CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Calyx samples of roselle were obtained from UKM. A total of 11 roselle samples were planted in Experimental Plot at UKM. Completely random experimental design with 10 replications was used to plant these samples. List of all roselle samples used for this study is as shown in Table 3.1. Fruits were harvested and decored to obtain calyces, and washed before use. Samples of Asam keping *(Garcinia atroviridis)* and *G. cambogia* known to have high contents of HCA were used as controls for comparison. *G. atroviridis* used in this study was purchased from the local market. *G. cambogia* sample was obtained from Agricultural Park ARC Tenom, Sabah.

Table 3.1: List of roselle samples used in the study

Sample	Description
1) Accession 6 (Terengganu variety)	
2) Accession 21 (Arab variety)	
3) Accession 2	
4) HS0275-30-4-4-1-1-1	Red calyx Mutant Acc. 2
5) HS0275-30-3-3-1-1-1	White calyx Mutant Acc. 2
6) Accession 3	
7) HS03100-29-2-1-17-15-1	Red calyx Mutant Acc. 3
8) HS03100-29-7-1-6-3-1	White calyx Mutant Acc. 3
9) Accession 12	
10) HS1250-18-18-1-1-1-1	Red calyx Mutant Acc. 12
11) HS1250-1-18-1-1-1-1	White calyx Mutant Acc.12
12) Asam keping (*Garcinia atroviridis*)	Controls
13) Garcinia cambogia	

3.1.2 Chemicals

All solvents used were of analytical reagent grade. Methanol used for HCA extraction was obtained from R&M Chemicals, UK. Potassium hydroxide used for the treatment was from E. Merck, Germany. Activated charcoal powder (7440-44-0) used was from R&M Chemicals, UK. HPLC grade solvents used for chromatographic separation which were sulphuric acid and methanol were from Fisher, UK. Potassium hydroxycitrate standard was obtained from Sabinsa Corporation, India. Double-distilled water was used for HPLC analysis.

3.1.3 Instrumentation

Roselle methanolic extracts were refluxed using electromantle from MTOPS, Korea. High performance liquid chromatography was conducted using the model Shimadzu LC-10ATVP from Shimadzu. Regenerated cellulose-type filter (pore size 0.45µm) was obtained from Fisher Scientific, UK. pH meter used was from Mettler-Toledo,

USA. HPLC facilitiy used in this study was courtesy from HPLC Lab, Universiti Malaya Kuala Lumpur.

3.2 Moisture Content

The moisture content was recorded for every sample prepared for use in HCA extraction. The decored and washed calyces were weighed before being dried in the oven. The drying process was done at the temperature of 55-60°C for about 72 hours. After the calyces were fully dried, the dry weight was taken. Normally the dry weight is about one tenth from the fresh weight measured. The calculation of moisture content is as followed:

$$\text{Moisture (\%)} = \frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \times 100\%$$

3.3 Extraction Methods

Samples used for HCA extraction was in the form of dried roselle calyces. The fully dried calyces were then grounded using an electric kitchen blender until fine powder. These samples were ready to be used in HCA extraction.

3.3.1 HCA Extraction (Majeed et al. 2005)

The procedure of extracting HCA using procedure employed from Majeed et al (2005) is referred to as the original method. Hydroxycitric acid exists in two forms, the free acid form and the lactone form. The free acid form is biologically active and the lactone form is inactive. The free acid form is unstable and gets converted to its stable form but becomes inactive (Majeed et al., 2005). It is important to isolate HCA in a form that is both stable and biologically active. The extraction method employed isolate and stabilize free acid form of HCA as potassium salt to retain its bioactivity. Flow chart of HCA extraction method to produce potassium hydroxycitrate is showed in Figure 3.1. HCA was extracted from dried calyces in the form of potassium salt of HCA (potassium hydroxycitrate). 100g of dried roselle calyx was extracted with

300ml of methanol at reflux temperature (65°C) for 3 hours. The extract was filtered and additional 300ml of methanol was added and refluxed for another 3 hours. The second extract was filtered and additional 300ml of methanol was again added and refluxed. This was filtered and the three extracts collected were combined. The combined extracts were treated with 1M methanolic potassium hydroxide to produce potassium hydroxycitrate. It was then refluxed to obtain the precipitated potassium hydroxycitrate. The salt was filtered, dried and stored in a dessicator for further use.

