CHAPTER III

RESEARCH METHODOLOGY

3.1 Materials and Equipment

Materials used were Java tea leaves and sappan wood (both from Biofarmaka Study Center LPPM IPB), elephant ginger, lime, kaffir lime, and Javanese turmeric that were obtained from Sinpasa Modern Market Serpong. Red fruit oil (long red fruit) from CV. Made Mulya Asih Papua, and Carboxymethylcellulose (CMC) (PT. Hade Putra Persada). To make the drink, there were some ingredients added, including sucrose (Gulaku), synthetic sugar (Acesulfame and Sucralose) from PT. Chemco Prima Mandiri, IMP:GMP (PT. Ajinomoto), xanthan gum (PT. Chemco Prima Mandiri), lemon flavor (PT. KH Roberts Indonesia), and distilled water. For analysis, the materials were Folin-Ciocalteu (Merck), gallic acid (Merck), sodium carbonate (Merck), acetone (Merck), sodium hydroxide, and methanol (Merck).

Equipment used were heat plate stirrer (Barnstead Thermolyne Cimarec), Heidolph RZR-1 Overhead Stirrer, chromameter (Konika Minolta), UV-Vis spectrophotometer (Hitachi U-1800), viscometer (Brookfield Digital Model DV-II), centrifuge (Boeco M-240R), vortex (Barnstead Thermolyne), oven (Memmert), cabinet dryer, dry blender (Philips), analytical balance, pH meter, thermometer, evaporating dishes, beaker glass, cuvette quartz (Hellma), centrifuge tubes, dropping pipette, graduated cylinders, Mohr pipet, volumetric flask, stirring rod, spatula, funnel, sterile bottles, and test tubes.

3.2 Research Procedures

The flowchart of the research methodology can be seen in Figure 3.2.

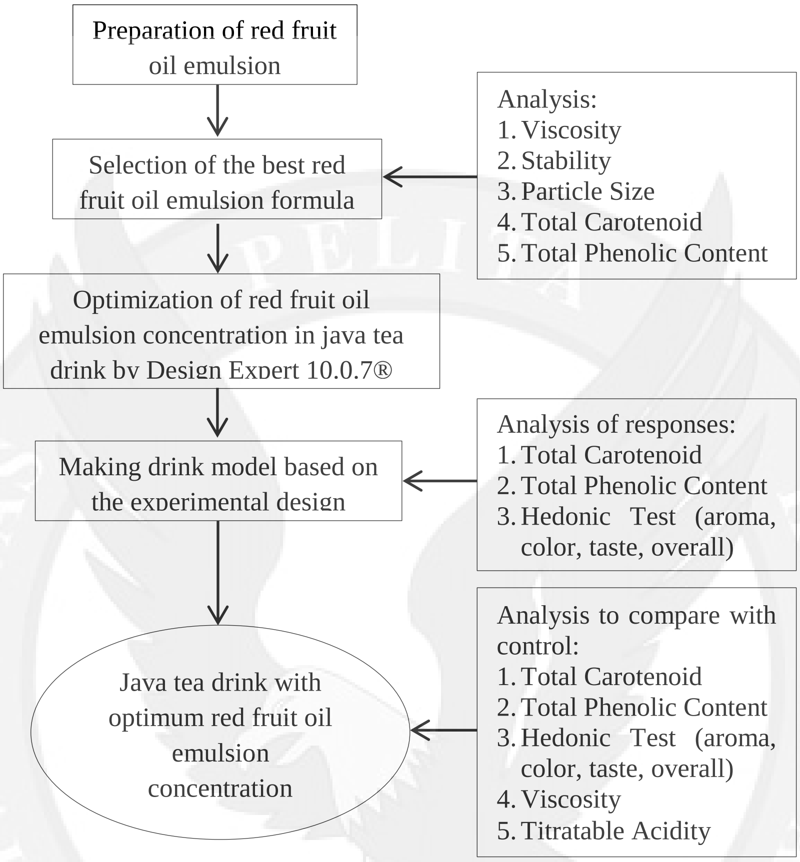

Figure 3.1 Flowchart of research methodology

3.2.1 Research Stage I

Research stage I was done to obtain a stable, low viscosity, high phenolic, and carotenoids content red fruit oil emulsion that would be used in stage II for functional drink optimization. The red fruit oil emulsion in this research was prepared by the method from Cahyadi (2011), in which the carboxymethylcellulose (CMC), as a stabilizer (1.00% and 1.50%), was firstly diluted in water by heat plate stirrer (1000 rpm, 10 minutes), then red fruit oil (10%, 15%, and 20%) was added and stirred by using overhead stirrer at scale 5

(±1000 rpm, 10 minutes). The flowchart of emulsion processing can be seen on Figure 3.2 and the formulation of red fruit oil emulsion can be seen on Table 3.1. The emulsions were characterized in terms of its stability, viscosity, particle size, phenolic content, and total carotenoids content as shown in Figure 3.2 above.

