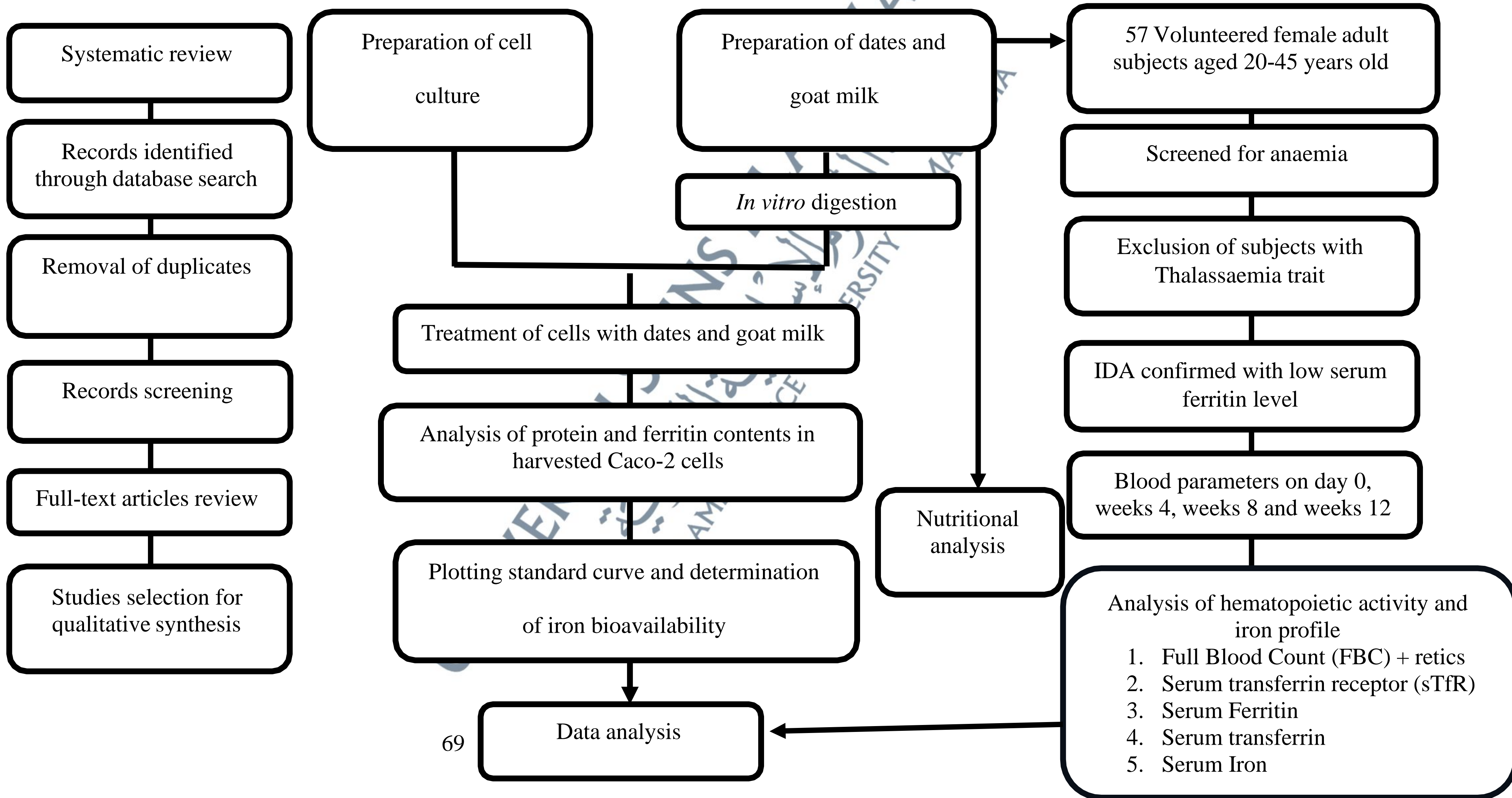
CHAPTER 3

MATERIALS AND METHODS

Research Flow Chart



3.1 SYSTEMATIC REVIEW

3.1.1 Data Sources and Search strategy

a) Date palm: Reports eligible for inclusion in the review were located from the Medline, Ovid, PubMed and Scopus with unlimited publication date. Our search strategy involved using a combination or broad indexing terms for each databases. The following keywords were used for dates palm (*dact*l*fera,* palm* and date*) and anaemia (h*moglobin, an*mia, iron, iron deficiency and blood) respectively. The Medline search strategy was developed and adapted for other databases as required. These are available in supplementary information from the authors upon request. Bibliographies of screened studies and relevant reviews and manuscripts were also searched for eligible studies and a search was conducted by using Google scholar.

b) Goat milk: Studies eligible for inclusion in the review were located from the Medline, Ovid, PubMed and Scopus with unlimited publication date. Our search strategy involved using a combination or broad indexing terms of each databases. The following keywords were used for goat (hircus, hircine, capra and caprine), milk (dairy, whey, cheese, yogurt, butter and cream) and anaemia (h*moglobin, an*mia, Iron, Iron deficiency and blood) respectively. The Medline search strategy was developed and adapted for other databases as required. These are available from the authors upon request. Bibliographies of screened studies and relevant reviews and manuscripts were also searched for eligible studies and a search was conducted by using Google search engine.