Preparation of 1 M methanolic potassium hydroxide:

$$\text{Mass (g)} = M \times MW \times \frac{x}{1000} \times \frac{100}{\text{purity}}$$

Whereby:

M = Molarity

MW = Molecular weight

x = volume to prepare in mL

3.3.2 Modified HCA Extraction

The modified procedure of extracting HCA using charcoal is referred to as the modified method. HCA was extracted from dried calyces in the form of potassium salt of HCA (potassium hydroxycitrate). HCA extraction method was according to Majeed et al., 2005 HCA extraction method but involved a modification step with additional treatment of activated charcoal. Steps involved in the extraction method were repeated like the original HCA extraction method which involved refluxing of methanol as solvent with roselle samples repetitively for three times and extracts were collected each time. These extracts were combine finally. The combined extracts were treated with activated charcoal at temperature 40-45°C and constantly stirred. At the range of 40-45°C, the amount of charcoal treated was fully dissolved, thus the temperature selected. The amount of activated charcoal used was according to the total volume of refluxed methanol collected. The weight of activated charcoal used was 1/3 from the total volume of methanol collected. Activated charcoal used to eliminate any presence of anthocyanins that constituted to the colouring of furher extracts produced. It was

then filtered to obtain the clear filtrate. The process was repeated several times until clear filtrate was obtained. The clear filtrate was later treated with 1M methanolic potassium hydroxide to produce potassium hydroxycitrate. It was then refluxed to obtain the precipitated potassium hydroxycitrate. The salt was filtered and dried to be kept in a dessicator for further use. Figure 3.2 showed the complete modified extraction method.

Figure 3.1: HCA extraction method used to produce potassium hydroxycitrate (Majeed et al., 2005)

