub>e</sub>	0.13	1/h
t <sub>1/2</sub> (k <sub>a1</sub> )	0.18	1/h
t <sub>1/2</sub> (k <sub>a2</sub> )	9.80	1/h
t <sub>1/2</sub> (k <sub>e</sub> )	5.40	1/h
T <sub>max1</sub>	0.91	h
C <sub>max1</sub>	2.13	ng/mL
T <sub>max2</sub>	15.2	h
C <sub>max2</sub>	3.73	ng/mL
AUC <sub>0-t</sub>	108	ng/mL•h
AUC <sub>0-∞</sub>	118	ng/mL•h

Frac	fraction of drug absorbed via site 1
T <sub>lag</sub>	time delay of absorption via site 2
k <sub>a</sub>	absorption rate constant
k <sub>a1</sub>	k <sub>a</sub> for site 1
k <sub>a2</sub>	k <sub>a</sub> for site 2
k <sub>e</sub>	elimination rate constant
t <sub>1/2</sub>	half-life
T <sub>max</sub>	time to reach maximum serum concentration
T <sub>max1</sub>	T <sub>max</sub> for peak 1
T <sub>max2</sub>	T <sub>max</sub> for peak 2
C <sub>max</sub>	maximum serum concentration
C <sub>max1</sub>	C <sub>max</sub> for peak 1
C <sub>max2</sub>	C <sub>max</sub> for peak 2
AUC	Area under the serum concentration versus time curve
AUC <sub>0-t</sub>	AUC calculated from time zero to final sampling time
AUC <sub>0-∞</sub>	AUC calculated from time zero to extrapolated infinite time

The double-peak phenomenon provides a plausible explanation for the range of onset times for clinical signs/symptoms of toxicity (0.5 to 17 h) observed in the 2008 poisoning incident. The findings from this pharmacokinetic study, and the subsequent analytical identification of tutin glycosides in honey, provide a plausible explanation for the long delay in the onset of severe adverse effects following consumption of tutu honeydew honey, namely a delayed and sustained release of tutin from glycoconjugates resulting in a large broad peak in the serum concentration versus time profile with T<sub>max2</sub> ≥ 12 h. It is currently not known how or where in the gastrointestinal tract these tutin glycoconjugates are hydrolyzed to release tutin, but preliminary studies suggest that microflora in the gastrointestinal tract play only a very minor role in this process (Appendix 2). Attempts to release tutin from tutin glycosides using acid and enzymatic hydrolysis were unsuccessful (Appendix 3).

It is considered that the various tutin glycoconjugates are likely to produce pharmacokinetic profiles similar to those observed for sustained release drug formulations. The possibility of “flip-flop” kinetics was therefore considered, in which the rate of absorption is the rate limiting step in the sequential processes of drug absorption and elimination (Yáñez et al 2011). Under flip-flop conditions the terminal phase of a concentration-time curve reflects the absorption process and not, as is usually the case, the elimination of the drug. To

convincingly demonstrate flip-flop kinetics, the elimination rate ( $k_e$ ) calculated from intravenous (IV) dosing should ideally be compared with the  $k_e$  following oral dosing. Although there are no kinetic data for tutin or tutin glycoconjugates following IV dosing, visual inspection of Figure 3 suggests that the decline curves following peaks 1 and 2 differ sufficiently to indicate that flip-flop kinetics may be occurring. This contention is supported by the parameters derived from the two-site absorption model which show that  $k_e$  is approximately twice the value of  $k_{a2}$  (Table 2). The prolonged apparent half-life of tutin in serum observed in the pharmacokinetic study is therefore consistent with the slow release of tutin from tutin glycosides in the gastrointestinal tract, not because of intrinsically slow systemic elimination of tutin.

Safety information obtained from this pharmacokinetic study is described in Section 2.2.9.

### **2.2.3 Acute Toxicity**

Published acute toxicity studies are available for a number of animal species using various routes of administration. Results from these studies are shown in Table 3. These studies are of limited value due to the uncertain impurity profile of the administered tutin. Palmer-Jones (1947b) reported an oral (gavage)  $LD_{50}$  for rats of 20 mg/kg. Tutin was more acutely toxic when administered by the subcutaneous (SC) and intraperitoneal (IP) routes which gave  $LD_{50}$  values of approximately 4 and 5 mg/kg bw, respectively. No other oral  $LD_{50}$  data are available. Palmer-Jones (1947b) reported  $LD_{75}$  values for guinea pigs of 1.2 mg/kg bw (gavage) and 0.75 mg/kg bw (SC). For rabbits, minimal lethal doses of 1.25 mg/kg bw (IV), 1.5-2.5 mg/kg bw (SC) and approximately 6 mg/kg bw (oral gavage) were reported.

**Table 3: Acute toxicity of tutin - results from published studies<sup>†</sup>**

Species	Route	Parameter	Value (mg/kg bw)	Reference
Mouse	Intracerebral	LD <sub>50</sub>	0.014	Swallow et al 1989
	Intracerebral	LD <sub>50</sub>	0.01	Clinch 1966
	Subcutaneous	MLD	4	Swanson 1940
	Intraperitoneal	LD <sub>50</sub>	3	Jarboe et al 1968
Rat	Oral, gavage	LD <sub>50</sub>	~20	Palmer-Jones 1947b
	Subcutaneous	LD <sub>50</sub>	~4	
	Intraperitoneal	LD <sub>50</sub>	~5	
Guinea pig	Oral, gavage	LD <sub>75</sub>	1.2	Palmer-Jones 1947b
	Oral, gavage	MLD	>1.5	Fitchett 1908
	Subcutaneous	LD <sub>75</sub>	0.75	Palmer-Jones 1947b
	Subcutaneous	MLD	2	Ford 1910
	Intraperitoneal	LD <sub>50</sub>	0.7	Palmer-Jones 1947b
Rabbit	Subcutaneous	MLD	1.5	Swanson 1940
	Subcutaneous	MLD	2.5	Fitchett 1908
	Subcutaneous	MLD	1.7	Ford 1910
	Intravenous	MLD	1.25	Swanson 1940
	Oral, gavage	MLD	~6	Fitchett 1908
Cat	Subcutaneous	MLD	~0.38	Fitchett 1908
	Oral, gavage	"Lethal dose"	~0.54	Easterfield 1900
Pig	Oral, feeding	"Lethal dose"	~8	Easterfield 1900

<sup>†</sup> This Table was adapted from McNaughton and Goodwin (2008). MLD: Minimum lethal dose; LD<sub>50</sub>: Lethal dose for 50% of the dosed animals; LD<sub>75</sub>: Lethal dose for 75% of the dosed animals.

A series of unpublished acute toxicity studies conducted over the period 2008-2011 are described below.

Munday R (2008a) Approaches toward risk assessment of the honey contaminant, tutin. Unpublished report included as an Appendix in McNaughton and Goodwin (2008) **Lab**: AgResearch, Hamilton, New Zealand. **Date**: April 2008. **GLP**: No.

It was stated that this study was conducted according to OECD Guideline 425: Acute oral toxicity – up and down procedure (OECD 2001). LD<sub>50</sub> values and confidence intervals were calculated with the statistical programme accompanying the guideline. Tutin (purity > 95%) in aqueous solution was administered at various dose levels by gavage to female Swiss albino mice, 6-7 weeks old. Non-fasted mice were allowed free access to food at all times while for fasted mice food was withdrawn at 4 p.m. on the day before dosing, and replaced 10 min after administration of tutin. Mice were observed and weighed each day for 7 days after dosing. Mice dying during the experiment and those killed at its termination were necropsied.

In non-fasted mice, the LD<sub>50</sub> for tutin was 4.7 mg/kg bw, with 95% confidence limits of 3.6 and 6.8 mg/kg bw. At lethal doses, abdominal breathing was noted within 5 min of dosing, and the mice were lethargic. After 15 min, the hind legs of the mice were slightly extended and animals were inactive. After 15-30 min, tremors were noted when the mice moved. These were first apparent in the head, but later progressed to the whole body. The hind legs of the animals were stiff. Tremors were subsequently observed in some animals even when at rest. After between 40 min and 2 h, the mice fell on their side and exhibited

rapid running movements for a few seconds. The hind legs then became fully extended and rigid, and the mice died. No macroscopic lesions were observed at necropsy. At sub-lethal doses, hind leg extension and tremors were observed soon after dosing. These persisted for 2-3 h after which the mice were hunched and lethargic. Clinical signs resolved at 4-5 h after dosing. Mice appeared normal throughout the subsequent one-week observation period and no abnormalities were observed at necropsy.