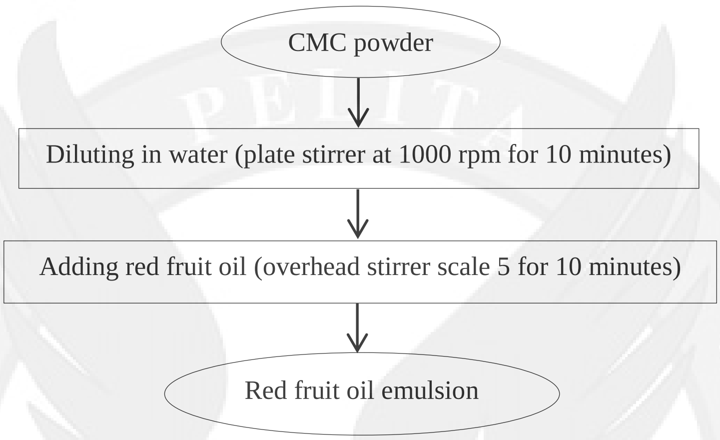

Figure 3.2 Flowchart of red fruit oil emulsion preparation
Source: Cahyadi (2011) with modifications

Table 3.1 Formulation of red fruit oil emulsion

Ingredients	Formula					
	A1B1	A1B2	A1B3	A2B1	A2B2	A2B3
Red fruit oil (ml)	10	15	20	10	15	20
Demineralized water (ml)	90	85	80	90	85	80
CMC	1.00%	1.00%	1.00%	1.50%	1.50%	1.50%

Source: Cahyadi (2011) with modifications

3.2.2 Research Stage II

Research stage II was aimed to obtain optimum concentration of red fruit emulsion to be added to Java tea-based functional drink so that the antioxidant activity and sensory acceptance (aroma, color, taste, and overall) of the drink were optimum.

3.2.2.1 Preparation of Java Tea-based Functional Drink

3.2.2.1.1 Extraction of Herbs

Java tea, ginger, sappan wood, and Javanese turmeric were extracted simultaneously. After being weighed according to formula, the herbs were mixed in a beaker glass and solubilized with the addition of 95^{o}C distilled water. The mixture was stirred for 30 minutes by using plate stirrer. The herbs extract was then filtered and the filtrate was bottled. The bottled extract was then pasteurized at 80^{o}C for 15 minutes and cooled before storage. The procedure of the extraction is shown at Figure 3.3.

