Chapter Five: Discussion

5.1 FRAP assay

The results obtained in the present study support the stated hypotheses. All bee products demonstrated antioxidant activity when evaluated using the FRAP assay. At 1.0mg/ml (Table 1), the bee product available to the general public with the greatest FRAP value was ethanolic extract of propolis tablet (95μmol/l) (pure propolis resin had a FRAP value of 746μmol/l). The honey with the greatest FRAP value was manuka 30+ (9 μmol/l). In the present study antioxidant properties increased as the concentration of the samples increased indicating a dose response effect (Figure 4).

Interestingly, bee pollen produced a higher FRAP value (15 μmol/l) than manuka 30+ and royal jelly produced a FRAP value (8.0 μmol/l) which was greater than the other honeys evaluated. Royal jelly and bee pollen were used only for comparison in this study but future studies may focus solely on these products.

The artificial honey, representing the typical sugar contents of honey showed no antioxidant activity. This shows constituents of the honeys, other than sugars, have antioxidant activity, with respect to the FRAP assay. These constituents may include flavonoids such as those tested in the present study (quercetin, cinnamic acid, naringenin) which all displayed high antioxidant activity (Table 2). Quercetin produced the highest FRAP reading compared with other pure compounds and the bee products at 0.1mg/ml dilution and was extrapolated to have the highest antioxidant activity of all substances tested (Table 2). The exact flavonoid content of the bee products was not investigated in the present study but further studies could determine flavonoid content of bee products and compare it with observed antioxidant activity.

It was hypothesised that antibacterial and antioxidant properties of the manuka and tea tree honeys would increase as the unique manuka factor (UMF) increased. As seen in Table 4, the highest UMF honey (manuka 30+) produced the highest FRAP value of all the honeys tested. Standard honey, gave the lowest FRAP value. However, the order of FRAP values was inconsistent between honeys based on the strength or activity stated by the manufacturer and antioxidant activity actually observed. In fact the honey with the second highest UMF (18+) never produced the second highest FRAP value, but manuka 5+, the lowest UMF, in some (but not all) assays, produced the second highest FRAP value. The reason for this inconsistency may be due to error in the preparation of the products by the researcher; the assay procedure or even the products themselves could have been damaged or contaminated in some way. Additionally, the UMF rating indicates only the antibacterial activity of manuka or tea tree honeys, not antioxidant activity. In order for more specific and reliable results to be obtained, several tubs of each honey would have to be tested to account for batch variations.

Mohammadzadeh et al. (2007) evaluated the reducing power of Iranian propolis using the FRAP assay. The FRAP values obtained for ethanolic extracts of propolis were 1650±72 µmol/l at a concentration of 2 mg/ml. This is approximately 825,000 µmol/l at a concentration of 1.0g/ml. This was 1100 times the potency of propolis resin found in the present study (746µmol/l) and 8700 times that of propolis tablet (95 µmol/l). This large difference in results could reflect the different method of processing the samples. Specifically, Mohammadzadeh et al. (2007) concentrated raw propolis samples after extraction in a rotary evaporator. Mohammadzadeh et al. (2007) proposed that strong

antioxidant activity occurs in propolis with high phenolic content and weak activity in propolis with low phenolic content. Further research is needed to evaluate the composition of commercially available propolis using chromatograph methods such as HPLC in order to identify components which potentially contribute to antioxidant activity.

When utilising the FRAP assay to evaluate the reducing power of a selection of honeys, Küçük, Kolaylı, Karaoğlu, Ulusoy, Baltacı, and Candan (2007) found the reducing power of all honeys tested showed a concentration dependent effect. This reflects the findings of the present study. However, Küçük et al. (2006) stated that the honeys tested could be concluded to have a high level of antioxidant activity. However, it was not possible to compare these results with the literature due to a lack of standardised methods. Therefore, although the findings of the present study could be compared qualitatively with other studies, quantitative comparison is limited.