In fasted mice, the LD<sub>50</sub> was 3.2 mg/kg bw, with 95% confidence limits of 2.4 and 4.6 mg/kg bw. Clinical signs were the same as those in non-fasted mice, and their onset was similarly rapid. At necropsy, small haemorrhages were observed in the glandular stomach of two out of the three mice receiving fatal doses of tutin. No other macroscopic abnormalities were observed in fasted mice.

In order to estimate an acute no observed adverse effect level (NOAEL), mice were dosed with tutin at levels below the LD<sub>50</sub> and their subsequent clinical signs were monitored and compared with control mice dosed with water. Three mice were tested at each dose-level, set at 2, 1, 0.5 and 0.25 mg/kg bw. Non-fasted mice were used in these experiments.

At an oral dose of 2 mg/kg bw, tutin induced obvious toxic effects with mice displaying abdominal breathing, tremors and hind limb rigidity. After 1 h, these mice were less active than controls and were hunched with slight piloerection. Their condition rapidly improved and by 2 h after dosing they were indistinguishable from control animals. At 1 mg/kg bw, lethargy, abdominal breathing, hunching and hind leg stiffness were again observed. Clinical signs resolved 1-1.5 h after dosing. At 0.5 mg/kg bw, slight hunching was observed, and the mice were less active than control animals. These clinical signs had resolved by 1 h. At 0.25 mg/kg bw, no effects were observed and this dose was considered the NOAEL.

The New Zealand Food Safety Authority (NZFSA) established an Acute Reference Dose (ARfD) for tutin of 2.5 µg/kg bw by application of a 100-fold uncertainty factor to this NOAEL (NZFSA 2008).

Munday R (2008b) Acute toxicity of hyenanchin in mice. Unpublished report. <b>Lab:</b> AgResearch, Hamilton, New Zealand. <b>Date:</b> November 2008. <b>GLP:</b> No.
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This study examined the acute oral toxicity of the tutin metabolite hyenanchin and also examined the acute toxicity of tutin administered to non-fasted female Swiss albino mice by the IP route. The LD<sub>50</sub> for tutin (purity > 95%) was 3.0 mg/kg bw (IP). The 95% confidence interval of 2.3 to 4.3 mg/kg bw overlaps with the corresponding oral interval noted above (3.6 to 6.8 mg/kg bw; Munday 2008a). The clinical signs of toxicity observed after IP injection were similar to those observed with orally administered tutin. At lethal doses, abdominal breathing was observed within minutes of dosing and the mice became lethargic. Whole body tremors were noted when the mice were disturbed, and their hind legs became stiff and extended. Death occurred between 40 min and 1.7 h after dosing. At sub-lethal doses, abdominal breathing, piloerection, tremors and hind leg extension were initially observed, but after 2-2.5 h the clinical signs had resolved and mice remained normal throughout the subsequent 2-week observation period. No macroscopic abnormalities were reported in mice that died or those that were killed at the end of the observation period. Organ weights were stated to be within the normal range.

Results for hyenanchin are described in Section 2.2.10.



Munday R (2010) Acute toxicity of tutin and its derivatives found in toxic honey. Unpublished report. (Report no. AGFH 01245) **Lab:** AgResearch, Hamilton, New Zealand. **Date:** December 2010. **GLP:** No.

An acute toxicity study in mice dosed with honey containing tutin and hyenanchin as natural toxicants or with honey spiked with tutin (purity > 95% w/w; Larsen and Sansom 2009) was conducted in order to address the question of whether an unidentified toxic compound or compounds may be present in tutin/hyenanchin-contaminated honey. The spiked honey and the naturally-contaminated honey contained tutin at a concentration of 40 mg/kg. The hyenanchin concentration in honey was not stated. Groups of non-fasted mice received a single oral gavage dose of 400 mg of naturally contaminated honey or spiked honey resulting in a tutin dose of 0.79 mg/kg bw for each group. A control group received 400 mg of uncontaminated honey.

The appearance and behaviour of mice dosed with honey spiked with tutin or with naturally-contaminated honey were normal, and did not differ from those of the control animals. Body weight gains of the test animals during the 14-day observation period did not differ from those of controls and no statistically significant differences ( $p < 0.05$ ) in bodyweight-relative organ weights, weights of stomach contents or blood packed cell volumes were observed between the treatment groups.

## 2.2.4 Short-Term Repeat-Dose Toxicity Studies

Munday (2009) Cumulative toxicity of tutin in mice. Unpublished report. **Lab:** AgResearch, Hamilton, New Zealand. **Date:** October 2009. **GLP:** No.

The same purified tutin sample that was used in the Munday (2010) study, above, was administered daily by gavage at a dose of 1 mg/kg bw to female Swiss albino mice for one to five days. The dose of 1 mg/kg bw is approximately 5-fold lower than the LD<sub>50</sub> and 4-fold greater than the acute NOAEL determined by Munday (2008a). Mice, of initial body weight between 19 and 24 g, were randomly allocated to 7 treatment groups, each containing 5 mice, as follows:

- Group 1: Vehicle control (daily doses of water for 5 days).
- Group 2: One dose of tutin per day for 5 days.
- Group 3: One dose of tutin per day for 4 days.
- Group 4: One dose of tutin per day for 3 days.
- Group 5: One dose of tutin per day for 2 days.
- Group 6: A single dose of tutin.
- Group 7: Untreated control.

During the dosing period, all mice were examined and weighed daily. Mice in Group 1 received 200 µL of water by gavage. Mice in Groups 2-6 were dosed by gavage with tutin solution (0.4 mg/mL in water) at a dose volume of 50 µL per 20 g body weight, made up to 200 µL with water. This equates to a tutin dose of 1 mg/kg bw. Mice in Group 7 were weighed but not dosed. After the period of dosing, mice were weighed each day for 14 days. They were then killed and necropsied. After gross examination, the liver, kidneys, spleen, heart, lungs and brain of each mouse were weighed. Histological examination of the organs was not conducted.

After the first dose of tutin, the mice exhibited increased activity, but after 5-7 min they became lethargic and exhibited abdominal breathing. The mice showed little movement and little response to stimulation over the next hour. Their condition subsequently improved and

by 1.5 h after dosing the clinical signs had resolved and all animals remained normal throughout the day of observation. Control mice gavaged with water showed hyperactivity after dosing, but this resolved within 10 min. The following day, all mice appeared normal, and the body weights of the mice dosed with tutin were not significantly different from those of control mice.

Animals receiving a second dose of tutin showed signs of intoxication similar to those observed after the first dose. There were no perceptible differences in the severity of the effects or in the time to recovery. After recovery, the appearance and behaviour of the animals were normal throughout the day of dosing and on the next day, and there was no effect on body weight.

Mice dosed three times with tutin showed effects of similar severity and duration to those seen after a single dose, and after recovery their appearance and behaviour were normal throughout the day of dosing and on the following day. In contrast, the effects observed in mice dosed with tutin on 4 or 5 consecutive days were reported to be noticeably less than those in animals receiving a single dose or two to three consecutive doses, and recovery occurred within an hour after dosing. Again, the appearance and behaviour of the animals was subsequently normal, and their body weights did not differ from those of control mice.

There were no unscheduled deaths during the study. The appearance and behaviour of the mice during the 14-day observation period after dosing were normal, and there were no significant differences in body weight among the test and control animals at any time point. No abnormalities were observed at necropsy. There were no differences among the treatment groups with regard to relative organ weights.

The observation of decreased signs of toxicity in mice receiving 4 or 5 consecutive doses of tutin suggests that tolerance to tutin may develop after repeated oral exposure.

## 2.2.5 Sub-chronic Toxicity Studies

No data are available.

## 2.2.6 Chronic Toxicity and Carcinogenicity Studies

No data are available.

## 2.2.7 Genotoxicity Studies

Pagnon J, Karunasinghe N, Ferguson LR (2009) Genetic Toxicology Report. Ames bacterial mutagenicity tests for tutin and hyenanchin + dihydrohyenanchin. Unpublished report. **Lab:** Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, University of Auckland, New Zealand. **Date:** 11 September 2009. **GLP:** No.