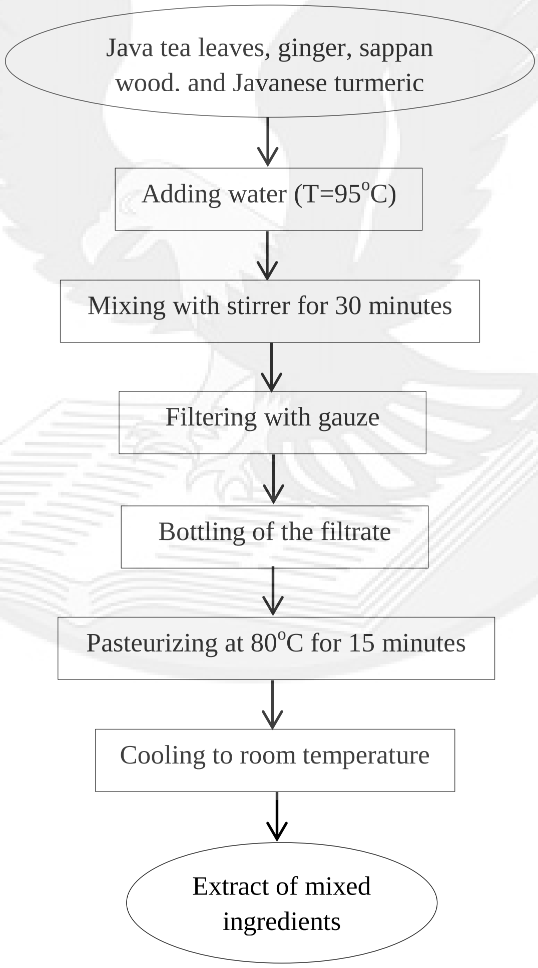

Figure 3.3 Flowchart of herbs extraction
Source: Ng (2016) with modification

3.2.2.1.2 Extraction of Citrus (Ng, 2016 with modification)

The citrus used in the formulation of the drink were lime and kaffir lime. To extract, both lime and kaffir lime were firstly washed, then sliced into two parts and squeezed and filtered separately. The extracts were then weighed according to the formula and mixed into a beaker. The mixture of lime and kaffir lime extracts were put into a sterile bottle and pasteurized at 80°C for 15 minutes.

3.2.2.1.3 Preparation of 1% Acesulfame Stock Solution (Febriani, 2012)

Acesulfame stock solution was made by dissolving 5 g of acesulfame powder into 500 ml of drinking water, stirred until fully dissolved, and stored at refrigerator before usage.

3.2.2.1.4 Preparation of 1% Sucralose Stock Solution (Febriani, 2012)

Sucralose stock solution was made by dissolving 1 g of sucralose powder into 100 ml of drinking water, stirred until fully dissolved, and stored at refrigerator before usage.

3.2.2.1.5 Preparation of 1% Xanthan Gum Stock Solution (Ng, 2016)

10 grams of hydrocolloid powder was added to 1000 ml 65 °C water and stirred with magnetic stirrer on 70-80°C hotplate until all powder was dissolved completely. The stock solution was filled into sterile bottle and kept in room temperature for a night. On the next day, the solution was stored in refrigerator.

3.2.2.2 Procedure of Java Tea-Based Functional Drink (Herold, 2007)

The procedure of Java tea-based functional drink making was shown in Figure 3.4 and the ingredients were shown in Table 3.2. To make the drink, all the ingredients were mixed according to formula and added with 100°C distilled water, then stirred until homogenous while being heated to reach the temperature of 80°C. The solution was then poured into a sterile bottle and pasteurized at 80°C for 15 minutes. The drink was cooled down to room temperature before storage.

Table 3.2 Ingredients of Java tea-based functional drink

No	Ingredients	Amount (in 100ml)
1.	Mixed herbs extract	*
2.	Mixed citrus extract	*
3.	Sweetener (sucrose, acesulfame, and sucralose)	*
4.	IMP:GMP	*
5.	Xanthan gum stock solution	*
6.	Salt	*
7.	Lemon flavor	*
8.	Water	*