5.2 Disc Diffusion assay

All honeys inhibited growth of bacteria. Inhibition showed a dose response relationship using percentage dilutions. However, serial dilutions (100mg/ml, 10mg/ml, 1.0mg/ml, and 0.1 mg/ml) gave inconsistent results. This was also the case for royal jelly. For example, royal jelly inhibited the growth of *E.coli* at dilution 10mg/ml but not at a higher concentration (100mg/ml). This result suggests constituents in the royal jelly may have diffused more readily into the nutrient agar, providing an explanation for this unexpected finding. Similarly at dilution 1.0mg/ml, manuka 5+ was found to inhibit growth of *E.coli* but at dilution 0.1mg/ml inhibited growth more than standard honey and itself at the

previous dilution and 100mg/ml (2.17mm±0.76 at 100mg/ml; 2.16mm±1.25 at 1.0mg/ml; 3.83mm ±0.76 at 0.1mg/ml). For viscous solutions such as honey, the disc diffusion assay may be only relevant for samples that are considerably diluted. This was consistent for different bacterial strains. For example, at 10 mg/ml, manuka 5+ inhibited growth of *S.aureus* having not inhibited growth at 100mg/ml. Again, dilution creates a less viscous solution allowing the honey solution to diffuse across the bacterial lawn farther when placed on the disc, thus enabling the active components to inhibit growth. At dilution 0.1mg/ml only the standard honey exhibited antibacterial activity but was shown to have no corresponding activity at the higher concentrations (100mg/ml, 10mg/ml). Therefore, when using serial diluted products against *S.aureus*, the results were inconsistent and no conclusion could be drawn as to which product was the most potent.

Ethanolic extracts of the propolis tablet did not inhibit growth of any of the bacteria tested. This may have been due to the way the tablets were prepared (dissolved in ethanol and then placed on to discs which were left to dry over night in sterile petri dishes to ensure it was not the ethanol inhibiting growth). Alternatively, the commercially available propolis tablets may not have contained sufficient antibacterial activity to elicit an effect in the disc diffusion assay. Propolis resin was not tested in the present study. Further studies are needed to evaluate the antibacterial activity of propolis resin as it has been shown to have a much higher antioxidant activity (FRAP assay) compared with the propolis tablet.

Note *S.mutans*, the tea tree honeys and the manuka 30+ honey were not available for comparison at the time the serial dilutions were evaluated using the disc diffusion assay.

When comparing honeys the largest mean zone of inhibition was using undiluted manuka 30+ against *S.epidermidis* (32 mm± 5.25). The second largest zone of inhibition observed was using undiluted tea tree 18+ against *S.mutans* (31mm ±3.32). No products were observed to inhibit *E.coli* beyond 50% dilution and *S.mutans* beyond 25% dilution. None of the bacteria were inhibited below 12.5% dilution. Other results were also found to be inconsistent when using the disc diffusion assay. Manuka 5+ produced a larger mean zone of inhibition than manuka 30+ (34mm vs. 13mm) against *E.coli* when applied undiluted to the Whatmann disc (Table 5). For *S.aureus*, manuka 30+ produced the largest mean zone of inhibition (24mm) which was found to be significant ($p<0.05$). However, at 50% dilution tea tree 18+ produced a significantly larger average zone of inhibition compared with manuka 30+. It appeared that between dilutions, different products produced the largest mean zone of inhibition; manuka 30+ did not always produce the larges zones of inhibition as one may have expected. These results suggest that a more expensive product marketed is not necessarily superior to the cheaper, standard product using the disc diffusion assay to estimate antimicrobial activity.

Studies by Lusby, Coombes and Wilkinson (2005) found there was a progressive increase in inhibition of bacteria as honey concentration increased. This was inconsistent with the findings of the present study which showed some honeys produced larger zones of inhibition when applied in a more diluted form. However, some data agrees with the study by Lusby et al. (2005) which showed at higher concentrations most honeys become more potent inhibitors of bacterial growth. For examples, when tea tree 18+ was diluted

by 50%, the inhibition of growth of *S.aureus* reduced by 50% (Table 6). However, standard honey produced a larger mean zone of inhibition of *S.aureus* at 50% dilution compared with undiluted. As previously discussed, this may have occurred because the diluted honey was more viscous and more able to spread across the plate to inhibit a larger zone of bacterial growth.