3.1.2 Criteria included in the review

a) Date palm: Only experimental studies were eligible for inclusion; only study that used date palm fruit or seed were chosen while study on other part of date palm such as leaves, shoot and root were excluded and the subjects of interest selected were only human or animal population. To be eligible for the review, a study must report validated measures of blood parameters in reducing anaemia after date palm treatment. This systematic review focused on the contributions of iron in date palm toward significantly increased haemoglobin level and reduce anaemia. Besides that, reports on chemical compositions in date palm other than iron (Fe) or irrelevant to IDA were ruled out from the database searching.

b) Goat milk: A study has to meet the following criteria to be eligible for inclusion: Randomised controlled trials, controlled before and after studies, and experimental studies; only study that used goat milk product were chosen and the subjects of interest selected were only human or animal. To be eligible for the review, a study must report validated measures of blood parameters in reducing anaemia after goat milk supplement. Reviews were focused only on the contributions of Fe toward significantly increased in haemoglobin level and reduce Fe deficiency state.

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3.1.3 Identification of Relevant Studies and Data Extraction

All manuscripts from searches were downloaded into an Endnote library. Potentially relevant papers were selected by screening the titles (first step), abstracts (second step) and the entire article (third step) retrieved from the database searches. Two researchers independently conducted this screening. Disagreement about eligibility between the reviewers was solved through discussion with a third researcher. Records of reasons for rejection were kept.

Data was extracted from the included studies into a table. Each study's details including study design, sample size, outcomes, parameters studied, extraction method, duration of exposure, sample collection and details of the study design were listed in the table. Data entry was checked for each study after completing data extraction.

3.1.4 Data reporting

Data reporting involved a descriptive summary. Report of the study was done according to PRISMA guidelines (Moher *et al.*, 2009)

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3.2 Nutritional Analysis

Mariami dates, Ajwa dates, goat milk and goat milk fed with ajwa seed sample was analysed for vitamin C and sugar profile (galactose, glucose, fructose, lactose, maltose and sucrose) using High Performance Liquid Chromatography (HPLC). Each component in a sample combination can be identified, separated, and quantified using the HPLC technique. It uses pumps to move a column of solid adsorbent material through a pressurised liquid solvent containing the sample mixture. Each component in the sample mixture interacts with the adsorbent material differently, resulting in varying flow rates for the various components and allowing the components to separate as they exit the column. Minerals (iron, calcium, magnesium, zinc, copper and manganese) was analysed by using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Chemical element detection is done using the analytical method ICP-OES. It is a sort of emission spectroscopy that creates excited atoms and ions that emit electromagnetic radiation at wavelengths specific to a particular element using an inductively coupled plasma. The intensity of this emission is indicative of the concentration of the element within the sample.

One bottle containing 250 ml goat milk; 250 ml of goat milk fed with ajwa seed; 100 gram Mariami dates; 100 gram Ajwa dates were analysed by PERMULAB SDN BHD located at Petaling Jaya, Selangor.

3.3 Iron Bioavailability Study Using Caco2 Cell Model

The amount of iron that is absorbed from the food and utilised for normal body functions is known as iron biavailability. To evaluate the bioavailability of human iron using in vitro technique, cell culture has been widely used. Human colon adenocarcinoma cell line Caco-2 cell has shown a numerous of enterocyte morphological and biochemical characteristics. These cells spontaneously differentiate into polarized monolayers with a well-developed brush border and associated enzymes (Pinto *et al.* 1983). The mechanisms and regulation of iron absorption and iron bioavailability have been studied using this cell model in a wide range of nutritional research (Au *et al.,* 2000). Using a Caco-2 cell model as a physiological method for evaluating mucosal cell iron uptake seems to be a good approach. This method is a simulation of digestion and absorption in the human intestine using in vitro gastric and intestinal digestion in combination with cultivated Caco-2 cells (Glahn *et al.,* 1998).

3.3.1 Preparation of Media

3.3.1.1 Complete Media (Dulbecco's Modified Eagle Media (DMEM))

DMEM (Sigma Aldrich, USA) complete media was prepared by adding 16% of foetal bovine serum (Sigma Aldrich, USA). Prior to use, 1% of penicillin-streptomycin (100 IU/mL) (Biowest, USA) was added into the DMEM. The media was filtered sterilized using 0.22 µm membrane filter under vacuum condition and stored at 4 ºC prior to use.

3.3.1.2 Cryopreservation of Media

Into DMEM (Sigma Aldrich, USA), 20% foetal bovine serum (Sigma Aldrich, USA), 1% penicillin-streptomycin (100 IU/mL) (Biowest, USA) and 10% dimethyl sulfoxide (DMSO) (Merck, USA) was added. The media was filtered sterilized using 0.22 µm membrane filter under vacuum condition and stored at 4 ^{0}C prior to use.

3.3.2 Preparation of Reagents

3.3.2.1 Hydrochloric Acid, HCI (0.1 M)

Hydrochloric Acid, 0.1M HCL was prepared by diluting 2.05 ml of 12.178 M HCL stock solution into 200 ml volumetric flask with distilled water. After thorough mixing, the 0.1 M HCl were stored at room temperature.

3.3.2.2 Hydrochloric Acid, HCl (5 M)

Hydrochloric Acid, 5 M HCL was prepared by diluting 102.60 ml of 12.178 M HCl stock solution into 250 ml volumetric flaks with distilled water. After thorough mixing, the 5 M HCl were stored at room temperature.

3.3.2.3 Sodium Bicarbonate, $NaHCO_3$ (1 M)

1 M $NaHCO_3$ was prepared by adding 42 gram of $NaHCO_3$ powder (Amresco,USA: Lot no. 0164C410) in 500 ml deionized water. After thorough mixing, the solution were stored at room temperature.

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3.3.2.4 Pepsin solution

Pepsin solution was prepared by dissolving 0.08 mg of pepsin (Amresco, USA: Lot no. 0584C062) in 500 ml 0.1 M HCL. Then, this solution was stored at -20°C.