Source: IPB Patent p00200700564

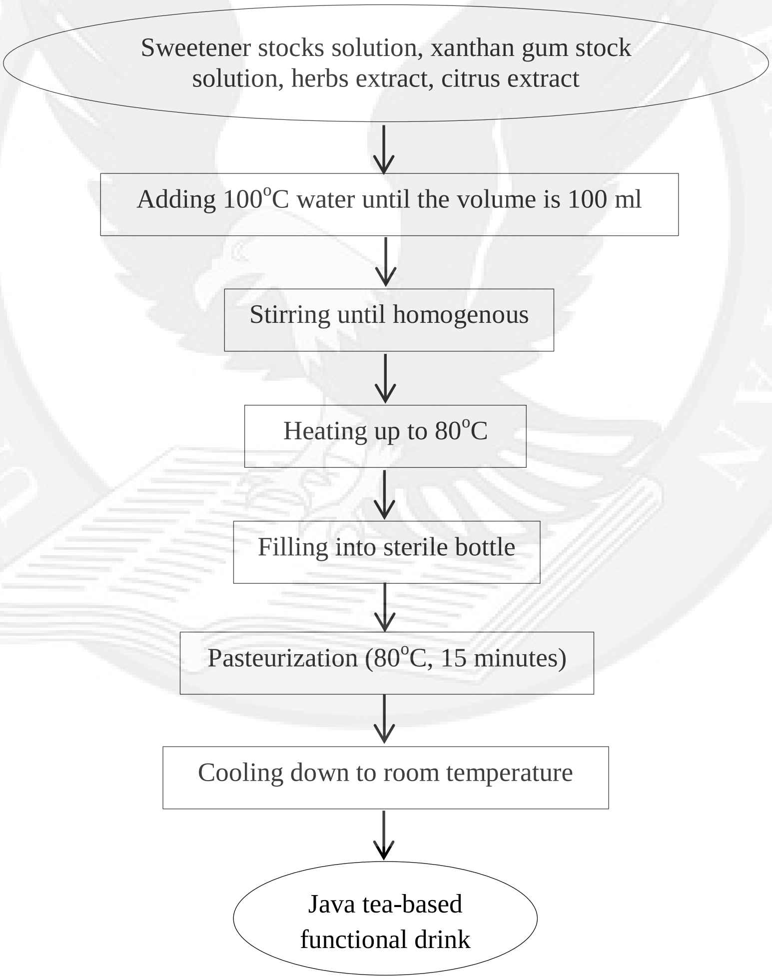

Figure 3.4 Flowchart of Java tea-based functional drink preparation
Source: Herold (2007)

The enriched functional drink was made simply by mixing the Java tea-based functional drink that had been prepared with red fruit oil emulsion. The procedure of functional drink enriched with red fruit oil emulsion was shown in Figure 3.5.

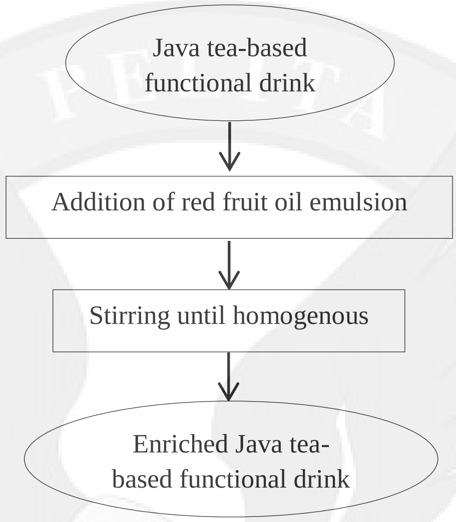

Figure 3.5 Flowchart of enriched Java tea-based functional drink preparation

3.3 Experimental Design

3.3.1 Research Stage I

In research stage I, the experimental design used was Completely Randomized Design with two factors, which were CMC concentration and red fruit oil concentration, and three replications. The variations of CMC concentrations were 1.00% (A_1) and 1.50% (A_2) and the variations of red fruit oil concentration were 10% (B_1), 15% (B_2), and 20% (B_3). Table 3.2 shows the experimental design of stage I research.

Table 3.3 Experimental design of research stage I

		Red Fruit Oil Concentration		
		10%(B_1)	15% (B_2)	20% (B_3)
CMC Concentration	1.00% (A_1)	$(A_1B_1)_1$	$(A_1B_2)_1$	$(A_1B_3)_1$
		$(A_1B_1)_2$	$(A_1B_2)_2$	$(A_1B_3)_2$
		$(A_1B_1)_3$	$(A_1B_2)_3$	$(A_1B_3)_3$
	1.50% (A_2)	$(A_2B_1)_1$	$(A_2B_2)_1$	$(A_2B_3)_1$
		$(A_2B_1)_2$	$(A_2B_2)_2$	$(A_2B_3)_2$
		$(A_2B_1)_3$	$(A_2B_2)_3$	$(A_2B_3)_3$

Linear method used in this research was:

$$Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + \mathcal{E}_{ijk}$$

Where:

i = level i (1, 2)

j = level j (1, 2, 3)

Y_{ijk} = observed value level one, with factors of CMC concentration at level i, red fruit oil concentration at level j, and replicate at level k

μ = actual mean value

A_i = effect of CMC concentration at level i

B_j = effect of red fruit oil concentration at level j

$\mathcal{E}_i$ = error factor

$(AB)_{ij}$ = effect of interaction of treatment A at level i and treatment B at level j

Hypothesis:

H_o:

1. There is significant effect of different CMC concentration towards the characteristics of emulsion.
2. There is significant effect of different red fruit oil concentration towards the characteristics of emulsion.

3. There is interaction between CMC concentration and red fruit oil concentration towards the characteristics of emulsion.

H_1:

1. There is no significant effect of different CMC concentration towards the characteristics of emulsion.
2. There is no significant effect of different red fruit oil concentration towards the characteristics of emulsion.
3. There is no interaction between CMC concentration and red fruit oil concentration towards the characteristics of emulsion.