When using honey with a UMF of 18+, Patton et al. (2006) found at 50% dilution a mean zone of inhibition of 19.6mm against *E.coli* and 20.8mm against *S.aureus*. In the present study, using the same strength UMF and same dilution, the mean zone of inhibition against *E.coli* was 9mm and 13mm against *S.aureus*. These values are less than those found by Patton et al. (2006) but this may be due different brands of honey or differences in methodology, for example Patton et al (2006) used 8.2mm diameter Whattman discs compared with 14mm in the current study. Additionally, Patton et al. (2006) placed the Whattman discs in the honey samples for ten minutes before applying them to the agar plate. In the present study the Whattman discs were dipped in the samples for a few seconds until they were visibly saturated.

Taormina et al. (2001) determined if six honeys from six different floral sources were lethal to, or inhibited the growth of, six food borne pathogens. These researchers also assessed the influence of the presence of hydrogen peroxide and level of antioxidant activity against survival and growth of the pathogens, which included *E.coli* and *S.aureus*. The disc diffusion assay was used to assess honeys of different dilutions for inhibition of growth of the pathogens. The study found inhibition was most evident around the discs soaked in the highest concentration of honey. *S.aureus* was most

sensitive to the range of honeys tested. In the present study, the largest zone of inhibition was observed against *E.coli* (34mm using artificial honey and 34mm using manuka 5+ undiluted (Table 5)). *S.aureus* was inhibited down to a dilution of 6.25% whilst the other three bacteria were not inhibited beyond 25% dilution. Taormina et al. (2001) also found strains of five of the six pathogens exhibited sensitivity to one of more honeys at concentrations of 25% or less. Three of the unprocessed honeys evaluated in the study were more inhibitory than processed honeys to growth of five of the six food borne pathogens tested.

Lee, Churrey and Worobo (2008) compared the antimicrobial activity of domestic honeys from the USA and two manuka honeys (UMFs not stated) against various bacterium including *S.aureus* and *E.coli*. Each honey was tested for the presence of endogenous antimicrobial activity. When tested against two foodborne pathogen indicator strains, (*L.monocytogenes* and *S. aureus*), the six varieties of honey harvested in North America exerted relatively lower activity than the two manuka honeys from New Zealand (statistical significance not reported).

A number of previous studies have demonstrated that various honeys, both commercially and locally produced, have antimicrobial activity. A study of six commercial honeys by Nzeako and Hamdi (2000) (as cited by Lusby et al., 2005) found inhibition of Gram positive *S.aureus*, and Gram negative *E.coli* did not occur at honey concentrations less than 40%. This differs from the findings of the present study where Gram positive *S.aureus* was inhibited up to 25% dilution (manuka 30+) and Gram negative *E.coli* was

also inhibited up to 25% dilution (manuka 5+). The results of another study by Basson and Grobler (2008) confirmed that honeys from different countries and regions have a wide variability in their antimicrobial activity, although no statistical significance was found. The honeys tested had antimicrobial activity in the 17% to 25% range against the organisms tested, except for *S.oralis* which showed sensitivity at 12.5%. The bacteria *S.aureus* (Gram positive) and *E.coli* (Gram negative) were both inhibited in the 25% to 50% range which also occurred in the present study (see tables 5 & 6). However ,other studies have found these species to be sensitive at concentrations as low as 1.8% to 11% for some honeys. (Wilkinson & Cavanagh, 2005; Wilix, Molan & Harfoot, 1992, both as cited by Basson & Grobler, 2008). This shows that the honeys tested in the present study do not have the highest antibacterial properties available and other products are superior.

The present study showed some correlation between high antioxidant activity (FRAP) and potency of antibacterial activity of honeys. For example at 25% dilution the honeys rank as follows for FRAP values: manuka 30+ >manuka 5+ >tea tree 16+ >tea tree 18+ > tea tree 12+ > standard honey. In the present study the most potent antibacterial effect was observed against using manuka 30+ against *S.epidermidis* (33mm mean zone of inhibition when applied undiluted, see table 7) and manuka 30+ also produced the largest zone of inhibition against *S.aureus* when applied to the Whatmann disc undiluted (24mm mean zone of inhibition, see table 6). However results were not always consistent. Although manuka 30+ consistently exhibited the highest antioxidant activity (FRAP value), it was not always the most potent against the bacteria tested. For example, manuka 5+ inhibited the growth of *E.coli* significantly more than manuka 30+ when