3.3.2.5 Pancreatin solution

Pancreatin solution was prepared by dissolving 0.8 g of pancreatin (Sigma Aldrich, USA: Lot no. SLBJ8147V) in 200 ml distilled water. Then, this solution was stored at -20°C.

3.3.2.6 Preparation of standard

Ferrous sulphate tablet (Feosol original) containing 65 mg elemental iron was crushed by using mortar crucible and made to the volume of 100 ml using distilled water. From this stock solution (650 µg/mL), the serial dilution of 10.0 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.63 µg/mL, 0.31 µg/mL and 0.16 µg/mL were prepared.

3.3.2.7 Preparation of 1 X RIPA buffer

1 X RIPA buffer was prepared by diluting 1 mL of 10 X RIPA buffer (Nacalai Tesque, Japan) with 8 mL of deionized water and 1 mL of SDS solution in 15 mL sterilized falcon tube. Mixed the solution and stored at -20°C.

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3.3.3 Preparation of Caco-2 Cell Line

3.3.3.1 Thawing of Cells from Frozen Storage

Caco-2 cells in passages 3 to 8 were purchased from American Type Culture Collection. The cryopreserved Caco-2 cells stored in liquid nitrogen were taken out and rapidly thawed at 37°C in a water bath. 1 mL suspension cells were transferred carefully to 25 cm^2 tissue culture flasks containing 4 mL of DMEM media (Sigma Aldrich, USA) for Caco-2 cells with 16% of foetal bovine serum (Sigma Aldrich, USA) and 1% penicillin-streptomycin (Biowest, USA). Caco-2 cells were then incubated at 37°C with 5% CO_2 in an incubator (Thermo Forma, 310 Series) for overnight. Media was discarded to totally remove the DMSO (cryoprotecting agent) and replaced with new complete media. Cells were then allowed to grow in incubator for several days until confluent (80 – 100%).

3.3.3.2 Sub-Culturing of the Cells

The adherent Caco-2 cell was harvested using trypsin-EDTA (0.2% trypsin in 0.5% EDTA) (Gibco, USA). The harvested cells were suspended in complete media and then was centrifuged at 2000 x g for 2 minutes. The media supernatant was carefully discarded. The cells were washed with Dulbecco's Phosphate Buffer Saline (DPBS) (Biowest, USA) twice and tube was centrifuged again for 2 minutes. The cells were resuspended with 3 ml of DMEM (Caco-2 cells) complete media. Cell number and viability were determined to ensure 95% viability. 1 ml of the cell suspension was then aliquoted into new flasks containing 4 ml complete media. Caco-2 cell were then incubated back in incubator at 37°C in 5% CO_2 incubator.

3.3.3.3 Cell Counting

For in vitro cell cultivation, counting cells is an important step. Consistent cell concentrations allow accurate and reproducible experimentation. Cell counts are crucial for assessing the health and rate of cell proliferation, seeding cells for subsequent studies, and preparing for cell-based assays (Ongena *et al*., 2010).

Hundred microliter of cell suspension was mixed with 100 µl (ratio 1:1) 0.4 % trypan blue solution (Amresco, USA). 100 µl of this suspension was put onto a haemacytometer and left for few seconds. Then, counter chamber containing stained cells was observed under inverted microscope using 10X objective lens. Cells were counted from right to left. Characteristic of counted cells were as follows: single cell counted as one, for a group of cell, only the cell which displayed clear nucleus and cytoplasm (consider as life cell) counted as one. Numbers of cells were counted using standard formula as given below:

Cells in suspension (cells/ml) = average number of cells x dilution factor x 10^4

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3.3.3.4 Cryopreservation of the Cells

Cryopreservation was practised for longer storage of cells. Cells at log phase of growth were chosen to be frozen. The cell suspension was centrifuged at 2000 x g for 5 minutes. Then, the cell was resuspended with DMEM (Sigma Aldrich, USA) containing 20% FBS (Sigma Aldrich, USA), 1% penicillin-streptomycin (Biowest, USA) and 10% DMSO (Merck, USA). 1 ml of cell suspension was then transferred into each cryovial. Subsequently, tubes were placed on ice for 15 minutes prior transferring to -20°C for 1 hour. Then, the cryovials were placed into -80°C overnight for short term storage and liquid nitrogen for long term storage.

3.3.4 Preparation of date palm and goat milk for iron bioavailability study

a) Ajwa dates originated from Iran supplied by Saudagar Kurma, Penang and Mariami dates originated from Iran supplied by Pershia Green Fruit Sdn Bhd, Ampang was used in this study. The dates were completely dried by keeping in incubator at 70°C for 5 hours. Then, they were grinded into small particles using a domestic blender and pooled.

b) Pasteurized goat milk from Lembah Paya Goat Farm, Sepang and goat milk obtained from a group of goats fed with Ajwa dates seed (group 5 NRGS) were used in this study. The goat milk was stored at -20°C until further use.