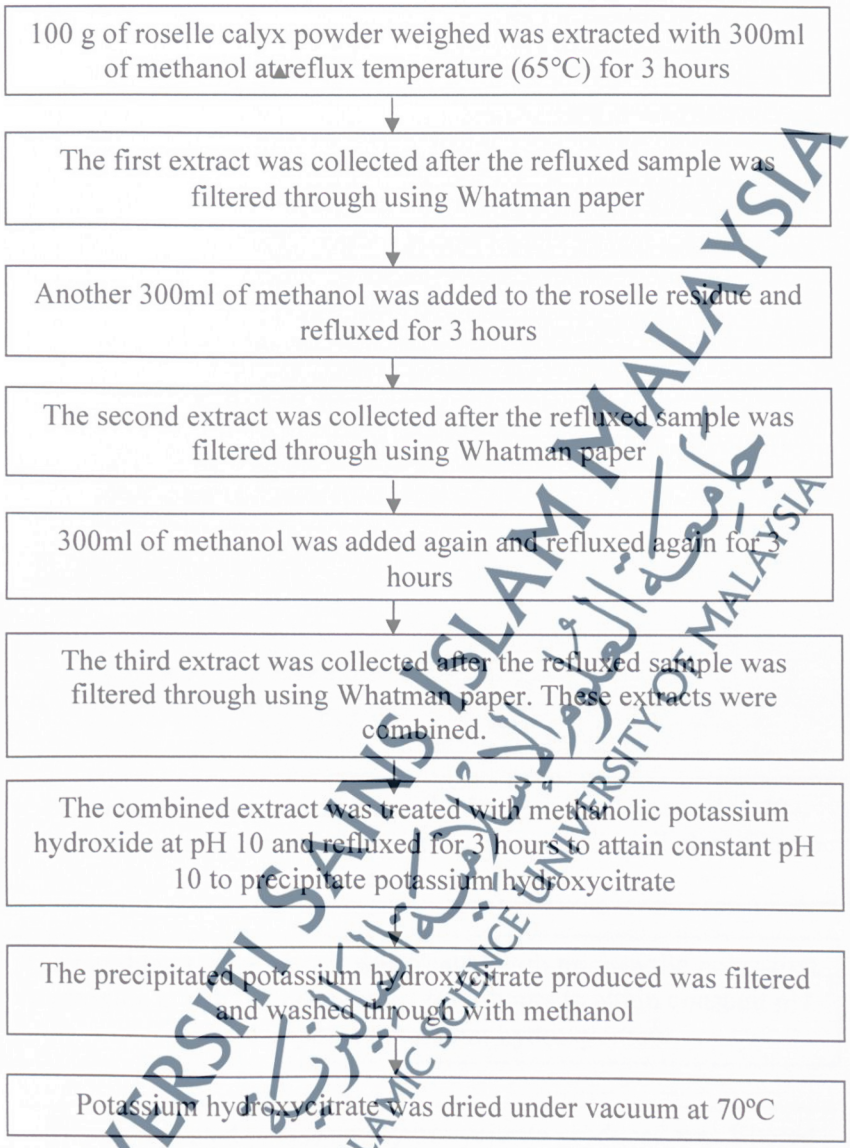
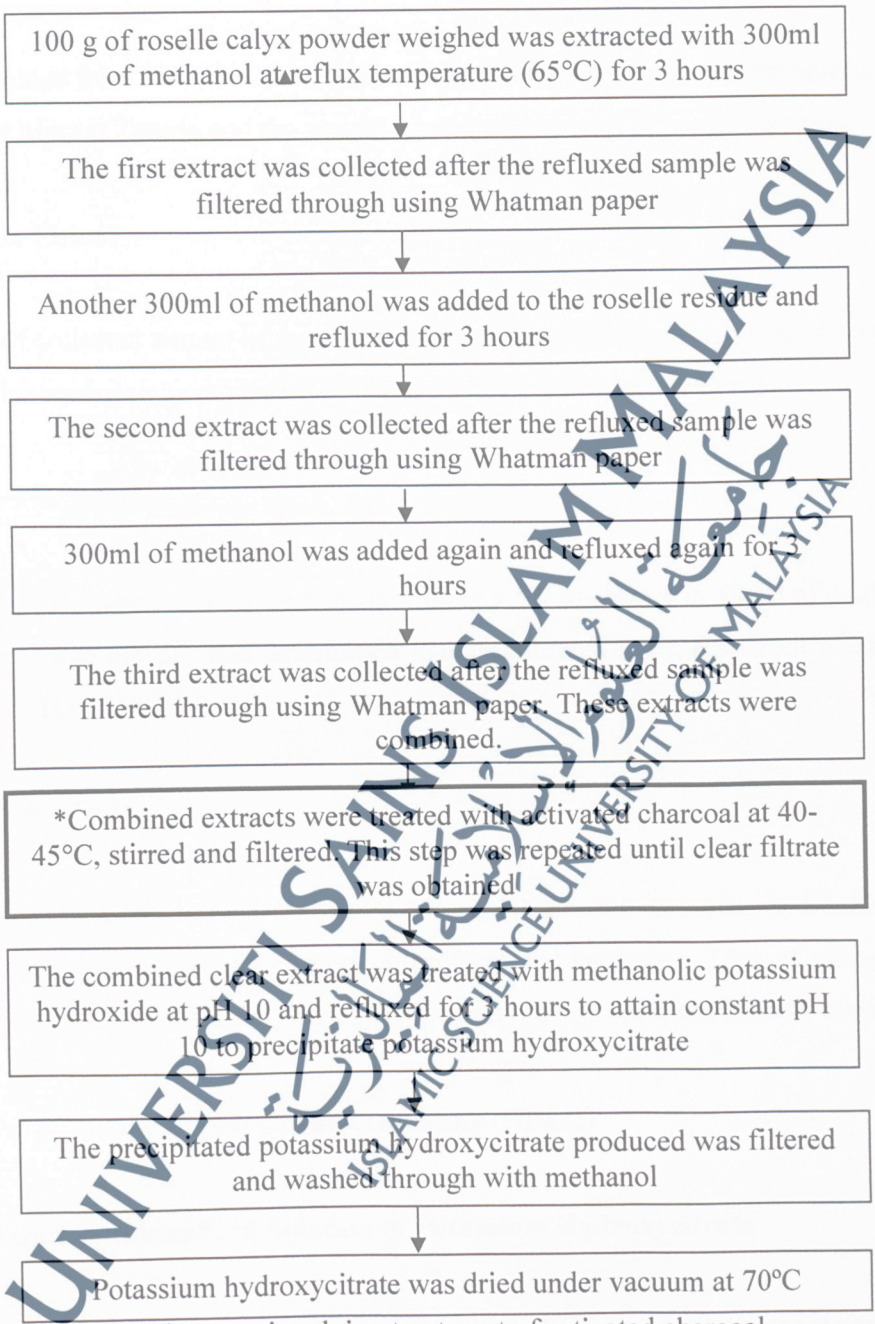


Figure 3.2: Modified* HCA extraction method developed to produce potassium hydroxycitrate



*Modification step involving treatment of activated charcoal

3.4 Physical and Chemical Identification of Potassium Hydroxycitrate

3.4.1 Extract Yield (%)

Extracts obtained from two extraction methods were weighed using an electronic scale model AL54 Mettler Toledo and the weight is recorded in term of percentage (%).

3.4.2 Extract Colour

The colour of collected extract of extracts from two extraction methods were observed visually and recorded to be compared to the extracts obtained from controls.

3.4.3 pH in 5% Solution

5% solution of extracts was prepared by dissolving 2.5 g of extract in 50 ml of double-distilled water and the pH was determined using calibrated pH meter from Mettler-Toledo, USA. (Lewis, 1969).

3.4.4 Solubility Tests

The extract was used to test for its solubility in solvents such as water, hydrochloric acid (HCl), aqueous methanol, benzene, chloroform and methanol. 2.5 g of extract is dissolved in 50 mL solvent and the clarity was observed and recorded (Lewis, 1969).