Tutin was tested for mutagenic activity in bacterial reverse mutation assays (Ames tests) using *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537. Plate incubations were conducted both in the absence and presence of S9 metabolising mixture produced from the livers of Aroclor 1254-induced rats (male Sprague-Dawley). Mutagenicity assays were conducted in triplicate. For all *S. typhimurium* strains, tutin gave no significant increase in the number of revertant colonies compared to background levels both in the absence or presence of S9. The maximum tested concentration of tutin and hyenanchin + dihydrohyenanchin was 86.7 µg/mL, which corresponds to 375 µg per plate for the small plates used (3 cm diameter). This amount equates to 3 mg per plate for a standard 10 cm plate (8-times the volume of a 3 cm plate) which is similar to the recommended maximum of

5 mg per plate for soluble non-cytotoxic compounds (OECD 1997a). No cytotoxicity was observed at any of the tutin concentrations tested. Positive control compounds exhibited an expected degree of mutagenic activity except for 2-aminoanthracene used as a positive control for strain TA102 in the presence of S9.

It is concluded that: (i) results for strain TA102 in the presence of S9 cannot be interpreted because the positive control did not give expected results; and (ii) tutin did not exhibit mutagenic activity for the remaining strains ( $\pm$ S9) or for strain TA102 (-S9).

Ellett S, Zhu W, Han DY, Karunasinghe N, Munday R, Ferguson LR (2011) Genetic toxicology report. Mouse bone marrow micronucleus tests for tutin, hyenanchin +dihydrohyenanchin and pure hyenanchin. Unpublished report. **Lab:** Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, University of Auckland, New Zealand; and AgResearch Ruakura Research Centre, East Street, Hamilton, New Zealand. **Date:** 31 January 2009. **GLP:** No.

A bone marrow micronucleus test in Swiss albino mice was negative for tutin, hyenanchin and a mixture of hyenanchin and dihydrohyenanchin (80:20 w/w). It was stated that the OECD Guideline for the Mammalian Bone Marrow Erythrocyte Micronucleus Test No 474 (OECD 1997b) was the methodology used in this experiment. However, the dose level tested for hyenanchin and hyenanchin:dihydrohyenanchin (15 mg/kg bw twice daily) was much lower than the acute LOAEL of 640 mg/kg bw (Munday 2011; see Section 2.2.10). In order to maximise the probability of inducing micronuclei formation, Guideline 474 recommends doses that are sufficiently high to result in evidence of toxicity.

## 2.2.8 Reproductive and Developmental Toxicity Studies

No data are available.

## 2.2.9 Human Data

### *Isolated tutin*

McNaughton and Goodwin (2008) reviewed several reports on the human toxicity of tutin isolated from the tutu plant. One early paper stated that 'a small [oral] dose, estimated to be about 0.01 grain [ $\sim$ 0.65 mg], caused sickness and incapacity for work extending over 24 hr in a full-grown man' (Easterfield 1900). In a subsequent paper, self-administered oral doses of 1 or 2 mg were reported to have no adverse effects, however a 2 mg dose followed several hours later by an additional 1 mg resulted in increased blood pressure, loss of appetite, "knee jerks", and decreased mental acuity. A single oral dose of 3 mg resulted in elevated BP, nausea, loss of appetite, and impaired coordination (Corban 1929a). Subcutaneous injections of 1-2 mg tutin were reported to have no effects apart from one case in which tremors in the knees and slight twitching of the fingers were observed (Corban 1929b). Muscular twitching was observed after five subcutaneous doses of 0.5 mg/kg, given at hourly intervals, and convulsions occurred in an individual after the third of three doses of 1 mg tutin, given over a 36-hour period (Corban 1929b). A limitation of these reports is the unknown purity/composition of the administered tutin preparations.

### *Poisoning from plants containing tutin*

Human poisoning from the ingestion of tutu (*Coriaria arborea*) berries appears to be rare. Three cases in New Zealand were documented in 2012, however the previous case in the medical literature was over 40 years earlier (Belcher & Morton 2013). Each of the three cases from 2012 reported eating "hundreds" of berries. At around two hours post ingestion (2-HPI) the trio developed nausea. At 5:30-HPI, patient A (26 yo male) had a tonic-clonic convulsion which lasted two minutes and then spontaneously resolved. He was tachycardic

(110 bpm), however neurological exam was unremarkable with normal reflexes and no tremor. He was given diazepam (10 mg oral). At 9-HPI, he had a second 2-min tonic-clonic convulsion and received a second dose of diazepam. Patient B (21 yo female) developed an elevated heart rate (100 bpm), became highly anxious, then at 6:30-HPI had a 2-minute, self-resolving tonic-clonic convulsion. She too was given diazepam (10 mg oral). Patient C (20 yo female) experienced mild nausea and no other adverse effects. An early report indicated that the tutin content of seeds from tutu berries ranged from 0.1 to 0.6% w/w and that there was no evidence of tutin in berries from which seeds were removed (Malcolm 1919). This is consistent with reports that tutu berries are safe to consume if the seeds are removed, as appears to be the case with *Coriaria japonica* berries (Kinoshita et al 2005).

### *Honey poisoning*

Toxic honey was known to occur sporadically in New Zealand dating back to the 19<sup>th</sup> century (Goodwin 2013). Following the 2008 poisoning incident, NZFSA published a discussion paper which included a tabulation of reported cases of toxicity from consumption of New Zealand honey (NZFSA 2008). At least 25 separate poisoning incidents have occurred since the late 19<sup>th</sup> century with more than 180 individuals affected resulting in over 40 hospitalisations and four deaths.

There have been several reviews on toxic New Zealand honey (Palmer-Jones 1965; Love 1990, Sutherland 1992, Goodwin 2013). Palmer-Jones (1965) stated that some people have been severely affected by as little as 5 g of honey, however the concentrations of tutin and hyenanchin in such honey samples are not known. Conversely, in cases where the concentrations of tutin and hyenanchin in consumed honey were measured, albeit using the relatively insensitive methods available at the time (thin layer chromatography and animal bioassays), the amount of honey consumed was not reported. It was therefore concluded by Palmer-Jones that the '*exact level of hyenanchin and tutin which affects humans cannot yet be determined accurately*'.

A review published in 1990 included a discussion of improved analytical methods for the measurement of tutin and hyenanchin in honey, namely those utilising gas chromatography (GC) and high performance liquid chromatography (HPLC) (Love 1990). A GC method published in 1980 was used to analyse four honey samples responsible for human poisonings (Swallow et al 1980). Tutin concentrations ranged from 34 to 95 mg/kg and hyenanchin concentrations ranged from 63 to 170 mg/kg. Love (1990) stated that the HPLC method was more convenient and reliable than the GC method, but that it '*would have to be further developed if levels of toxin below about 2 mg/kg were considered of significant concern*'.

The most recent poisoning incident occurred in early 2008, in which there were 11 confirmed and 9 probable cases of intoxication attributed to tutin with the main clinical signs and symptoms being nausea, headache, vomiting and convulsions (NZFSA 2008 and unpublished data; Beasley 2008; Chancellor 2013). All of the cases of intoxication were associated with the consumption of comb honey produced by one supplier. Confirmed cases were defined as those who experienced vomiting or any neurological symptom within 24 hours of eating comb honey, and for which analysis of left over honey confirmed the presence of tutin. Probable cases were those which experienced vomiting and any neurological symptom within 24 hours of eating comb honey, but for which no honey remained for analysis or tutin was not detected in remaining honey. Analysis of honey samples associated with the 11 confirmed cases revealed the presence of tutin at levels ranging from 29 to 49 mg per kg honey. Hyenanchin was present at levels approximately 6-times those of tutin (i.e. 180 to 300 mg/kg).

Case details from the 2008 incident were provided by NZFSA (unpublished data). Clinical

signs and symptoms for confirmed cases included nausea, vomiting, headache, dizziness, delirium, convulsions, and in one case, coma. There was little information recorded on the severity of particular signs and symptoms in affected individuals. For the 11 confirmed cases (7 male) reported amounts of honey consumed ranged from one teaspoon to *'four pieces of toast smeared thickly with honey'*. Ten of the 11 confirmed cases were adults ranging in age from 32 to 74 y) with the remaining confirmed case a 3 year old boy. The 9 probable cases (7 male) comprised 8 adults (34-76 y) and a boy aged 12 years.