applied undiluted to a Whatmann disc (table 5) and tea tree 18+ inhibited the growth of *S.mutans* significantly more than manuka 30+ when applied undiluted to a Whatmann disc (table 8). Additionally, standard honey exhibited the lowest antioxidant activity (FRAP values) but at times produced the largest zones of inhibition in the disc diffusion assay (see tables 4, 6 & 7). Furthermore, artificial honey did not produce a FRAP reading at any time but was observed to inhibit bacterial growth suggesting sugar content also has a role in honeys antibacterial properties. This may further suggest that sugar contents of honeys are largely responsible for antibacterial properties.

Scazzocchio et al. (2006) investigated the antimicrobial activity of ethanolic extracts of propolis against Gram positive bacteria by determining minimal inhibitory concentration of ethanolic extracts of propolis. Their findings suggested components of propolis including flavonoids such as quercetin and cinnamic acid probably act on the microbial membrane or cell wall site causing functional and structural damage. However, in the present study, the propolis tablet demonstrated no significant antimicrobial activity against Gram positive or Gram negative bacteria despite producing the highest FRAP value of the bee products (95 µmol per 1mg/ml (95,000 µmol per 1 gram tablet)). This differs from the results obtained by Kujumgiev et al. (1999) who found propolis extracts inhibited growth of Gram positive bacteria such as *S.aureus* and Koo et al. (2000) who found propolis extracts significantly inhibited growth of *S.aureus* and *S.mutans* but agrees with the finding by Kujumgiev et al. (1999) that inhibition of the growth of Gram negative bacteria, such as *E.coli*, did not occur. However, if the active components of the propolis had not been properly extracted, the result from the present study may not reflect

its actual antibacterial potential. Furthermore, Scazzocchio et al. (2006) evaluated raw propolis which may have been more representative of the antibacterial properties of propolis, unlike the tablets which may have lost antibacterial properties during processing. Castaldo and Capasso (2002) also comment that the antibacterial activity of propolis largely depends on the site of its collection. This would imply further comparisons with other studies may be complicated if the propolis evaluated is sourced from different geographical locations. Flavonoids are thought to account for much of the biological and pharmacological activities in propolis, although other compounds are implicated (Grange& Davey, 1990). In the present study the total flavonoids and polyphenol content of the ethanolic extracts of the crude propolis and propolis tablets were not determined. Mohammadzadeh et al. (2007) used aluminium nitrate and Folin-Cicoalteu calorimetric methods to quantify total flavonoid and polyphenol contents of crude ethanolic extracts of Iranian propolis samples. The estimation of phenolics content can be used for evaluation of propolis quality and its properties. Future studies of products available to the public could identify and quantify the flavonoid and polyphenol contents of crude propolis and products available to the public.

5.3 Limitations of the present study

Increasing the scale of the investigation may increase data reliability. Testing a wider range of brands and a larger number of product batches may show which products have the highest antioxidant and antimicrobial activities.

The FRAP procedure is relatively simple and quick; the reagents are inexpensive and readily available (the equipment was available in the university laboratories) and the

results obtained are highly reproducible over a wide range of concentrations. Prior and Cao (1999) state an advantage of the FRAP assay is its ability to indirectly measure antioxidant activity without using pro-oxidants, so, despite the FRAP assay not being the most specific of the antioxidant assays, it has a role as a general, indirect measure of antioxidant activity. However, the FRAP assay does not involve an oxidisable substrate. Further studies are required utilising assays which use an oxidisable as no antioxidant method is without potential for errors.

The ORAC (oxygen radical absorbance capacity) assay method is sometimes seen as a "gold" standard of antioxidant testing and is used to illustrate the antioxidant value of consumer products such as Welch's grape juice (http://www.welchsjuice.co.uk/).The ORAC assay is based on scavenging of the peroxyl radical, a free radical produced by lipid peroxidation *in vivo*. The peroxyl radical is generated from the organic molecule AAPH (2,2'-azobi(2-amidinopropane)dihydrochloride) and attacks a fluorescent molecule. This generates a decrease in the emission of fluorescence which is monitored. The area under the curve is then measured and is interpolated into a Trolox curve and results are expressed as Trolox equivalents (Perez-Jimenez & Saura-Calixto, 2006).