3.5 High Performance Liquid Chromatography (HPLC)

3.5.1 Preparation of Standard Solution of Potassium Hydroxycitrate

Potassium hydroxycitrate was used as a working standard. Standard stock solution of potassium hydroxycitrate was prepared using deionised water and this stock was used for a generating calibration curve. Concentration range was 50, 80, 100, 200 and 400µg/mL. HCA present in roselle samples was quantified using the calibration curve. HCA lactone was also provided as standard to check for its presence in samples

injected. Two standards were used: HCA lactone standard and potassium hydroxycitrate standard from Sabinsa Corporation.

3.5.2 Sample Preparation

50mg of the extract is accurately weighed, dissolved in double-distilled water and diluted to 25ml with double-distilled water.

3.5.3 HPLC Analysis

Shimadzu liquid chromatograph consisting of two LC-10AT *VP* pumps equipped with a SPD-M10A *VP* dual diode array detector and a SCL-10A *VP* system controller was used for HPLC. Chromatographic separation was conducted using an ODS Hypersil column (5µm, 250x4.6 mm²) from Thermo Electron Corporation. System was equipped with a manual injection system from Rheodyne with 20-µL sample loop. 10 µL of sample was injected. HPLC is coupled with DAD for quantitative determination of HCA. Detection was done by wavelength detector at a wavelength of 210 nm. The elution was carried out with 0.01M sulphuric acid and flow rate was 1.0 ml/min under isocratic condition (Jayaprakasha & Sakariah, 1998). All standards and samples were filtered through 0.45-µm regenerated cellulose syringe filter from Fisher Scientific. The compound was quantified using Shimadzu LC Solution software.

3.5.4 Calibration and Linearity

The linearity of the method was evaluated by analyzing a series of potassium hydroxycitrate standards. Aliquots of 10 µL of each of the five working standard solutions were injected on to the HPLC elution was carried out as mentioned above and peak area responses were obtained. The calibration curve for potassium hydroxycitrate was prepared by plotting concentration of potassium hydroxycitrate versus peak area with the average of three runs (Jayaprakasha & Sakariah, 1998).

3.5.5 Quantification of Potassium Hydroxycitrate in Samples

A known volume (20 µL) of each prepared sample was injected to the HPLC and the concentrations of potassium hydroxycitrate were obtained directly from the peak area and by application of the dilution factor. Potassium hydroxycitrate was calculated using a calibration curve generated using Curve Expert software. The concentrations of potassium hydroxycitrate in the sample were expressed as mg / 100g of sample (Jayaprakasha & Sakariah, 1998).

3.6 Statistical Analysis

Statistical tests are crucial to know whether means for data recorded have similarity or differences between them. Special tests are used to isolate a set of significant means to the same mean subsets (Mohamad, 2001). The test used was the Student's t-test in SAS software whereby this test was intended to see the comparison between means from variation source existed in the analysis. The results were reported as means (standard deviations from three repeated determinations). Statistical differences on the data of extracts yield and potassium hydroxycitrate concentration from both extraction methods test was conducted were analyzed according to Student's t-test wherein the differences were considered to be statistically significant at $P \leq 0.05$.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Moisture Content

The moisture content of all samples are shown in Table 4.1. From the thirteen samples used including two controls, sample Acc. 21 showed higher moisture content of 94%. This was not surprising to note because comparing with other roselle accessions, Acc. 21 had bigger calyx size and heavier calyx weight, thus higher moisture content. In contrast, the mutant HS03100-29-2-1-17-15-1 showed lower moisture content of 85%. Other roselle samples had moisture content ranging from 85% to 89%. Their respective moisture contents were as follows: Acc. 6 (89%), Acc. 2 (88.8%), roselle red mutant HS0275-30-4-4-1-1-1 (86%), HS0275-30-3-3-1-1-1 (86%), Acc. 3 (86.4%), HS03100-29-7-1-6-3-1 (85.5%), Acc. 12 (86.4%), HS1250-18-18-1-1-1-1 (86.8%) and HS1250-1-18-1-1-1-1 (85.9%). As for controls, *Garcinia atroviridis* had 95% and *G. cambogia* had 95.5%. Since HCA is the main source used as weight loss agent in many slimming products, the quality evaluation in samples is an important step. The moisture content of fruit is an important parameter for the stability of product keeping for commercial purposes. Higher moisture content enhances microbial growth. The importance of knowing the moisture content in samples can give better handling procedure in the processing steps toward the development of products. Generally, to ensure the quality of samples before processing for storage, the samples should be dried at moisture content less than 8% as a precaution to avoid from any microbial attack.