3.3.2 Research Stage II

The experimental design used for the stage II research was Response Surface Methodology (RSM). The design used was One Factor Design with red fruit emulsion concentration as the factor. To do the optimization, firstly lower and upper limits of red fruit emulsion addition were determined (by using One-way ANOVA for one-factorial Completely Randomized Design). The range of the upper and lower limit was then input into Design Expert 10.0.7® trial program, as well as responses aimed to be optimized. The program established seven runs and predicted one optimum formulation with highest desirability value based on the actual response values of the seven runs. Then the optimum formulation woukd be verified (prediction value of optimum formula was compared to actual value) and the selected one will be compared to Java tea-based functional drink without the addition of red fruit emulsion (control) by using Completely Randomized Design (One-Way ANOVA).

3.3.2.1 Determination of Lower and Upper Limit

To determine the lower and upper limits of red fruit emulsion concentration, One-way ANOVA was used. The variations of the emulsion concentration in the drink were 2%, 4%, 6%, and 8% ($^{w}/_{w}$). The response measured was overall sensory acceptability.

Linear method used in this stage was:

$$Y_i = \mu + A_i + \varepsilon_i$$

Where:

i = level i (1, 2, 3, 4)

Y_i = observation value of treatment i

A_i = emulsion concentration at level i

ε_i = error factor

Hypothesis:

H_0 = concentration of red fruit emulsion gave no significant effect to overall sensory acceptance of Java tea-based functional drink

H_1= concentration of red fruit emulsion gave a significant effect to overall sensory acceptance of Java tea-based functional drink

3.3.2.2 Determination of Optimum Emulsion Concentration

After the lower and upper limits were obtained, they were inputted into Design Expert 10.0.7® trial program software for formula optimization. The responses or variables for the optimization included total carotenoid, total phenolic content, and hedonic test values (acceptance) in terms of color, aroma, taste, and overall. The experimental design is shown on Table 3.3, which consisted of two factorial points (-1, 1), one center point (0), and two level of axial points (-α, +α). The design included four factorial points, two axial points, and one central point. Therefore, the software established seven runs (seven concentrations of red fruit oil emulsion in the drink) for optimization by One Factor Design. All responses aimed to be optimized were needed to be determined and input to the software. Based on the inputs, the software suggested one optimum formulation with the highest desirability value.

Table 3.4 Experimental design for formula optimization

Run	Codified	Actual value
1	-1	x%
2	-1	x%
3	0.5	x%
4	1	x%
5	-0.5	x%
6	0	x%
7	1	x%

3.3.2.3 Verification Test

Design Expert 10.0.7® trial program software suggested an optimum formulation with predicted responses' values. Verification test was done to verify the optimum formulation suggested, whether the predicted responses were compatible to the actual values. The result would be accepted if the actual values of the responses were in the range of Confidence Interval (CI) or Prediction Interval (PI).

3.3.2.4 Product Profile and Comparison

The verified optimum formulation was further analyzed and compared to Java tea-based functional drink with no emulsion addition in terms of titratable acidity, viscosity, degree of color, total carotenoid, total phenolic content, and sensory acceptability in terms of aroma, color, taste, and overall. The experimental design is shown at Table 3.4.

Table 3.5 Experimental design of optimum product comparison

Functional drink	Repetition
Control (A_1)	$(A_1)_1$
	$(A_1)_2$
	$(A_1)_3$
Final optimum product (A_2)	$(A_2)_1$
	$(A_2)_2$
	$(A_2)_3$

Hypothesis:

H_0= red fruit emulsion addition gave no significant effect to the drink in terms of titratable acidity, viscosity, and sensory acceptance (color, aroma, taste, and overall).

H_1= red fruit emulsion addition gave significant effect to the drink in terms of titratable acidity, viscosity, and sensory acceptance (color, aroma, taste, and overall).

3.4 Analysis Procedures

3.4.1 Physical Analysis

3.4.1.1 Viscosity (Ali *et al.*, 2014 with modification)

Red fruit emulsion and Java tea-based functional drink viscosity were measured by a viscometer at a velocity of 10 rpm. The measurement of all samples was done at room temperature.

3.4.1.2 Particle Size (Frascareli *et al*., 2011 with modification)

The emulsions were observed for its particle size by observing the sample under microscope with 400X magnification that was connected to Olympus Stream software.