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3.3.5 *In vitro* digestion

In vitro digestion is an assays simulate the physiological conditions of digestion in vivo and are useful tools for studying and understanding changes, interactions, as well as the bioaccessibility of nutrients (González *et al.,* 2018).

a) Ajwa and Mariami date palm

Dried sample of dates were subjected to the extraction method as described by Scheers *et al.* with minor modifications (Scheers *et al.,* 2014). One gram of dates was dissolved in 10 ml of distilled water. The pH was adjusted to 2 using 5.0 M HCl using pH meter (Hanna Instruments). Then, 0.3 ml pepsin solution was added into the solution. The tubes were then kept on incubator shaker (New Brunswick Scientific, Innova 40) at 37°C and 2000 g for 60 minutes. To simulate the intestinal digestion, the sample's pH was adjusted to 7 by addition of 1 M $NaHCO_3$ into the solution and the sample was further digested with 1.7 ml pancreatin for 30 minutes at 37°C. The digested sample were filtered through a membrane with a 10-kD cutoff (Millipore) at 37°C and 2000 g for 45 minutes to imitate the filtering effect of the mucus layer and stored at 4°C for further use.

b) Goat milk and Goat milk obtained from goats fed with Ajwa seed

Goat milk were subjected to the extraction method as described by Scheers *et al.* with minor modifications (Scheers *et al.,* 2014). Ten millilitre of pasteurized goat milk sample was prepared by transferring into clean 15 mL screw cap tubes, and the pH was adjusted to 2.0 using 5.0 M HCl. Then, 0.3 ml pepsin solution was added to the solution. The tubes were then kept on incubator shaker (New Brunswick Scientific, Innova 40) at 37ºC and 2000 g for 60 minutes. To simulate the intestinal digestion, the sample's pH was adjusted to 7 by addition of 1 M $NaHCO_3$ into the solution and the sample was further digested with 1.7 ml pancreatin for 30 minutes at 37ºC. The digested sample was centrifuged at 12,000 g for 30 minutes to remove debris (pellet) and fat (supernatant). Then, the solution were filtered through a membrane with a 10-kD cutoff (Millipore) at 37ºC and 2000 g for 45 minutes to imitate the filtering effect of the mucus layer and stored at -20ºC for further use.

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3.3.6 Cell viability

A population's percentage of healthy, alive cells is known as cell viability. Cell viability assays are used to evaluate the general condition of cells, perfect experimental or culture settings, and monitor cell survival after drug treatment. Cell viability assays are important tools for assessing cellular responses to experimental compounds of interest (Cell Signaling Technology, 2022)

Cytotoxicity assay was determined by Cell Titer 96® $_{\text{Aqueous}}$ One Solution Cell Proliferation assay (Promega, USA). The solution contains tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and phenazine ethosulfate (PES). PES has enhanced chemical stability which stabilized the MTS solution when combined. The MTS compound is bio reduced by cells into a coloured formazon product. This formazon product is soluble in tissue culture medium, a characteristic which can eliminate solubilisation step normally required for MTT assays before recording the absorbance. The number of living cells is directly proportional to quantity of formazon product as measured by the absorbance at 490 nm.

Caco-2 cell line was maintained in DMEM medium (Sigma Aldrich, USA) supplemented with 10% foetal bovine serum (Sigma Aldrich, USA) and 1% penicÿillin-streptomycin (Biowest, USA). Number of cell was measured as described (in **Section 3.3.3.3**) and diluted in complete media to reach a density of 1.0 x 10^5 cells/mL. One hundred microliter of this cell suspension was seeded per well of 96-well plate. Several wells containing media without cells were also included in the 96-well plate. Then, this 96-well plate was incubated at 37ºC in a humidified 5% CO_2 atmosphere for 7 days. On day 7th, the cells were treated where

100 µL of date palm extract and goat milk were added to the respective wells. For control and untreated cells, 100 µL of media was added into respective wells containing media only (control) and the cells (untreated cells). The final volume of each well is 200 µL. Date palm extract in complete media were started at concentration of 0 mg/mL, 100 mg/mL, 500 mg/mL and 1000 mg/mL while goat milk were started at concentration of 0 v/v%, 10 v/v%,, 50 v/v%, and 100 v/v%. The treated, untreated cells and control were incubated for 24, 48 and 72 hours prior to addition of 40 µL per well of Cell Titer 96® $_{\text{Aqueous}}$ One Solution reagent. Each experiment was performed in three replicates. The plate was further incubated at 37°C in a humidified 5% CO_2 atmosphere for another 4 hours before measuring the absorbance at 490 nm by (Infinite M200 Pro, Tecan) ELISA Microplate Reader.

The cell viability was determined by MTS assay since formazan produced is directly proportional to the number of viable cells. The absorbance values of 96-well plate for cytotoxicity analysis were measured by using Tecan Sunrise ELISA Microplate Reader equipped with Magellan Data Analysis Software. First, the average absorbance value of wells containing media only (without cells) was determined and assigned as baseline value (blank). Average absorbance of treated cell for respective concentration as well as untreated cells (without treated extract) were also determined. The mean values and the percentage were calculated using Microsoft Office Excel 2010. The cell viability is calculated as follow:

$$\text{Cells viability (\%)} = \frac{(\text{Average absorbance value of treated cells} - \text{baseline value})}{(\text{Average absorbance value of untreated cells} - \text{baseline value})} \quad X\ 100$$

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3.3.7 Cell experiment for ferritin/protein analysis and iron uptake

For bioavailability studies, The Caco-2 cells (1.0 x 10^5 cells/mL) were cultured in 6 well plate designed together with transwell chamber (SPL Life Sciences Co., Ltd, Korea). Each transwell chamber was designed with the inside wall being treated for uniform cell attachment (**Figure 3.1**). The surface area of the membrane is 0.4 µm pore size. Caco-2 cell were incubated and maintained in DMEM (Sigma Aldrich, USA) containing 10% FBS (Sigma Aldrich, USA), 1% penicillin-streptomycin (Biowest, USA) at 37 ºC in a humidified 5% CO_2 atmosphere. At 12–14 days post seeding, cells reached 90–100% confluency and were used for iron uptake experiments.