The onset of toxicity for confirmed cases, as indicated by the first clinical sign or symptom evident following honey consumption, was highly variable, ranging from 0.5 to 17 h after honey consumption, with a mean of 7.3 h and median of 7.5 h. Vomiting, which was reported for 8 of the 11 confirmed cases, showed relatively low variability in onset time (4 to 9 h after consumption; mean 6.8 h; median 7.5 h). Six of the confirmed cases reported headache, however no information on severity or duration was recorded. Time of headache onset was recorded for one case (headache at 3 h and again at 16 h post-consumption). "Giddiness" was reported by 6 of the confirmed cases with onset time ranging from 0.5 to 24 h post-consumption. Delirium was reported for 4 of the confirmed cases with an onset time of 7 to 24 h post consumption. Four of the confirmed cases experiences one or more convulsions of duration reported as follows: case 1: 3 min; case 2: 5 min; case 3: 4 min and 1.5 min (two separate convulsions); and case 4: 30 sec, 3 min and 1 min (3 separate convulsions). There was minimal information on the duration of other signs and symptoms.

#### *Human pharmacokinetic study with honey containing tutin*

Background information, methods and pharmacokinetic results from this study are described in Section 2.2.2. Safety parameters investigated in this study were vital signs (body temperature, heart rate, blood pressure), laboratory values for haematology, biochemistry and complete urinalysis, electrocardiograms (ECG), and adverse events (AEs). The following haematology and biochemistry parameters were assessed: haemoglobin, haematocrit, red blood cell count, white blood cell count, neutrophils, basophils, eosinophils, lymphocytes, monocytes, platelets, sodium, potassium, chloride, bicarbonate, urea, creatinine, glucose, calcium, phosphate, total protein, albumin, bilirubin (total and direct), alkaline phosphatase, gamma-glutamyl transpeptidase, aspartate aminotransferase and alanine aminotransferase.

There were no clinically significant findings for haematology, clinical chemistry, urinalysis and ECG parameters, nor were there any clinically significant conditions or changes from baseline noted on physical examinations. No serious adverse events were observed in the study. Two subjects each reported two adverse events; these were mild in severity, resolved within 2 h and were not associated with any sequelae (Table 4). Subject 5 reported the onset of mild light-headedness 103 min post dose. No associated symptoms were reported, in particular there was no vertigo, no postural component to symptoms, and no features to indicate a vasovagal basis for the event. Heart rate and blood pressure recordings, performed while symptomatic, were unremarkable. The event resolved without intervention at approximately 123 min post dose (event duration 20 min, maximum intensity = mild). Subject 5 also reported the onset of bi-temporal headache approximately 6 h post dose. No associated clinical signs or symptoms were present. One gram of paracetamol was administered orally, with complete resolution of headache by 8 h post dose (event duration 2 h, maximum intensity = mild). Subject 6 reported the onset of mild light-headedness 79 min post dose. This was described by the subject as feeling 'very slightly light-headed' and 'spacey'. The symptoms were intermittent and predominantly present when the subject was having blood collected from his cannula. No associated symptoms were reported, nor were there features to indicate a vasovagal response or postural basis for the symptoms. Heart rate and blood pressure, recorded during the event, were unremarkable. The event resolved without intervention approximately 174 min post dose (event duration 95 min, maximum intensity = mild). Subject 6 later reported the onset of bi-temporal headache approximately

12 h post dose. No associated symptoms were present. The headache resolved without intervention approximately 45 min later (maximum intensity = mild).

Initially it was considered likely that the mild transient light-headedness and headaches reported by subjects 5 and 6 were unrelated to tutin exposure. However, the concordance of time to headache onset with respective tutin  $T_{max2}$  levels (i.e. 6 h onset with a  $T_{max2} \sim 8$  h; 12 h onset with  $T_{max2} \sim 14$  h) for subjects 5 and 6 respectively, suggested that the headaches may be related to treatment (Table 4). A similar argument for light-headedness could be proposed for subjects 5 and 6 on the basis that their serum tutin concentrations at  $T_{max1}$  were the two highest reported in this study. Given that headache and light-headedness are symptoms known to be associated with tutin toxicity a causal relationship is plausible.

**Table 4: Adverse events,  $C_{max}$  and  $T_{max}$  values for tutin in serum**

Adverse Event	Subject 5	Subject 6
Light-headedness		
Time of onset (h:min post-dose)	1:43	1:19
Duration (h:min)	0:20	1:35
Maximum intensity	Mild	Mild
$C_{max1}$ (ng/mL)	3.2	2.5
$T_{max1}$ (h)	0:30	0:30
Headache		
Time of onset (h:min post-dose)	6:00	12:00
Duration (h:min)	2:00	0:45
Maximum intensity	Mild	Mild
$C_{max2}$ (ng/mL)	5.3	3.3
$T_{max2}$ (h)	8:00	14:00

## 2.2.10 Hyenanchin

Hyenanchin differs from tutin only by the presence of an additional hydroxyl group (Appendix 1). Hyenanchin, or 4-hydroxytutin<sup>2</sup>, was also detected in honey samples associated with poisoning in the 2008 New Zealand incident (NZFSA 2008). The levels of hyenanchin in these honey samples ranged from 180 to 300 mg/kg which are 6-fold higher, on average, than the tutin levels (30 – 50 mg/kg honey). Subsequent analysis of 15 honey samples covering a large range of tutin levels (0.13 to 52 mg/kg honey) resulted in hyenanchin:tutin ratios ranging from 3 to 13 (Appendix 4). It is reported that hyenanchin is not present in the tutu plant, but is formed as a metabolite of tutin by the vine hopper *Scolypopa australis* (Palmer-Jones and White 1949). Hyenanchin was first isolated from the southern African plant *Hyaenanche globosa*. Tutin has also been isolated from *H. globosa* (Momtaz et al 2010).

Prior to the 2008 honey poisoning incident there were inadequate data to enable an assessment of the relative acute oral toxicities of tutin and hyenanchin in animals. The only oral toxicity data located for hyenanchin were from acute toxicity studies that reported  $LD_{50}$  values of 40-90 mg/kg for rats and 12 mg/kg for guinea pigs (Palmer-Jones 1947b). The same paper reported an oral  $LD_{50}$  for tutin in rats of approximately 20 mg/kg, (i.e. 2- to approximately 4-fold lower than the value for hyenanchin). For guinea pigs, the only other

<sup>2</sup> Other numbering systems give 8-hydroxytutin or 9-hydroxytutin (Sutherland 1992).

species tested for acute oral toxicity, the LD<sub>75</sub> was 1.2 mg/kg bw (the LD<sub>50</sub> was not reported). For non-oral routes of administration, published reports suggest that the acute toxicity of hyenanchin is 5- to 10-fold lower than that of tutin.

Munday R (2008b) Acute toxicity of hyenanchin in mice. Unpublished report. Report no. DST 89076 **Lab:** AgResearch, Hamilton, New Zealand. **Date:** November 2008. **GLP:** No.

An acute oral toxicity study of hyenanchin in mice was commissioned by NZFSA in 2008 following the honey poisoning incident (Munday 2008b). Hyenanchin, isolated from *Hyaenanche globosa* and purified<sup>3</sup>, was administered as an aqueous solution by gavage to fasted female Swiss albino mice, 6-7 weeks old, of initial body weight 18-22 g. The dose levels were 50, 100, 200 and 320 mg/kg bw. The number of mice dosed was only reported for the high dose group (n = 3). No effects due to hyenanchin were reported at any of the doses during the day of dosing or during the subsequent two-week observation period. It was stated that the mice rapidly regained the weight lost during the overnight fast and continued to gain weight over the observation period. At necropsy, it was stated that no abnormalities were detected, and that weights of liver, spleen, heart, lungs, intestine and brain were in the normal range.

The acute toxicity of hyenanchin administered to mice by the IP route was also investigated. No effects were observed at doses ≤ 30 mg/kg bw<sup>4</sup>. At doses of 120, 159, 200 and 250 mg/kg bw, abdominal breathing was observed soon after dosing and the mice became lethargic, with stiffness evident in the hind legs. Clinical signs resolved 1.5-2 h post-injection. At 300 mg/kg bw<sup>5</sup>, the mice were lethargic and abdominal breathing and tremors occurred soon after dosing. The mice returned to normal 3-3.5 h post-dose and remained normal throughout the subsequent observation period. Thus, in contrast to tutin, hyenanchin appears to be substantially more acutely toxic by the IP route relative to the oral route. It is possible that, for the oral route, hyenanchin is subject to appreciable first pass metabolism, potentially arising from participation of the additional hydroxyl group in phase II metabolic reactions.

Munday R (2011) Acute toxicity to mice of an hyenanchin-dihydrohyenanchin mixture in mice. Unpublished report. **Lab:** AgResearch, Hamilton, New Zealand. **Date:** February 2011. **GLP:** No.