Table 4.1: Moisture content (%) of 13 samples used in the study

Samples	Moisture Content (%)
Accession 6	89.0^{bc} ±2.65
Accession 21	94.0^{ab} ±1.00
Accession 2	88.8^{c}±1.31
HS0275-30-4-4-1-1-1 (Red calyx)	86.0^{c} ±1.00
HS0275-30-3-3-1-1-1 (White calyx)	86.0^{c} ±1.00
Accession 3	86.4^{c} ±3.33
HS03100-29-2-1-17-15-1 (Red calyx)	85.0^{c} ±2.65
HS03100-29-7-1-6-3-1 (White calyx)	85.5^{c} ±0.50
Accession 12	86.4^{c} ±0.53
HS1250-18-18-1-1-1-1 (Red calyx)	86.8^{c} ±1.30
HS1250-1-18-1-1-1-1 (White calyx)	85.7^{c} ±3.84
Asam keping (*Garcinia atroviridis*)	95.0^{a} ±1.10
Garcinia cambogia	95.5^{a} ±1.32

4.2 Comparison of Extraction Methods

The existent of HCA in free acid form is known to be biologically active but unstable which have the tendency to be converted to its lactone form which is stable but yet inactive (Majeed et al., 2005). Therefore, it is important to extract HCA in the form that is both stable and biologically active for nutraceutical use. In this study, the original extraction method used to extract HCA is employed from Majeed et al., (2005) which used *Garcinia* as its sample then isolate and stabilize free acid form of HCA as potassium hydroxycitrate which is a water soluble salt. Aside from potassium salt, other form of HCA salts suffer from problems in assimilation, a fact attested to by poor performance in controlled weight loss trials. For instance, the free acid form of HCA is extremely ionic and does not pass readily through the gut membranes. Free HCA has several further disadvantages. It undergoes rapid lactonization and again the lactone form has no appreciable physiological activity. As a comparison, the calcium and magnesium salts of HCA were reported to be poorly absorbed by gastrointestinal tract because they are poorly soluble in aqueous media and because both of these

minerals are saponified by bile acids and fats in the gut or are bound up by soluble or insoluble fibers or other substances in the diet or secreted during digestion (Heymsfield et al., 1998). Moreover, there is no evidence that merely making calcium and calcium salts more soluble such as can be accomplished by adding small amounts of potassium and/or sodium and/or lactone will solve the problem of assimilation.

HCA is known to have three separate binding points and simple chemical experimentation quickly shows that divalent ions such as those of calcium and magnesium cannot be readily separated by the application of other acids such as human gastric acid from the HCA once these minerals have been reacted with it. The action of stomach acid however may free one of the two valences of calcium or magnesium for attachment to fats, bile acids, gums, fibres and pectins which is an undesirable outcome. Sodium salt has other disadvantages whereby for long-term administration, both because sodium possesses no positive metabolic effects with regard to obesity and because sodium has potential hypertensive actions. Potassium as a ligand for HCA does not possess the disadvantages associated with sodium, calcium or magnesium. Moreover, the absorption of potassium salt of HCA is considered to be superior to that of the sodium salt owing to its greater rate of uptake of potassium in relation to sodium in most tissues (Majeed et al., 2005).

Potassium is an ion primarily found in the cell cytoplasm and it can easily cross from outside the cell to inside the cell. The cell membrane permeability for potassium is 100 times higher than sodium and 25 times higher than for chloride. Potassium salt of HCA acts as a transporter of HCA inside the cell, where the biochemical action of HCA is exerted. Thus, producing HCA in the form of potassium hydroxycitrate is the most ideally suited form for its use as nutraceutical use in terms of its bioavailability and bioefficacy. The solvent used to produce potassium hydroxycitrate in the extraction is methanol. Methanol is a polar solvent. Methanol used would take out HCA together with other water soluble compounds as well from the samples. Supposedly, there would be traces of other compounds but their presences were minor (Majeed et al., 2005).

According to Majeed et al., (2005) using HCA extraction method, potassium hydroxycitrate was successfully produced but requiring certain adjustments. The pH during the treatment of potassium hydroxide into methanolic extract was set to be between pH 10 to 11. The yield showed to be the most optimum within this range. The pH plays critical role in the production of the salt. The method claimed to produce a chemically stable product which will not convert to lactone form, which will not be hygroscopic and which is soluble in aqueous solutions (Majeed et al., 2005). Potassium hydroxycitrate produced according the method however, was not completely physically stable and non-hygroscopic as claimed. The potassium salt has a tendency to form lumps during storage due to its highly hygroscopic nature, thus reducing the shelf life of the HCA salt (Figure 4.1).