After the cell reach 100% confluency, the medium was removed from the well and cell were washed with 1 mL of DPBS (Biowest, USA) twice on both apical and basal chambers. Then, 1.5 mL of digested sample were transferred to the apical chamber. The standard and samples were tested in triplicates, and one well in each plate was used as blank by transferring complete media instead of digested sample to the apical chamber of the well . The plate was lidded and incubated at 37°C with 5% CO^2 for 1 hour, 2 hours and 3 hours. After incubation, the apical and basal chamber medium were aspirated. The cells were rinsed with 1 mL DPBS (Biowest, USA) and incubated in complete media for another 21 hours to allow ferritin formation in the Caco-2 cells. Each experiment was performed in three replicates

After 21 hours, the complete media were removed and the cells were washed with 1 mL of DPBS (Biowest, USA) twice. After removing the buffer, 1 mL of 1 X RIPA buffer was added to the culture plate and stirred slowly for 5 minutes. Then, the cells was scraped completely with a scraper. The lysate with a

pellet were transferred to new microtube. The culture plate was rinsed with 400 µL 1 X RIPA buffer and the solution was transferred to the microtube. Then, the samples was incubated for 15 minutes on ice. After 15 minutes, the lysate was transferred to a new microtube and centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant containing total proteins was transferred to a new tube and kept at -80°C for further analysis.

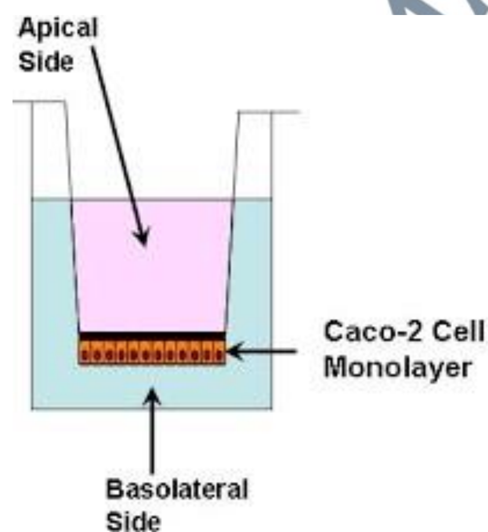

Figure 3.1: Caco-2 cell attachment used in iron bioavailability study model

3.3.8 Iron calorimetric assay

The iron content in the samples was measured using iron colorimetric assay kit (Bioversion, USA) which was reported to give comparable result with atomic absorption spectroscopy (Hosseinimehr *et al.*, 2007).

The iron standard was prepared by diluting 10 µL of the 100 mM iron standard with 990 µL deionized water to generate 1 mM standard iron. The serial dilution of

iron standard and 50 µL of samples was added into each 96 well plate. The assay buffer was added into the well to bring the volume into 100 µL. Then 5 µL of iron reducer was added to each standard and samples well. The standard and samples were incubated for 30 minutes at 25°C. Then, 100 µL of iron probe was added into each well and incubated, protected from light for 60 minutes at 25°C. The absorbance was measured at 593 nm in a Tecan Sunrise microplate reader. Each experiment was performed in three replicates.

3.3.9 Ferritin assay

Ferritin was measured using human ferritin ELISA kit (Sigma-Aldrich, USA: Product no. RAB0197). Standard solution was prepared by diluting 200 ng/mL ferritin solution with 1 X assay dilution to produce a dilution series. Then, 100 µL of each standard and sample was added into appropriate wells. Microplate was covered and incubated for 2.5 hours at room temperature with gently shaking using microplate shaker (Major Science Rocking Shaker). The solution was discarded and washed 4 times with 300 µL of 1 X wash buffer. After completion of the last wash, the plate was blotted against clean paper towels and 100 µL of detection antibody was added to each well. The plate was incubated for 1 hour at room temperature with gentle shaking. The solution was discarded and the wash procedure was repeated. Hundred microliter of streptavidin solution was added into the well and incubated for 45 minutes at room temperature with gently shaking. After 45 minutes, the solution was discarded and the wash procedure was repeated. Hundred microliter of TMB one-step substrate reagent was added into each well and incubated for 30 minutes at room temperature with gentle shaking. Lastly, 50

µL of stop solution was added into each well and the absorbance was measured at 450 nm immediately using microplate reader (Infinite M200 Pro, Tecan)

3.3.10 Protein assay

Protein concentration of the lysate was assayed using protein assay CBB solution (Nacalai Tesque, Japan: Product no. 11617). Standard solution was prepared by diluting 2 mg/mL albumin solution with 1 X RIPA buffer. Ten microliter of serial dilution of albumin and samples was added into each 96 well plate. Then, 200 µL of CBB solution was added into each well. Standard and sample were mixed with a microplate shaker (Major Science Rocking Shaker) for 10 minutes. The absorbance was measured at 595 nm using Tecan Sunrise microplate reader after 1 hour incubation. Each experiment was performed in three replicates.