A subsequent oral acute toxicity study in mice with an 80:20 w/w mixture of hyenanchin and dihydrohyenanchin resulted in no deaths at doses of 320, 640, 900, 1280 or 2000 mg/kg bw, the highest dose tested. No effects were observed at a dose of 320 mg/kg bw. At 640, 900 and 1280 mg/kg bw, abdominal breathing and lethargy were observed soon after dosing. After 3-3.5 h these mice showed normal appearance and behaviour which persisted throughout the remainder of the 14-day observation period. At 2000 mg/kg bw, immobility was observed 10-20 min after dosing and respiratory rate was decreased (90-110 breaths/minute *cf* the normal rate of ~130 breaths/minute). Respiration rates normalised within 1-1.5 h, and the mice showed normal appearance and behaviour after 2.5-3.5 h. The body weights of all the mice increased during the observation period, and their appearance and behaviour remained normal during this time. No abnormalities were detected at necropsy, and the bodyweight-relative weights of examined organs (liver kidneys, spleen, heart, lungs) were within the normal range.

<sup>3</sup> It was stated that semi-purified hyenanchin (~84%) was further purified, however the final purity was not stated.

<sup>4</sup> All doses tested were not presented in the report.

<sup>5</sup> The number of mice dosed was only reported for the 300 mg/kg bw group (n = 3).

## Genotoxicity

Pagnon J, Karunasinghe N, Ferguson LR (2009) Genetic Toxicology Report. Ames bacterial mutagenicity tests for tutin and hyenanchin + dihydrohyenanchin. Unpublished report. **Lab:** Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, University of Auckland, New Zealand. **Date:** 11 September 2009. **GLP:** No.

A mixture of hyenanchin and dihydrohyenanchin, was tested for mutagenic activity in bacterial reverse mutation assays (Ames tests) using *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537. Plate incubations were conducted both in the absence and presence of S9 metabolising mixture produced from the livers of Aroclor 1254-induced rats (male Sprague-Dawley). Mutagenicity assays were conducted in triplicate. The maximum tested tutin concentration was 86.7 µg/mL, which corresponds to 375 µg per plate for the small plates used (3 cm diameter). This amount equates to 3 mg per plate for a standard 10 cm plate (8-times the volume of a 3 cm plate) which is similar to the recommended maximum of 5 mg per plate for soluble non-cytotoxic compounds (OECD 1997a). No cytotoxicity was observed at any of the concentrations tested. Positive control compounds exhibited an expected degree of mutagenic activity except for 2-aminoanthracene which was used as a positive control for strain TA102 in the presence of S9.

The hyenanchin + dihydrohyenanchin mixture showed no increase in the number of revertant colonies compared to the background levels for strains TA98, TA102, TA1535 and TA1537, both in the absence and presence of S9. For strain TA100, and only at the maximum concentration tested, a greater than two-fold increase in the number of revertant colonies was observed, both in the absence (2.7-fold increase) and presence (2.2-fold increase) of S9. The assay was repeated and again a greater than two-fold increase in the number of revertant colonies was observed, also only at the maximum concentration (-S9: 2.2-fold; +S9: 2.3-fold). However, there was large variation in the triplicate analyses and the observed increases may therefore not be statistically significant (analysis of statistical significance was not performed). In addition, the positive controls sodium azide and 2-aminoanthracene gave revertant colony counts which were 6- to 7-fold greater than those observed for hyenanchin + dihydrohyenanchin at the maximum concentration.

It is concluded that: (i) the hyenanchin + dihydrohyenanchin mixture did not exhibit mutagenic activity in *S. typhimurium* strains TA98, TA1535 and TA1537 ( $\pm$ S9) or for strain TA102 (-S9); and (ii) the increases in revertant colony counts for hyenanchin + dihydrohyenanchin observed for *S. typhimurium* strain TA100 may not be statistically significant.

## 2.3 Discussion

*In vitro* and animal toxicity studies using purified tutin and hyenanchin became available within 3 years after the 2008 honey poisoning incident in New Zealand. The findings from these studies are summarised above. Studies from the 1940s, in which the composition/purity of the administered tutin and hyenanchin preparations was unknown, indicated that tutin was about 10-fold more acutely toxic than hyenanchin based on oral LD<sub>50</sub> values in guinea pigs, while in rats this ratio was approximately 2-4-fold. However, in recent mouse studies using well characterised, purified substances, the lower oral toxicity of hyenanchin is more pronounced with no deaths or clinical signs observed at hyenanchin doses 100 times the tutin LD<sub>50</sub>.

In an oral acute toxicity study using purified tutin the LD<sub>50</sub> in non-fasted mice was 4.7 mg/kg bw, while in fasted mice the LD<sub>50</sub> was somewhat lower at 3.2 mg/kg bw. Non-fasted mice receiving a tutin dose of 0.25 mg/kg bw (i.e. approximately 20-fold lower than the



LD<sub>50</sub>) showed no clinical signs of toxicity. This dose was considered an acute no observed adverse effect level (NOAEL). The lowest observed adverse effect level (LOAEL) in non-fasted mice was 0.5 mg/kg bw. The New Zealand Food Safety Authority (NZFSA) established an Acute Reference Dose (ARfD) for tutin of 2.5 µg/kg bw from the NOAEL of 0.25 mg/kg bw with application of a 100-fold uncertainty factor (NZFSA 2008).

Information on tutin toxicity following repeated dosing was lacking prior to the 2008 incident. In a subsequent study, decreased signs of toxicity were observed in mice administered gavage doses of 1 mg/kg bodyweight/day for 4 or 5 consecutive days compared to those receiving a single dose, or two to three consecutive daily doses, suggesting that tolerance to tutin may develop after repeated oral exposure. For humans, there is no available evidence indicating that chronic exposure to tutin from honey consumption causes adverse effects other than those consistent with repeated acute toxicity.

An acute toxicity study in mice gavaged with honey containing tutin and hyenanchin as natural toxicants, or with uncontaminated honey spiked with tutin, resulted in no observed adverse effects, despite a tutin dose (0.79 mg/kg bodyweight) that was greater than the LOAEL of 0.5 mg/kg bodyweight obtained in the study above using purified tutin dissolved in water. It is possible that the rate of tutin systemic absorption is decreased when administered in a honey matrix. This would result in a lower maximum blood concentration ( $C_{max}$ ) compared to that arising from administration of an aqueous solution of tutin. Because the acute central nervous effects of tutin are likely to be related to  $C_{max}$  rather than the area under the concentration versus time curve (AUC), a reduction in  $C_{max}$  would result in reduced acute toxicity.

An *in vitro* mutagenicity study on tutin was negative while hyenanchin gave indications of weak mutagenicity in one of the five bacterial strains tested, however there was large variability in the data and the observed differences may not be statistically significant. A chromosomal aberration study in mice was negative for tutin, hyenanchin and a mixture of hyenanchin and dihydrohyenanchin.

Information available from the animal studies summarised above did not provide an understanding of the highly variable onset time of clinical signs and symptoms of toxicity in humans following honey consumption. In the 2008 poisoning incident the reported onset time of toxicity for the 11 confirmed cases ranged from 0.5 to 17 h with a median of 7.5 h. To investigate the basis of this variability, a pharmacokinetic study was conducted in 2012 in which 6 healthy male volunteers each received a single oral dose of honey containing tutin as a natural toxicant. The tutin dose received by the volunteers (1.8 µg/kg body weight) was equivalent to that received by a high consumer of honey (97.5<sup>th</sup> percentile: 0.9 g honey/kg bodyweight) that contains tutin at the current ML of 2 mg/kg.

The serum concentration versus time curve for all volunteers exhibited two discrete peaks, with the first at 0.5 to 1.5 hours post-dose, and the second and higher serum level occurring at 8 to 16 hours post-dose. Transient mild light-headedness was reported by two out of 6 subjects at a time post-dose corresponding to peak 1, while transient mild headache was reported by the same two subjects at approximately peak 2. No other adverse effects were observed in the study, which included monitoring of blood pressure, heart rate, electrocardiogram, clinical chemistry and haematology. There were no associated clinical signs or symptoms typical of tutin intoxication such as nausea, vomiting, dizziness or convulsions. There would be considerable uncertainty in extrapolating the symptoms reported in this study to potential adverse effects in an entire population. Considering that a third of the test population in the small scale study were affected it is likely that more sensitive individuals would be present in the population and could experience more severe effects (e.g. nausea, vomiting, dizziness).

It was speculated that peak 1 was due to rapid systemic absorption of tutin while peak 2 was due to tutin released in a delayed and sustained manner from a hitherto unidentified source of additional tutin, such as conjugates of tutin. Subsequent chemical analysis revealed the presence of substantial amounts of various tutin glycosides in the administered honey sample.