3.4.1.3 Emulsion Stability (Nikzade *et al*., 2012 with modification)

The stability of emulsion was determined by centrifugation method, in which 10 ml of emulsion was put in a centrifuge tube and centrifuged at 5000 rpm for 20 minutes. The volume of separated oil was recorded and the percentage stability was calculated as below.

$$\text{Emulsion Stability (\%)} = \frac{V1-V2}{V1} \times 100\%$$

Where:

V_1 = initial volume of emulsion (ml)

V_2 = volume of separated oil (ml)

3.4.1.4 Degree of Color (Douglas, 2002)

The degree of color was measured by using Konika Minolta Chromameter, which works based on color differences resulted from sample surface. The sample was put inside a petri dish. The measurement was divided into L*, a*, and b* values. The "L" value represents lightness, which ranges from 0 (dark) to 100 (bright). The "a" value expresses reflected light that produces red-green color, with +a (positive) value of 0-100 for red and –a (negative) value of 0-(-80) for green color. Meanwhile, "b" value expresses chromatic color of blue-yellow with +b (positive) value 0-70 and –b (negative) value of 0-(-70) for yellow and blue, respectively. ^{o}Hue was calculated by the formula ^{o}Hue = arc tan (b/a). The color description by ^{o}Hue value can be seen on Table 3.5 below.

Table 3.6 Color description based on ^{o}Hue

^{o}Hue [arc tan (b/a)]	Color description
18-54	Red (R)
54-90	Yellow Red (YR)
90-126	Yellow (Y)
126-162	Yellow Green (YG)
162-198	Green (G)
198-234	Blue Green (BG)
234-270	Blue (B)
270-306	Blue Purple (BP)
306-342	Purple (P)
342-18	Red Purple (RP)

Source: Hutching (1999)

3.4.2 Chemical Analysis

3.4.2.1 Total Carotenoids (Kalaikandhan *et al*., 2014 with modification)

Total carotenoid was determined by extracting a hundred milligram of sample with 10 ml of 80% acetone. The mixture was then centrifuged at 3000 rpm for 15 minutes. The supernatant was taken and the pellet is re-extracted with 5 ml of 80% acetone until it became colorless. All the supernatant was pooled and the absorbance was measured at 480 nm, 645 nm, and 663 nm in UV/Vis spectrophotometer. Total carotenoid was calculated in mg/g by using the formula below.

$$\text{Total Carotenoid (mg/g)} = \frac{(A480 + 0.114A663) - 0.638A645}{a\ x\ 1000\ x\ w}\ x\ V$$

Where:

a = length of light path in the cell (1 cm)

V = volume of the extract (ml)

w = weight of sample (gr)

3.4.2.2 Total Phenolic Content (Alhakmani *et al*., 2013 with modification)

The total phenolic content is determined by using Folin-Ciocalteu method. Gallic acid was used as reference standard. 0.5 mL of sample with a concentration of 20 mg/mL for emulsion and 25 mg/mL for drink was mixed with 1.5 mL of Folin-Ciocalteu reagent (diluted 1:10 with deionized water) and neutralized with 2 mL of 7.5% w/v sodium carbonate solution after 5 minutes. The mixture was then incubated for 90 minutes at room temperature. The absorbance is measured at 725 nm using spectrophotometer. Total phenolic content is expressed in mg GAE/g.

3.4.2.3 Titratable Acidity (Nielsen, 2014)

The titratable acidity of the samples was determined by titrating 10 mL of sample with 0.1N NaOH to a phenolphthalein endpoint (pinkish color). The titratable acidity was determined by using formula below.

$$\% \text{ acid (w/v)} = \frac{N\ x\ V1\ x\ Eq.weight}{V2\ x\ 10}$$

Where:

N = normality of titrant (NaOH) (mEq/mL)

V1 = volume of NaOH (mL)

Eq. weight = equivalent weight of predominant acid (mg/mEq)

V2 = volume of sample (mL)

3.4.3 Sensory Analysis

3.4.3.1 Hedonic Test (Meilgaard *et al*., 2007 with modification)

Hedonic test was done to measure the degree of likeness of the drink that has been formulated. In this test, 70 panelists were asked to taste the sample and rinse the mouth with water in each tasting to neutralize, then were asked to give scores in terms of aroma, taste, color, aftertaste and overall acceptance by using 7-point scale hedonic test (1 = dislike extremely, 2 = like, 3 = like moderately, 4 = neutral, 5 = dislike moderately, 6 = dislike, and 7 = like extremely).

3.4.3.2 Scoring Test (Meilgaard *et al*., 2007)

Scoring test was performed on 70 untrained panelists to determine specific sensory intensity of product that is expressed in numbers. The parameters included aroma, color, taste, and aftertaste. Panelists were asked to give score from 1 to 6 (1 for the lowest and 6 for the highest).