3.3.11 Iron bioavailability

Iron bioavailability was calculated based on method produced by Nikooyeh and Neyestani (2016). The ferritin concentration was standardised against the total protein concentration and ferritin (ng)/protein (mg) was used as an indicator of iron uptake and absorption by the Caco-2 cells. After analysis of protein and ferritin contents in harvested Caco-2 cell, the standard curve was plotted using iron concentration and ferritin/protein ratio in X and Y axes respectively. Iron bioavailability from the samples was estimated by comparing the iron standard (serial dilution of ferrous sulphate) with similar iron concentration of samples in

the standard curve. Then, the linear equation was calculated using Excel 2010. The percentage of iron bioavailability was calculated as:

$$\frac{\textit{(ng ferritin/mg protein of the sample)}}{\textit{(ng ferritin/mg protein of the standard with same amount of iron)}} \times 100$$

3.4 Human Study

3.4.1 Study design

This is a quantitative study examining the effect of date palm and goat milk on haematological parameter and iron profile among IDA subject. The flow of the study is as depicted in **Figure 3.2.**

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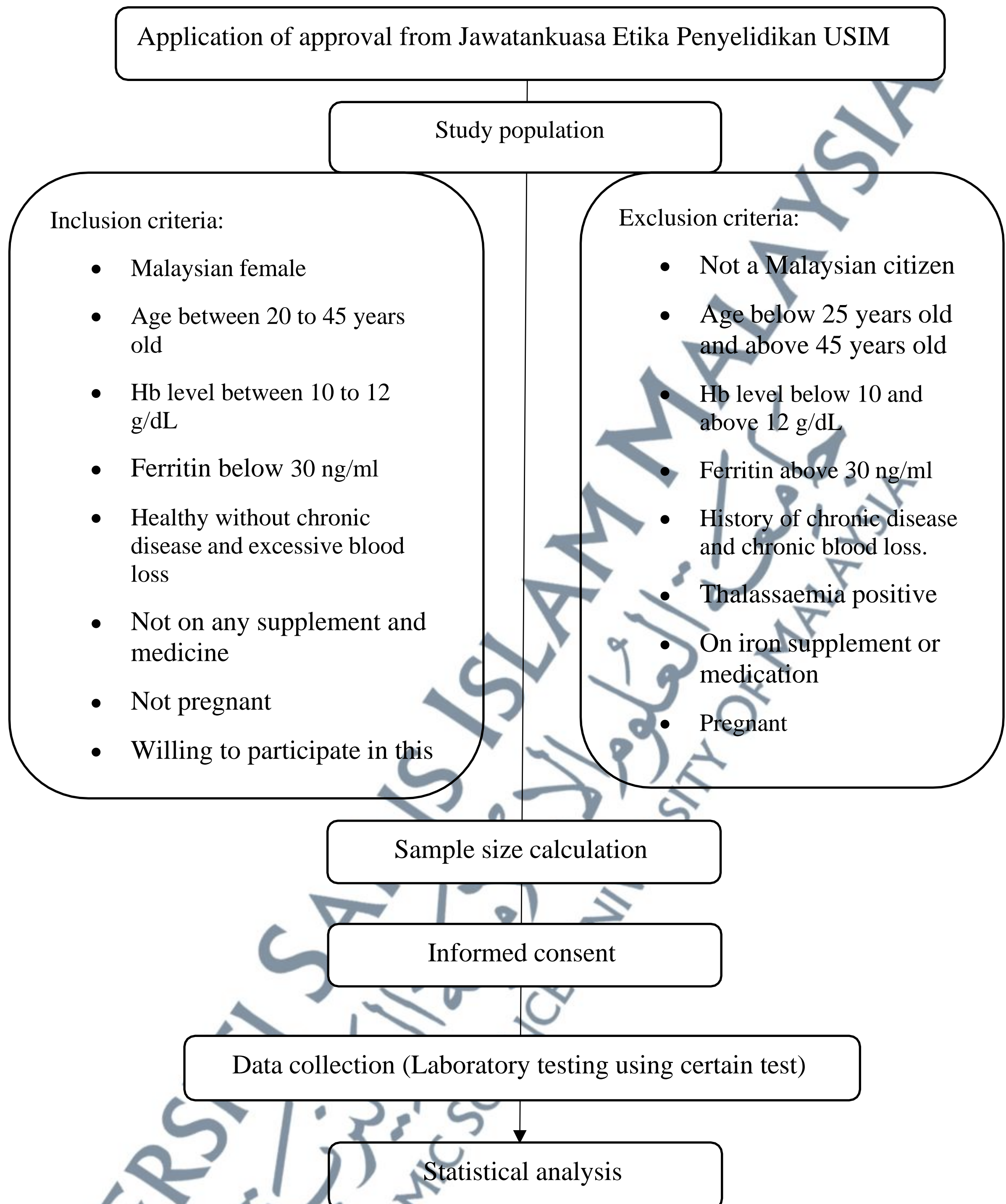

Figure 3.2: General view of study design

3.4.2 Study population

The study population was among female adult aged 20-45 years old with mild iron deficiency anaemia (Hb level between 10 to 12 g/dL).

3.4.3 Location of study

The study was conducted at Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, Pandan Indah, Ampang, Kuala Lumpur.

3.4.4 Ethical approval

For this study, approval to run the experiment was obtained from Jawatankuasa Etika Penyelidikan USIM (Code: USIM/JKEP-2016-5 (**Appendix 1**). Prior to the study, informed consent from the respondents was taken into account (**Appendix 2**). The respondents were informed about the purpose of the research, confidentiality and that participation is voluntary without any forces.

3.4.5 Sample size

Sample size calculation was done by using OpenEpi software, version 3. The sample size was calculated based on the reported IDA prevalence in Malaysia (Loh and Khor, 2010).

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Sample Size for Frequency in a Population

Population size (for finite population correction factor or fpc) (*N*):	450
Hypothesized % frequency of outcome factor in the population (*p*):	3.5%+/-5
Confidence limits as % of 100(absolute +/- %) (*d*) :	5%
Design effect (for cluster surveys-*DEFF*):	1

Sample Size(*n*) for Various Confidence Levels

Confidence Level (%)	Sample Size
95%	47
80%	22
90%	34
97%	56
99%	75
99.9%	111
99.99%	141

Equation

Sample size $n = [DEFF*Np(1-p)]/ [(d^2/Z^2_{1-\alpha/2}*(N-1) + p*(1-p)]$

Table 3.1: Sample size calculation using OpenEpi Software, Version 3.

