Chapter Two: Methods

2.1 Design

This research project was a laboratory investigation. The study followed a repeated measures design; measurements were taken in triplicate and means and standard errors calculated.

- Independent variables: the samples.
- Dependent variables: measurements of antioxidant and antimicrobial activities.
- Ethical approval was not required.
- Chemicals were disposed of in accordance with manufactures instruction the laboratory protocols developed at the University of Chester

2.2 Materials and Procedures

2.2.1 Materials.

An artificial honey (80% (w/v) sugar) was used as a control. It was prepared by dissolving the following in 100ml distilled water: 40g fructose, 30g glucose, 8g maltose and 2g sucrose. This formulation reflects the approximate sugar composition of honeys (White, 1979 as cited by Taormina et al., 2001). The sterility of the artificial honey was confirmed by spreading it on three nutrient agar plates and incubating for 24 hours at 37^{o}C. No bacterial growth was visible after this time and the artificial honey deemed sterile. All honeys were kept in a dark cupboard at room temperature and away from direct heat. Honeys and royal jelly were diluted using sterile water. Pure compounds and

bee pollen were diluted using 80% ethanol. Ethanol extracts of propolis tablet and resin were obtained using the method cited by Mohammadzadeh et al., (2007); the propolis (tablet/resin) was weighed, dissolved in 80% ethanol solution and placed on an automatic stirrer for 24 hours. The solution was then filtered through double folded filter paper and reweighed. The remaining solution was then kept in a cool, dark cupboard for further dilution to use in the two assays. One gram of propolis (tablet/resin) yielded 0.4g of non dissolvable constituents, therefore, results obtained related to the remaining 0.6g of dissolved constituents. The samples in this research project were bee products. Two sets of dilutions were evaluated: serial dilutions (100mg/ml, 10mg/ml. 1.0mg/ml & 0.1mg/ml) and percentage dilutions (50%, 25%, 12.5%, 6.25% & 3.125%).

Sigma-Aldrich, Gillingham, Dorset, UK supplied:

- propolis resin
- cinnamic acid
- naringenin
- quercetin

The products purchased from Holland and Barrett LTD (Golden Square Mall, Warrington, Cheshire) were:

- 500mg Propolis Capsules (Holland and Barrett ®);
- 500mg Royal Jelly Capsules (Holland and Barrett ®);
- 500mg Bee Pollen Tablets (Holland and Barrett ®);
- Clear Honey (standard) (Holland and Barrett ®);

- Manuka 5+ Honey (Medibee ®)
- Manuka 30+ Honey (Spirits Bay ®).

Xynergy health products (Elsted, Midhurst, West Sussex, GU29 0JT) supplied the following:

- Tea Tree 12+ Honey (Medigold ®);
- Tea Tree 16+ Honey (Medigold ®)
- Tea Tree 18+ Honey (Medigold ®)

The National Collection of Industrial, Food and Marine Bacteria (NCIMB) Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, Scotland, AB21 9YA supplied:

- *Streptococcus mutans (S.mutans) (Gram positive)*
- *Escherichia coli (E.coli) (Gram negative)*
- *Staphylococcus aureus (S.aureus) (Gram positive)*
- *Staphylococcus epidermidis (S.epidermidis) (Gram positive)*

2.2.2 Procedures

Two assays were utilised in this study: the FRAP assay and the disc diffusion method.

The FRAP assay:

The reducing power of propolis, honey, royal jelly and bee pollen were determined according to a method based on that described by Benzie and Strain (1996). The principle

of this method is based on the reduction of ferric-tripyridyltriazine complex to its ferrous, coloured form in the presence of antioxidants.

Materials:

The following chemicals were supplied by Sigma-Aldrich, Gillingham, Dorset, UK:

- Sodium Acetate Trihydrate
- Glacial Acetic Acid
- 40 mM HCL
- 2,4,6-tripyridly-1,3,5-triazine (TPTZ)
- Iron III Chloride Ferrous Iron II Sulphate

Equipment:

- Spectrophotometer (Pharmacia Biotech Novaspec II, data output 100m V, wavelength 325-900nm)

Procedure:

The method is a slight adaptation of that described by Benzie and Strain (1996). The assay was performed in three stages:

1. The constituent parts (A,B,C) of the FRAP reagent were prepared
2. The reagents were combined and incubated
3. The FRAP reagent was added to test samples and absorbance recorded at 593nm.

Preparation of FRAP reagents:

Reagent A: - Acetate Buffer (300m M, p H 3.6):

16ml of glacial acetic acid was added to 3.1g of sodium acetate trihydrate; the solution was then made up to 1 litre using distilled water. The p H of the solution was checked using a p H meter.

Reagent B: - TPTZ solution:

0.031g of TPTZ was added to 10ml of 40mM HCl and dissolved at 50^{o}C.

Reagent C: - Ferric Chloride solution:

0.054g of ferric chloride was dissolved in 10ml of distilled water.

Reagents B and C were freshly prepared each time the assay was performed.

Preparing the FRAP reagent:-

2.5ml of reagent B and 2.5ml of reagent C were added to 25ml of reagent A to make 30ml of the FRAP reagent. This was placed in a 37^{o}C water bath for a minimum of 10 minutes.

The FRAP assay procedure was as follows:

1ml of water and 80µl of test bee product sample was pipetted in to a standard 4ml plastic cuvette. 600µl of the incubated FRAP reagent was added to the cuvette, which was briefly inverted to mix the solutions. A reagent blank was also prepared as described above but 80µl of water was added instead of a test sample.

Change in absorbance at 593nm (a result of reduction of the Fe^{3+}-TPTZ complex to the blue Fe^{2+} TPTZ complex at low pH) was recorded at exactly four minutes using a spectrophotometer. Each test sample dilution was tested in triplicate to allow a mean absorbance to be calculated. A number of dilutions of each bee product were tested allowing dose response curves to be produced.

Standard Calibration curves:

A standard solution of 1mM ferrous sulphate was prepared by dissolving 0.139g of $FeSO_4 \cdot 7H_2O$ in 500ml of distilled water. Serial dilutions were made and the absorbance at 593nm measured by performing the assay as described above with ferrous sulphate in place of test samples.

The Disc diffusion assay:

The disc diffusion method is a slight adaption of that described by Maidment, Dyson & Haysom (2006). (Please refer to Appendix One for full laboratory method).

Materials:

- Microbes as stated above.

Chemicals supplied by Sigma-Aldrich, Gillingham, Dorset, UK:

- Ethanol
- Nutrient agar
- Glucose broth agar (for *S.mutans*)

Procedure:

Using aseptic technique 1ml of bacteria broth solution, prepared the day before, was added to the desired number of petri dishes.

A 13mm whatmann disc was then dipped into the test substance and placed in middle of petri dish. Plates were incubated at 37^{o}C overnight. Zones of inhibition were then measured around the disc with a ruler.

2.3 Data management and data analysis

Experiments were performed in triplicate and results expressed as means ± standard deviations.

Statistical analysis was performed on the mean absorbance/ zone of inhibition of each bee product using a univariate test. A Tukey post hoc analysis was performed. Statistical analyses was undertaken using SPSS version 16 and the level of significance was set to $p < 0.05$.