Further analysis of the pharmacokinetic honey sample and 14 other honey samples indicated that (i) four tutin glycosides accounted for the majority of tutin glycosides present in honey, (ii) the ratio of the sum of these four tutin glycosides to tutin aglycone ( $\text{tutin}_{\text{glyc}}:\text{tutin}_{\text{aglyc}}$ ) varied over an approximately 5-fold range, (iii) the ratio showed no apparent association with the tutin aglycone level, (iv) the highest  $\text{tutin}_{\text{glyc}}:\text{tutin}_{\text{aglyc}}$  ratio was for a honey sample associated with poisoning in 2008, (v) this ratio was 1.7-times greater than the ratio for the pharmacokinetic honey sample, and (vi) honey samples that contained no detectable tutin aglycone also contained no detectable tutin glycosides. As there are no analytical standards for the individual tutin glycosides identified in honey, the  $\text{tutin}_{\text{glyc}}:\text{tutin}_{\text{aglyc}}$  ratios determined for these honey samples are semi-quantitative only.

If the levels of tutin glycosides in the honey administered in the pharmacokinetic study were quantifiable it would be possible to set an Acute Reference Dose (ARfD), expressed as total tutin equivalents. However no direct or indirect method is currently available for the quantification of tutin glycosides in honey, therefore the continued use of an ML based on the level of tutin aglycone is necessary. The administered tutin dose in the pharmacokinetic study was based on the tutin ML, and all of this unconjugated tutin is expected to be present in the first peak in the pharmacokinetic profile. This peak tutin concentration, resulting from the absorption of tutin aglycone present in honey, was associated with mild, transient light-headedness in 2 out of 6 subjects. This tutin aglycone dose is therefore considered to be close to a NOAEL and resulted in a modelled  $C_{\text{max}1}$  of 2.1 ng/mL (Table 2), while the delayed  $C_{\text{max}2}$  of 3.7 ng/mL resulted from the release of tutin from tutin glycosides and was associated with mild, transient headache in the same 2 subjects.

In order to protect consumers from minor adverse effects such as those reported in the pharmacokinetic study, a reduction in the ML by a factor of 3 is proposed. This reduction factor is comprised of a factor of 1.5 to account for the relatively small inter-individual variability in maximum serum levels in the pharmacokinetic study, multiplied by a factor of 2 to account for use of the tutin aglycone level as a surrogate for total tutin equivalents (i.e. tutin + tutin glycosides). The factor of 1.5 is a chemical specific adjustment factor for human variability in toxicokinetics ( $\text{HK}_{\text{AF}}$ ) (IPCS 2005). This was derived from the variability in  $C_{\text{max}2}$  values observed in this study. Based on the pharmacokinetic data in Table 1, the standard error (standard deviation of the sample divided by the square root of the sample size) of  $C_{\text{max}2}$  is only 12% of the mean and is therefore acceptable as a measure of the central tendency. The  $\text{HK}_{\text{AF}}$  can therefore be calculated as 1.5 (mean plus two standard deviations divided by the mean =  $[4.2 + 2(1.1)] / 4.2 = 6.4 / 4.2 = 1.5$ ). The factor of 2 for honey variability is obtained by rounding up the value of 1.7 quoted above for the relative ratios of  $\text{tutin}_{\text{glyc}}:\text{tutin}_{\text{aglyc}}$  for the honey with the highest observed ratio and the honey administered in the pharmacokinetic study.

Application of an additional uncertainty factor to account for the possibility of accumulation of tutin following repeated (e.g. daily) honey consumption was considered unnecessary because the prolonged apparent half-life of tutin in serum observed in the pharmacokinetic study is consistent with the slow release of tutin from tutin glycosides in the gastrointestinal tract, not because of intrinsically slow systemic elimination (“flip-flip” pharmacokinetics).

## 2.4 Conclusions

Results from studies in mice with purified tutin did not provide any information about the

highly variable onset time of clinical signs and symptoms of toxicity in humans following tutu honeydew honey consumption. The results of a pharmacokinetic study in humans with tutu honeydew honey led to the conclusion that, in addition to tutin aglycone, the honey contained one or more tutin derivatives capable of undergoing conversion to tutin following ingestion. It was reasoned that tutin aglycone present in honey was rapidly absorbed following ingestion (peak concentrations 0.5 to 1.5 hours post-dose) followed by delayed absorption of tutin liberated from tutin derivatives (e.g. conjugates) several hours later. Subsequent studies confirmed the presence of several tutin glycosides at relatively high levels which provided a plausible explanation for the large delayed peak in the pharmacokinetic profile and the delayed onset of tonic-clonic convulsions in some individuals who consumed tutu honeydew comb honey in the 2008 incident.

No direct or indirect method is currently available for the quantification of tutin glycosides in honey, therefore the continued use of an ML based on the level of tutin aglycone is necessary. In order to protect consumers from minor adverse effects such as those reported in the pharmacokinetic study, a reduction in the ML by a factor of 3 is proposed, resulting in a revised ML of 0.7 mg/kg.

### **3. DIETARY EXPOSURE ASSESSMENT**

Dietary exposure assessments (DEAs) require data on concentrations of the chemical of interest in food and food consumption data. No dietary exposure modelling was possible for this risk assessment because quantitative concentration data are not available for tutin glycosides in honey, and, as indicated by the results of the human pharmacokinetic study, tutin glycosides are the major source of systemic exposure to tutin following consumption of honey.

As discussed in Section 2.2.2, data on honey consumption was used along with the current tutin ML of 2 mg/kg to select the tutin dose administered in the human pharmacokinetic study. The relevant honey consumption data was obtained from the 1997 New Zealand National Nutrition Survey which indicated that 97.5<sup>th</sup> percentile honey consumption for consumers aged 15 years and above was 0.9 g per kg bodyweight per day. Survey data from 2002 indicate that New Zealand children (5 to 8 years of age) who are high consumers (97.5<sup>th</sup>-percentile) of honey consume less honey than high consuming adults on an absolute basis (46 g/day compared to 66 g/day) but more on a bodyweight relative basis (2.2 g versus 0.9 g per kg bodyweight per day).

### **4. RISK CHARACTERISATION**

Based on the results of the human pharmacokinetic study, it is considered possible that adverse effects such as mild light-headedness and headache may be experienced following the consumption of honey containing tutin at the current ML of 2 mg/kg. Such adverse effects are more likely if a large amount of honey ( $\geq 0.9$  g of honey per kg bodyweight) is consumed in one sitting, as was the case in the pharmacokinetic study. The risk of adverse effects is increased if the ingested honey has a ratio of  $\text{tutin}_{\text{glyc}}:\text{tutin}_{\text{aglyc}}$  at the high end of the observed range.

As no method is currently available for the quantification of tutin glycosides in honey, the continued use of an ML based on the level of tutin aglycone is necessary. In order to protect consumers from minor adverse effects such as those reported in the pharmacokinetic study, a reduction in the ML by a factor of 3 is proposed. This reduction factor is comprised of a factor of 1.5 to account for the relatively small inter-individual variability observed in the pharmacokinetic study, multiplied by a factor of 2 to account for use of the tutin aglycone level as a surrogate for total tutin equivalents (i.e. tutin aglycone + tutin glycosides). This

factor of 2 is obtained by rounding up the value of 1.7 quoted above for the relative ratios of  $\text{tutin}_{\text{glyc}} : \text{tutin}_{\text{aglyc}}$  for the honey with the highest observed ratio and the honey administered in the pharmacokinetic study.

A risk characterisation for comb honey is not possible because there are insufficient suitable data on the variability of tutin levels across combs. It is conceivable that the tutin level in honey sampled from a specific portion of comb could differ markedly from the tutin level in another part of the comb. Similarly, the tutin level determined for a hive “drip” sample may differ markedly from that determined in a portion of comb taken from that hive.

## 5. RISK ASSESSMENT CONCLUSIONS

This assessment evaluated data relevant to the risk of consuming honey containing tutin and related substances. Specific consideration was given to the risk posed by the consumption of honey and comb honey containing tutin at the current MLs of 2 and 0.1 mg/kg, respectively.

### 5.1 Responses to Risk Assessment Questions

1. *Why do some consumers of toxic honeydew honey experience delayed onset of clinical signs and symptoms?*

<i>Section of report</i>	<i>Summary response/conclusion</i>
Sections 2 and 4	Delayed onset of clinical signs and symptoms can be explained by the presence of tutin glycosides in toxic honeydew honey. If honey contained tutin, but not tutin glycosides, a delayed onset of clinical signs and symptoms would not be expected.