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Forty-seven (47) subjects were recruited with a confidence level of 95%. By adding 20% of the possible dropout rate, a total of 57 subjects should be selected in this study.

3.4.6 Subject selection

Subjects comprised of female adult aged 20 - 45 years old with mild anaemia. Those with haemoglobin level between 10 to 12 g/dl were tested for ferritin levels to confirm iron deficiency anaemia. Potential subjects were screened for thalassaemia before final selection. Cases positive for thalassaemia was excluded from the subject pool. Selected subjects were then divided into 5 groups and assigned with different feeding regime.

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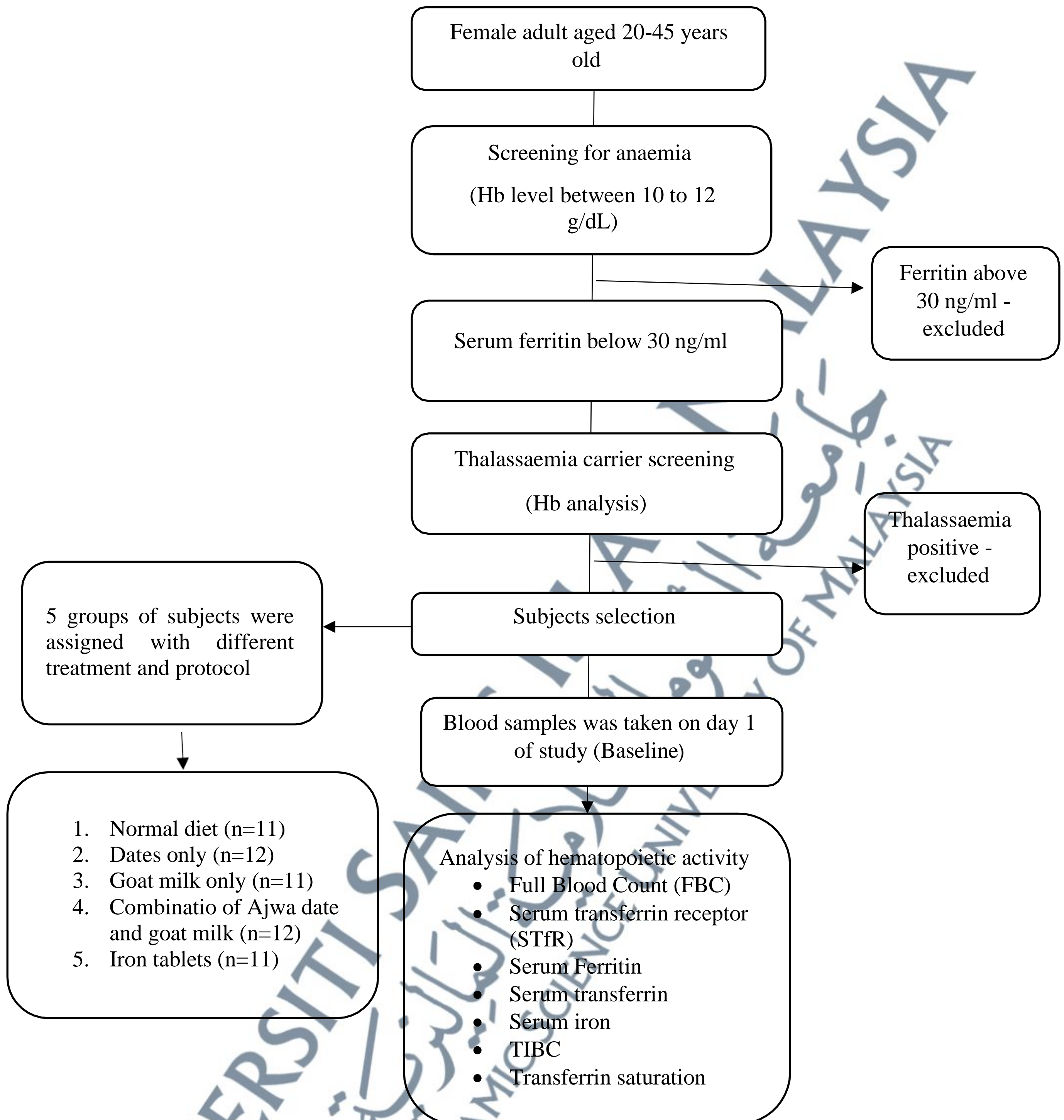

Figure 3.3: Illustration of the study workflow and subject selection

3.4.7 Treatment Protocol

Treatment protocol is based on simulation of the Prophet's practices. This simulation was performed on the subjects where they were supplemented with 5 pieces of Ajwa dates originated from Iran supplied by Saudagar Kurma in Penang, 250 ml of goat milk from Lembah Paya Goat Farm, Sepang and iron tablet (Hovid Fefur ferrous fumarate 100 mg) every morning for 12 weeks duration. Compliance was monitored closely by giving each of them a food diary (**Appendix 3**) to record daily food intake. Twelve subjects were assigned to each group (total of 5 groups) as the following:

- Group 1: Normal diet (Negative Control)
- Group 2: Dates only
- Group 3: Goat milk only
- Group 4: Combination of dates and goat milk
- Group 5: Iron tablets (Positive Control)

3.4.8 Sample Collection

Blood samples was taken on day 1 of study and repeated at 4 weeks, 8 weeks and 12 weeks of study. The blood samples was taken by venepuncture procedure. Three (3) mL of blood was transferred into each K_2 EDTA tube and plain tube. The sample were labelled with subject's unique requisition identifier.