The Fe^{3+} TPTZ complex used in the FRAP assay is not a pro-oxidant and would not cause oxidative damage. Therefore, results obtained give no indication of the protective properties of any antioxidants present, merely the reducing power of the whole sample. Furthermore, some substances within a test sample may be capable of reducing Fe^{3+} to Fe^{2+} but not actually have antioxidant activity. Similarly, some compounds within a test substance may be able to prevent oxidative damage but unable to reduce Fe^{3+}, for example, glutathione (Prior & Cao, 1999), and therefore go undetected in the FRAP

assay. Therefore, the use of one assay (FRAP) to measure total antioxidant activity based solely on the one characteristic (ability to reduce Fe^{3+} to Fe^{2+}) is over simplifying the complex processes that occur in nature. As most free radicals have individual reaction mechanisms in causing oxidative damage, it not appropriate perhaps to deploy a single assay. Furthermore, the pH at which the FRAP assay is performed, 3.6, is not the normal physiological pH and thus results obtained in the laboratory may not accurately represent *in vivo* antioxidant activity. Ideally, the protective properties of antioxidants should be measured under appropriate conditions that define the target of oxidation, for example, lipids, protein, DNA (Frankel & Meyer, 2000).

A number of studies have found data obtained using more than one assay can be inconsistent. However, Ou, Huang, Hampsch – Woodhill, Flanagan and Deemer (2002) found using the FRAP and ORAC assays, the results were generally comparable. In addition, Prior and Cao (1999) compared a number of methods for measuring antioxidant activity, including the ORAC and FRAP assays and found significant linear correlation between data obtained from the two assays. Therefore, the present study could be extended to use the ORAC assay to confirm the findings obtained using the FRAP assay before any definite conclusions can be made regarding the antioxidant activity of bee products. However, the complex composition of food and drink makes developing a reliable *in vitro* assay difficult (Frankel & Meyer, 2000). For example, vitamin C is a known antioxidant but is rarely found in isolation within foods. Compounds within foods may inhibit or promote antioxidant activity or may have no effect at all. Furthermore, results obtained in the present study may not reflect antioxidant actions *in vivo* as different compounds may not be absorbed or extensively metabolised.

Limitations also occurred when using honeys. Honey is not normally processed so can contain a diverse microbial population originating from flowers, plants and hives as well as the honeybees themselves.The artificial honey was tested sterile in the present study. However; bacterial growth was observed when all the natural honeys were spread on nutrient agar plates. This may have affected the results of the antimicrobial assay. The additional antimicrobial activity exhibited by selected honeys may be attributed to the production of antimicrobial compounds by bacteria present in honey (Lee et al, 2008). These compounds could have potential applications in foods to target pathogens and spoilage microorganisms to enhance the safety and quality of foods. Further screening of the antimicrobial activity produced by bacterial strains from honey against human pathogens may expand the application of these antimicrobial compounds to clinical applications (Lee et al, 2008).

The disc diffusion method is regarded as the method of choice for inhibition tests (Patton, Barrett, Brennan and Moran 2006). However, this method can be unreliable in certain situations as subjectivity is associated with visual determination of zones of inhibition. An autodata automatic zone reader is less subjective with a measure of ± 0.1mm would allow for more accurate measurements of zones of inhibition. Patton et al (2006) found a less time consuming and possibly cheaper test, the 96-well microtiter plate assay, which allows multiple samples to be tested on a single plate. Patton et al (2006) found the spectrophotometric assay offers a number of advantages compared with the disc and well diffusion methods. For example it is reported to have increased repeatability and sensitivity. The method requires test substances to be added to a 96 well microtiter plate

with a lid to prevent cross contamination. Optical density is determined in a spectrophotometer at 620 nm prior to incubation, T_0 and again after 24 hours, T_{24}. Zones of inhibition are measured using an autodata automatic zone reader with a tolerance of ± 0.1mm. The method continuously reports MIC values at greater dilutions compared with the diffusion assays and enables the subjective aspect of observations to be removed via the use of data analysis and real time data capture and interpretation. The method was found to consistently demonstrate an improvement over current methods and could therefore be considered for future projects.