2. *Does a delayed onset of clinical signs and symptoms following consumption of tutu honeydew honey have implications for the current tutin ML of 2 mg/kg?*

<i>Section of report</i>	<i>Summary response/conclusion</i>
Section 2 and 4	Yes, however no method is currently available for the quantification of tutin glycosides in honey, therefore the continued use of an ML based on the level of tutin is necessary. A reduction in the ML by a factor of 3 is considered sufficient to minimise the risk of adverse health effects from the consumption of honey containing tutin and tutin glycosides.

3. *Does comb honey containing tutin at the current ML of 0.1 mg/kg pose a health risk?*

<i>Section of report</i>	<i>Summary response/conclusion</i>
Section 4	There are insufficient data on the potential heterogeneity of tutin distribution to characterise the risk for comb honey.

4. ***If so, what magnitude of reduction in the ML for tutin in comb honey would be considered effective to minimise the health risk?***

<b>Section of report</b>	<b>Summary response/conclusion</b>
Section 4	An ML equivalent to the analytical limit of detection would minimise the health risk posed by comb honey.

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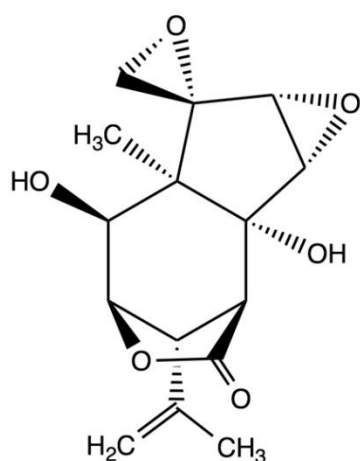
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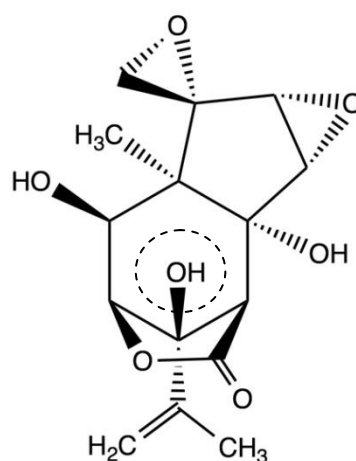
# Appendix 1

## Structural formulae of tutin and related compounds

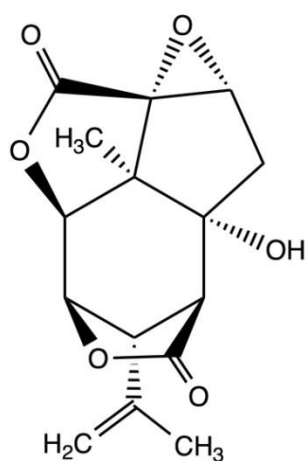
The structures of tutin, hyenanchin, picrotoxinin and picrotin are shown below. Tutin and hyenanchin differ only by the substitution of a hydrogen in tutin for a hydroxyl group (circled) in hyenanchin. Tutin and hyenanchin possess an additional epoxide group compared to picrotoxinin and picrotin. 'Picrotoxin' is an equimolar mixture of picrotoxinin (a GABA<sub>A</sub> receptor antagonist) and picrotin (inactive at the GABA<sub>A</sub> receptor).



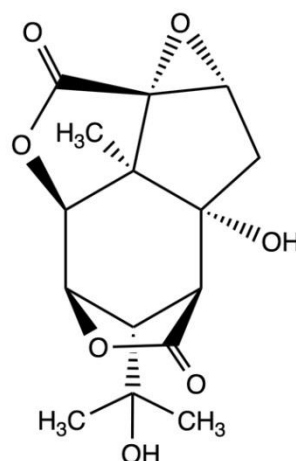
**Tutin**



**Hyenanchin**



**Picrotoxinin**



**Picrotin**

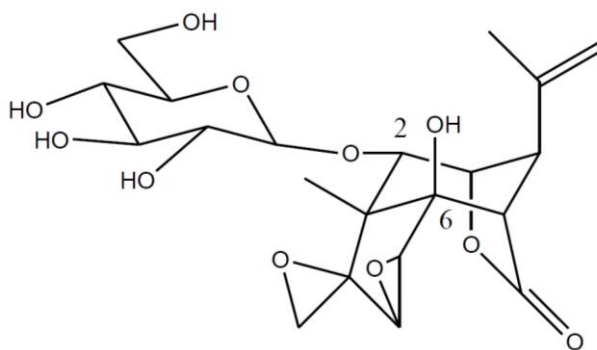
## Appendix 2

### Discovery of tutin glycosides in honey samples and attempts to release tutin from glycosides by incubation with human colonic microflora

Larsen, L, Joyce NI, Cooney JM, Jensen DJ, Tannock GW, Sansom CE and Perry NB (2014) Discovery of tutin metabolites in New Zealand toxic honey. The New Zealand Institute for Plant & Food Research Ltd. Report no. PFR SPTS No 9504. February 2014. Unpublished report.

Samples of *Coriaria arborea* leaf and three honeys known to contain tutin were extracted, fractionated and analysed using liquid chromatography-mass spectrometry (LC-MS). Use of an ion-trap LC-MS methodology resulted in the identification of two tutin monoglycosides, two tutin diglycosides and two tutin triglycosides in all three honey samples. A trace amount of a possible hyenanchin monoglycoside was found in two of the honey samples. One of the tutin monoglycosides was also detected in a *Coriaria arborea* leaf extract along with low levels (relative to tutin) of hyenanchin, dihydrohyenanchin and dihydrotutin.

The molecular weight of the tutin monoglycosides is consistent with glycosidation at one of the two hydroxyl groups of tutin, to give a structure such as that shown below (drawn as a glucoside at C2, however a tutin monofructoside is of equivalent mass).



This study also investigated whether incubation of the honey samples with human faecal material could result in the release of tutin from tutin glycosides. A positive result would support the hypothesis that tutin glycosides are metabolised to tutin by microflora in the human colon resulting in the delayed systemic absorption of additional tutin as observed in the human pharmacokinetic study. Honey samples were incubated with human faecal preparations under anaerobic conditions using a published protocol with modifications (Gardana et al 2003). Incubation experiments were conducted with the honey sample administered in the human pharmacokinetic study and with two honey samples associated with poisoning incidents (tutin concentrations 18 mg/kg and 50 mg/kg respectively).

Tutin and hyenanchin concentrations in the incubation culture were measured at the beginning of the incubation and after 24 h. Monitoring of tutin glycoside levels was by comparison of peak areas. Tutin concentrations in the incubation mixture were slightly lower or unchanged at 24 h compared to initial concentrations. In contrast, large changes were observed in the levels of some tutin conjugates. For example, peak areas at 24 h for three di-glycoside conjugates were only 1 to 4% of the areas at time zero, while peak areas for two mono-glycosides increased by 2.8- to 3.7-fold. Levels of two tri-glycosides were also observed to decrease, but to a lesser degree than that observed for the di-glycosides. A negative control incubation (no faecal microflora) resulted in no substantial changes in the levels of tutin glycosides. In conclusion, under the conditions of these experiments, tutin does not appear to be released from conjugates indicating that the microbial enzymes present in these incubations do not efficiently cleave tutin glycoside bonds, whereas di- and tri-glycosides appear to undergo cleavage resulting in increased levels of tutin mono-glycosides.

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Gardana C, Simonetti P, Canzi E, Zanchi R, Pietta P (2003) Metabolism of stevioside and rebaudioside A from *Stevia rebaudiana* extracts by human microflora. *J Agric Food Chem*, **51**:6618-6622.

## Appendix 3

### Attempts to hydrolyse tutin glycosides in honey using enzymatic and acid incubations

Sansom CE, Cooney JM, Perry NB, Jensen DJ (2013) Hydrolysis of tutin glycosides in toxic honey using enzymes and acid. The New Zealand Institute for Plant & Food Research Ltd. Report No. PFR SPTS No. 9137. October 2013. Unpublished report.