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3.4.9 Blood sample preparation

The plain tube was centrifuged (Hettich Rotofix 32 A) at 1500 g for 10 minutes to separate the serum from blood cell. The serum was later collected for further analysis.

3.4.10 Haematological analysis

3.4.10.1 Haemoglobin screening

For the anaemia screening purposes, a blood sample was taken from the tip of finger by a finger prick technique. The finger prick site was cleaned with an alcohol swab. The sterile lancet was used to make the skin puncture at the centre of the fingertip. Sufficient quantities of blood was collected using haemoglobin cuvette (DiaSpect Medical GmbH, Germany) and blood was analysed for haemoglobin level using haemoglobin analyser (DiaSpect Medical GmbH, Germany)

3.4.10.2 Full blood count

Full blood count was analysed using automated haematology analyser (Sysmex XE-2100). XE-2100 performs haematology analyses according to the RF/DC detection method, HydroDynamic Focusing (DC Detection), flow cytometry method (using the semiconductor laser) and SLS-haemoglobin method. It utilizes the technology of fluorescence flow cytometry to quantitate the standard five part differential, immature granulocytes (metamyelocytes, myelocytes and promyelocytes), nucleated red blood cells (NRBC), reticulocyte count, immature

reticulocyte fraction and "optical" fluorescent platelet count. The combination of side scatter (inner complexity of the cell), forward scatter (volume) and fluorescence intensity of nucleated cells gives a concise but precise image of each cell detected in the peripheral blood. The blood sample was mixed by vertical inversion for 10 times. Then, the tube cap was opened carefully and placed at the aspiration pipette and pressed [START]. After two beeps of sound, the tube was removed from the aspiration pipette. Two copies of results was automatically printed by the analyser.

3.4.10.3 Iron profile analysis

a) Serum iron, total iron binding capacity and transferrin

Serum iron, total iron binding capacity and transferrin was analysed by using automated analyser (Siemans Advia-2400). Siemans Advia-2400 performs analysis according to the spectrophotometry detection method. Spectrophotometry is a method to measure a chemical substance absorbs or transmit light over a certain range of wavelength by measuring the intensity of light as a beam of light passes through sample solution. The human sample was placed on the sample tray. The sample was aspirated and diluted by the dilution probe then dispensed into cuvettes in the dilution tray. The photometer then measures the absorbance based on the optical density of the cuvettes.

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b) Transferrin saturation

Transferrin saturation is a calculation representing the percentage of transferrin that is saturated with iron. The value of the transferrin saturation was calculated according to the formula below:

(Serum iron level / total iron-binding capacity) X 100

c) Ferritin

Ferritin was measured using human ferritin ELISA kit (Elabscience, USA: Cat no. E-EL-H2009). This ELISA kit uses Sandwich-ELISA method. The micro ELISA plate has been pre-coated with an antibody specific to human ferritin. Standard was prepared by diluting the reference standard with sample diluent to generate 200 ng/mL stock solution for serial dilution. Then, 100 µL of each standard, blank and sample was added into appropriate wells. Microplate was covered and incubated for 90 minutes at 37°C. Liquid of each well was removed and 100 µL of biotinylated detection antibody working solution was added immediately to each well. Microplate was covered with plate sealer and incubated for 1 hour at 37°C. Each well was aspirated and washed for 3 times with 350 µL of washing buffer. After a last wash, the plate was blot against clean absorbent paper. Hundred microliter of HRP conjugate was added into each well and incubated for 30 minutes at 37°C. The washing process was repeated for five times before 90 µL of substrate added into each well and incubated for 15 minutes at 37°C. Stop solution (50 µL) was added into each well and optical density was determined using microplate reader (Infinite M200 Pro, Tecan) at 450 nm wavelength.

d) Serum transferrin receptor

Serum transferrin receptor was measured using human transferrin receptor ELISA kit (Elabscience, USA: Cat no. E-EL-H2345) which uses Sandwich-ELISA method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to TFR/CD71. Standard was prepared by diluting reference standard with sample diluent to generate 10 ng/mL stock solution and a serial dilution was prepared. Then, 100 µL of each standard, blank and sample was added into appropriate wells. Microplate was covered and incubated for 90 minutes at 37°C. After that, liquid of each well was removed and immediately added 100 µL of biotinylated detection antibody working solution to each well. Microplate was covered with plate sealer and incubated for 1 hour at 37°C. Then, each well was aspirated and washed for 3 times with 350 µL of washing buffer. After a last wash, the plate was blot against clean absorbent paper. Hundred microliter of HRP conjugate was added into each well and incubated for 30 minutes at 37°C. Then, wash process was repeated for five times before adding 90 µL substrate into each well and incubated for 15 minutes at 37°C. Lastly, 50 µL of stop solution was added into each well and optical density was determined using microplate reader (Infinite M200 Pro, Tecan) at wavelength 450 nm.

3.5 Statistical analysis

Data was analysed using SPSS version 21.0 for windows (IBM Corp. Released 2012.Armonk, NY). Descriptive statistics were used to present mean and median. Non-parametric test such as Wilcoxon signed rank test was used to compare median before and after given a treatment. Besides that, Kruskal Wallis test followed by post hoc with Bonferroni correction were used to determine significant association between groups in human study. Iron uptake experiments were carried out in three times independently, with each treatment performed in triplicate. A mean of nine replicates was calculated for each treatment. The data is presented as the mean +/- SD and the differences between samples were analysed using one-way ANOVA followed by post hoc test. All statistical tests were two-tailed and P-value was set at 0.05 for significant level.

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