Figure 4.1: Comparison of extract's physical stability before and after exposure in open-air environment

Before exposure

After exposure
Extract becomes sticky due its hygroscopicity

The instabilised physical changes normally happen after a short period of time right after exposure to open-air environment. Therefore, a modification step was made in HCA extraction method to overcome this problem. The step involved an additional step whereby treatment of charcoal with the methanolic extracts prior to treatment with potassium hydroxide to produce the potassium salt. The modified HCA extraction method using charcoal successfully produced more chemically stable form of potassium hydroxycitrate which was not hygroscopic. Therefore, the shelf life of HCA salt can be prolonged. However, potassium hydroxycitrate produced after the modification demonstrated differences in term of its yield and physical characteristics.

4.2.1 Extract Yield

4.2.1.1 HCA Extraction Method Based on Majeed et al. (2005)

Extracts had successfully been extracted using methanol in the dried samples. HCA extraction method was repeated three times respectively. The average yields of extracts for 13 samples were recorded. HCA extraction method based on Majeed et al. (2005) is referred to as the original extraction method. Majeed et al., (2005) reported that the yields of the extract were between 12-30% using *Garcinia cambogia* as sample. From this study the control sample of *Garcinia cambogia* showed the highest yield of 20.7% while *Garcinia atroviridis* yielded slightly lower at 19.5%, but still these two *Garcinia* species were among the highest in producing extract when compared with the roselle samples.

Table 4.2 shows the yields of extracts from 13 samples extracted using the original method. Acc. 21 produced extract yield of 17.8%. In contrast, Acc. 12 produced the lowest extract yield of 10.1%. On average, the extract yield ranged between 11-15%. The extract yield of other samples were Acc. 6 (15.5%), Acc. 2 (13.2%), HS0275-30-4-4-1-1-1 (14.6%), HS0275-30-3-3-1-1-1 (13%), Acc. 3 (11.5%), HS03100-29-2-1-17-15-1 (12.3%), HS03100-29-7-1-6-3-1 (14.6%), HS1250-18-18-1-1-1-1 (11.3%) and HS1250-1-18-1-1-1-1 (13.2%). Roselle mutants generally produced slightly higher yield compare to their parents. It can be concluded that the use of induced mutations was able to generate roselle mutants with higher HCA content but this screening procedure is needed for the selection of the lines. Results of the ANOVA showed that the mean squares for methods, samples and interaction between methods and samples were significant in both extraction methods used in the study. From the ANOVA analysis, the samples used in the original extraction method were also significantly different. The different subsets of extract yield mean from this original extraction method were grouped with different alphabets as shown in Table 4.2. Different mean subsets were significantly different with different listed alphabets.

Table 4.2: Extracts yield of 13 samples used in the study based on original extraction method

Samples	Average Yield (%)
Accession 6	15.5^{bc} ±0.40
Accession 21	17.8^{b} ±0.17
Accession 2	13.2^{de}±0.35
HS0275-30-4-4-1-1-1 (Red calyx)	14.6^{cd}±0.38
HS0275-30-3-3-1-1-1 (White calyx)	13.0^{de}±0.31
Accession 3	11.5^{ef} ±0.47
HS03100-29-2-1-17-15-1 (Red calyx)	12.3^{ef} ±0.54
HS03100-29-7-1-6-3-1 (White calyx)	14.6^{cd}±0.57
Accession 12	10.1^{f}±0.26
HS1250-18-18-1-1-1-1 (Red calyx)	11.3^{ef} ±0.31
HS1250-1-18-1-1-1-1 (White calyx)	13.2^{de}±0.32
Asam keping (*Garcinia atroviridis*)	19.5^{a} ±0.51
Garcinia cambogia	20.7^{a} ±0.70

* weight recorded g/100g dry calyces weight

**The same alphabet connotes no significant differences

Clouatre et al. (2002) reported that extracts from *Garcinia* produced had the tendency to bind with water in the open air to form a non-palatable paste not suitable for use in dry form. Extracts obtained through this original extraction method were hygroscopic if left in open air. This is due to its anthocyanins content which still existed in the extracts. Anthocyanins are easily oxidized thus degrading the salt from its original physical form.

4.2.1.2 Modified HCA Extraction Method

The yields of extract produced after the modification of HCA extraction method using charcoal were reduced due to the elimination of water soluble compounds by activated charcoal which resulted in less impurity. Activated charcoal

used in the treatment prior to the treatment of potassium hydroxide in forming the salt of potassium hydroxycitrate led to the elimination of anthocyanins pigments which were the main cause for the instability of the salt physical form. This method is referred to as the modified extraction method. Table 4.3 shows the yields of extracts of all samples used in the study using modified HCA extraction method.