The experiments in this study were conducted to attempt to release tutin from conjugates using enzymatic methods and also using acidic conditions. In published studies, a range of glycosidases have been used for the release of aglycones from glycoconjugates including mycotoxins, flavonoids, steviol glycosides and glycosides of sesquiterpene lactones (see for example Kotsos et al 2008; Pricelius et al 2009; Beloglazova et al 2013). Typically, several glycosidases of varying specificity are evaluated in order to achieve maximal release of aglycones. For example, the extent of glucose release from a mono-glucoside of zearalenone differed markedly for glycosidases purified from *Aspergillus niger* (100% cleavage) and almonds (6% cleavage). A cellulase preparation from the fungus *Trichoderma viride* also completely released zearalenone from the conjugate; however cellulase from *A. niger* gave only 20% release (Beloglazova et al 2013). Differences in activity can be due to differences in innate activities of an enzyme across species, as well as differences in the presence of other glycosidases in the enzyme preparations.

Samples of the honey that was administered in the human pharmacokinetic study were used in these experiments. Enzymes tested were pectinase from *Aspergillus aculeatus*, glucosidase from *Aspergillus niger*,  $\beta$ -glucosidase from almond, cellulase from *Trichoderma viride* and  $\alpha$ -glucosidase from *Saccharomyces cerevisiae* (all from Sigma-Aldrich).

For the enzyme incubations, honey (1 g) was dissolved in appropriate enzyme buffer (9 mL) and 0.5 mL aliquots of this solution were mixed with 2 mg of enzyme and incubated at 37 °C for 24 h. Acid incubations were conducted with honey diluted 1:10 with water followed by addition of 10 M hydrochloric acid to give final HCl concentrations of 0.1 M and 1 M. Acid incubations were conducted at 37 °C for 1, 6 and 24 h and the solutions were neutralised before analysis.

LC-MS analysis of the incubation solutions indicated that none of the enzymes had any substantial effect on releasing tutin from tutin glycosides with the largest ratio of final versus initial tutin concentration being only 1.5x. Similarly, the largest increase observed in the acid incubations was 1.4x.

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## Appendix 4

### Variability in the ratio of tutin to tutin glycosides in honey samples

Cooney JM, Jensen DJ, Sansom CE, Perry NB (2013). Tutin to tutin glycoside ratios in various New Zealand honeys. The New Zealand Institute for Plant & Food Research Ltd. Report No. PFR SPTS No. 9239. November 2013. Unpublished report.

The concentrations of tutin and hyenanchin were determined by LC-MS/MS for 15 honey samples harvested at various times and locations in New Zealand. The relative concentrations of the four major tutin glycosides were also determined for these honey samples. These tutin glycosides are a mono-glycoside (designated gly-A), two di-glycosides (digly-C and digly-D) and a tri-glycoside (trigly-H). Chemical reference standards for these tutin glycosides are not available, therefore the concentrations of these analytes were calculated as 'tutin equivalents' by converting the mass spectral transition area for each glycoside to an equivalent tutin concentration using the tutin calibration curve and adjusted for honey weight. The tutin glycoside concentrations reported should not be regarded as true quantitative figures, however they do allow for comparisons of relative ratios of tutin to tutin glycosides across different honey samples.

Results are shown as the mean of duplicate measurements (Table 1). Ratios of analyte concentrations or peak areas are shown in Table 2. In three honey samples (T1, T2, T3), tutin, hyenanchin and tutin glycosides were not detected (limits of detection not stated). In the remaining 12 honey samples, tutin concentrations ranged from 0.14 to 51 mg/kg (mean 5.3 mg/kg), hyenanchin concentrations ranged from 1.0 to 386 mg/kg (mean 37 mg/kg), and the hyenanchin:tutin concentration ratio ranged from 3.4 to 12.7 (mean 6.7). The ratio of total glycosides:tutin ranged from 2.6 to 12.7 (mean 7.0). The 6 possible pairwise comparisons between the 4 major tutin glycosides indicates predominantly small variations across honey samples. The relative standard deviations (RSD) for 3 of the pairwise ratios were less than 30%. However the ratio gly-A:digly-D varied across a large range (0.07 to 1.09) with an RSD of 69%.

**Table 1: Concentrations of tutin and hyenanchin, and “tutin equivalent” concentrations of four tutin glycosides in 15 honey samples**

Honey sample	Concentration (mg/kg honey)		Ratio	“Tutin equivalent” concentration (mg/kg honey)				Total glycosides	Ratio
	Tutin	Hyenanchin	H:T	gly-A	digly-C	digly-D	trigly-H	(mg/kg honey)	Total glycosides: Tutin)
M1 (1 of 4)	0.18	1.89	10.5	0.23	0.87	0.53	0.08	1.71	9.5
M1 (2 of 4)	0.18	1.76	10.0	0.19	0.87	0.59	0.09	1.74	10.0
M1 (3 of 4)	0.18	2.28	12.7	0.24	0.94	0.62	0.10	1.89	10.5
M1 (4 of 4)	0.16	1.64	10.3	0.22	0.93	0.61	0.10	1.84	11.5
M2	0.14	1.01	7.5	0.14	0.65	0.41	0.06	1.26	9.3
P1	0.53	2.34	4.5	0.22	1.07	0.97	0.08	2.34	4.4
P2	0.71	4.73	6.7	0.32	1.71	1.54	0.18	3.74	5.3
P3	0.63	2.13	3.4	0.18	0.81	0.88	0.07	1.93	3.1
P4	0.55	2.19	4.0	0.15	0.56	0.65	0.06	1.40	2.6
P5	0.57	2.05	3.6	0.16	0.74	0.88	0.09	1.85	3.3
F1	4.85	18.59	3.8	2.71	11.22	8.30	1.21	23.43	4.8
F2	0.65	2.37	3.6	0.50	1.79	1.16	0.22	3.67	5.7
T1	ND	ND	NA	ND	ND	ND	ND	NA	NA
T2	ND	ND	NA	ND	ND	ND	ND	NA	NA
T3	ND	ND	NA	ND	ND	ND	ND	NA	NA
Honey 1	15.30	97.79	6.4	5.68	48.58	23.02	5.09	82.37	5.4
Honey 2 <sup>^</sup>	4.41	25.81	5.8	6.16	18.14	5.63	3.19	33.11	7.5
Honey 3 <sup>†</sup>	50.79	385.59	7.6	26.22	218.62	357.71	42.74	645.29	12.7

<sup>^</sup> Honey 2 was administered in the human pharmacokinetic study. <sup>†</sup> Honey 3 was associated with human poisoning in 2008.



**Table 2: Ratios of analyte concentrations/peak areas in the 12 honey samples containing detectable tutin**

Honey sample	Ratio							Ratio (Total glycosides: Tutin)
	Hyenanchin: Tutin	gly-A: digly-C	gly-A: digly-D	gly-A: trigly-H	digly-C: digly-D	digly-C: trigly-H	digly-D: trigly-H	
M1 (1 of 4)	10.5	0.26	0.44	2.88	1.66	10.88	6.56	9.5
M1 (2 of 4)	10.0	0.22	0.32	2.11	1.47	9.67	6.56	10.0
M1 (3 of 4)	12.7	0.26	0.39	2.53	1.53	9.89	6.47	10.5
M1 (4 of 4)	10.3	0.23	0.36	2.26	1.53	9.74	6.37	11.5
M2	7.5	0.21	0.33	2.25	1.59	10.83	6.83	9.3
P1	4.5	0.21	0.23	2.75	1.11	13.38	12.06	4.4
P2	6.7	0.19	0.21	1.78	1.11	9.47	8.53	5.3
P3	3.4	0.22	0.20	2.69	0.91	12.38	13.54	3.1
P4	4.0	0.26	0.22	2.64	0.86	10.09	11.73	2.6
P5	3.6	0.21	0.18	1.82	0.84	8.65	10.29	3.3
F1	3.8	0.24	0.33	2.25	1.35	9.31	6.88	4.8
F2	3.6	0.28	0.43	2.27	1.54	8.14	5.27	5.7
Honey 1 <sup>*</sup>	6.4	0.12	0.25	1.12	2.11	9.54	4.52	5.4
Honey 2 <sup>^</sup>	5.8	0.34	1.09	1.93	3.22	5.68	1.76	7.5
Honey 3 <sup>†</sup>	7.6	0.12	0.07	0.61	0.61	5.12	8.37	12.7
<i>Minimum</i>	3.4	0.12	0.07	0.61	0.61	5.12	1.76	2.6
<i>Maximum</i>	12.7	0.34	1.09	2.88	3.22	13.38	13.54	12.7
<i>Mean</i>	6.7	0.22	0.34	2.13	1.43	9.52	7.72	7.0
<i>Standard deviation</i>	3.0	0.06	0.23	0.62	0.63	2.14	3.11	3.3
<i>RSD</i>	0.45	0.25	0.69	0.29	0.44	0.22	0.40	0.47