Table 4.3: Extracts yield based on modified extraction method using charcoal

No	Samples	Average Yield (%)
1	Accession 6	5.5^{b} ±0.50
2	Accession 21	6.0^{b} ±0.15
3	Accession 2	5.4^{b} ±0.12
4	HS0275-30-4-4-1-1-1 (Red calyx)	5.3^{b} ±0.38
5	HS0275-30-3-3-1-1-1 (White calyx)	5.3^{b} ±0.46
6	Accession 3	5.0^{b} ±0.23
7	HS03100-29-2-1-17-15-1 (Red calyx)	5.5^{b} ±0.57
8	HS03100-29-7-1-6-3-1 (White calyx)	6.0^{b} ±0.26
9	Accession 12	5.0^{b} ±0.15
10	HS1250-18-18-1-1-1-1 (Red calyx)	5.0^{b} ±0.10
11	HS1250-1-18-1-1-1-1 (White calyx)	5.5^{b} ±0.47
12	Asam keping (*Garcinia atroviridis*)	8.3^{a} ±0.45
13	*Garcinia cambogia*	10^{a} ±0.26

* weight recorded g/100g dry calyces weight

* The same alphabet connotes no significant differences

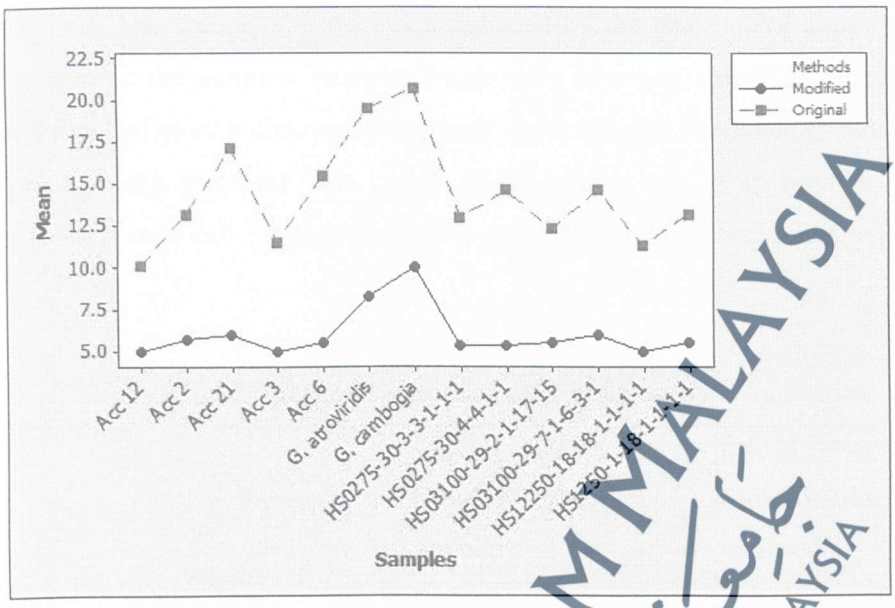
Extract yield was the highest in roselle Acc. 21 and HS03100-29-7-1-6-3-1 both at 6.0%. In contrast, Acc. 3, Acc. 12 and HS1250-18-18-1-1-1-1 produced the lowest extract yields at 5.0%. As for Acc. 6 extract yield was 5.5%, Acc. 2 (5.4%), HS0275-30-4-4-1-1-1 (5.3%), HS0275-30-3-3-1-1-1 (5.3%), HS03100-29-2-1-17-15-1 (5.5%) and HS1250-1-18-1-1-1-1 (5.5%). Controls produced the highest extract yields compared to all roselle samples, with *Garcinia cambogia* producing 10% extract and *G. atroviridis* producing 8.3% extract. Results of the ANOVA showed the

samples used in the modified extraction method were significantly different. The different subsets of extract yield mean from this modified extraction method were grouped with different alphabets as shown in Table 4.3. Different mean subsets were significantly different with different listed alphabets.

As previously mentioned, extracts obtained through original extraction method, if left in the open air outside of a humidity-controlled environment will begin to absorb moisture quickly. Thus, it is not suitable under normal circumstances for the production of dry delivery forms. In drawing moisture to itself, extracts will also tend to bind to available binding sites of compounds in its immediate environment and this action often later will markedly impede the assimilation of extracts from the gut (Clouatre et al., 2002). Thus, modification was made to overcome this problem. More stable extracts form had successfully been extracted through a modified extraction method with addition use of charcoal treatment.

Chemically unstable form of extracts obtained in previous extraction method was caused by the presence of anthocyanins which disturbed the consistency of the salt. Anthocyanins are water-soluble pigments in plants responsible for giving colour to the parts of the plants. Roselle is well known to contain anthocyanins which give its characteristic colour. The additional step in modified extraction method by treatment of charcoal had been able to eliminate the presence of anthocyanins in the salts. Charcoal acted as the decolouriser to the extract making it free from any colour. Thus, the extracts yield obtained after modification of HCA extraction method were lower.

Figure 4.2: Extract yield (%) trend of 13 samples used in the study grouped based on their respective extraction methods



ANOVA shows that both extraction methods used in the study were significantly different from each other. From Figure 4.2, it can be seen the trend of extract yield in both extraction methods. All samples showed significant decrease in extract yield from original extraction method to modified extraction method. The reduction rate of extract yield was as high as 50% and more in some samples. It can be concluded that extracts obtained from the original extraction methods produced extracts with significant impurities as the trend showed that after treatment of activated charcoal, the extracts yield was reduced thus proving the purity of extracts produced through the modified extraction method.

4.2.2 Colour Test of Extracts

Majeed et al. (2005) reported the colour of potassium hydroxycitrate extract to range from beige to pale brown. Table 4.4 shows the variation of extract colour observed in the samples. Figure 4.3 shows the colours observed based on the original extraction method, ranging from beige, brown to pale brown. Acc. 6, Acc. 21, Acc. 2, HS0275-30-4-4-1-1-1, HS03100-29-2-1-17-15-1, Acc. 12 and HS1250-18-18-1-1-1-1 had extract yields which were brown in colour. For HS0275-30-3-3-1-1-1, Acc. 3,

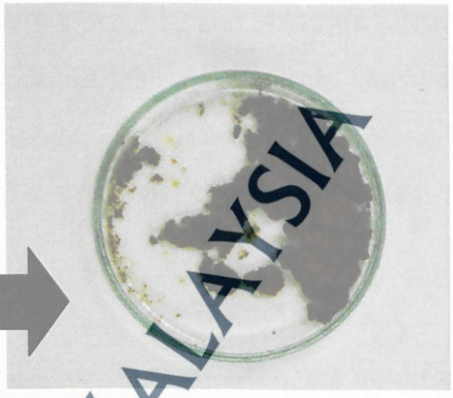
HS03100-29-7-1-6-3-1 and HS1250-1-18-1-1-1-1, their extracts were pale brown in colour. Only controls, *Garcinia cambogia* and *G. atroviridis* showed extracts to be beige in colour. The colour of extract is influenced by the amounts of anthocyanins pigment present in the samples. Extracts which were brown in colour were produced by samples that had more anthocyanins pigment in its samples compared to those with pale brown or beige in colour. The colour of the extract can be assumed from the colour of fresh roselle calyx which represents the intensity of anthocyanins content it possesses.

Table 4.4: Colours of extracts of 13 samples used in the study

Samples	Extract colour (original extraction method)	Extract colour (modified extraction method)
Accession 6	Brown	Offwhite
Accession 21	Brown	Offwhite
Accession 2	Brown	White
HS0275-30-4-4-1-1-1 (Red calyx)	Brown	White
HS0275-30-3-3-1-1-1 (White calyx)	Pale brown	Offwhite
Accession 3	Pale brown	White
HS03100-29-2-1-17-15-1 (Red calyx)	Brown	Offwhite
HS03100-29-7-1-6-3-1 (White calyx)	Pale brown	White
Accession 12	Brown	White
HS1250-18-18-1-1-1-1 (Red calyx)	Brown	Offwhite
HS1250-1-18-1-1-1-1 (White calyx)	Pale brown	White
Asam keping (*Garcinia atroviridis*)	Beige	White
Garcinia cambogia	Beige	White

Figure 4.3: Colours of extracts of 13 samples used in the study using the original extraction method

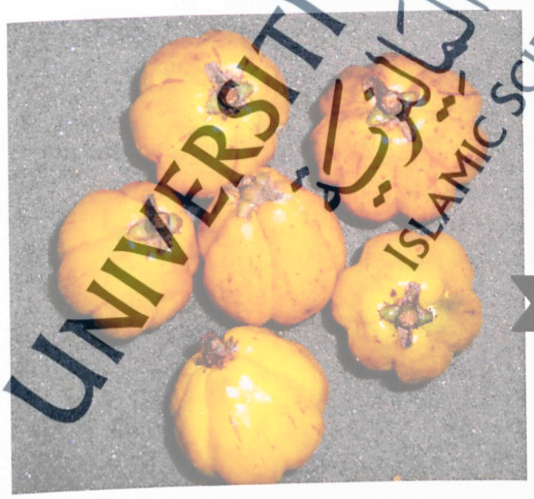
Roselle calyces with high contents of anthocyanins



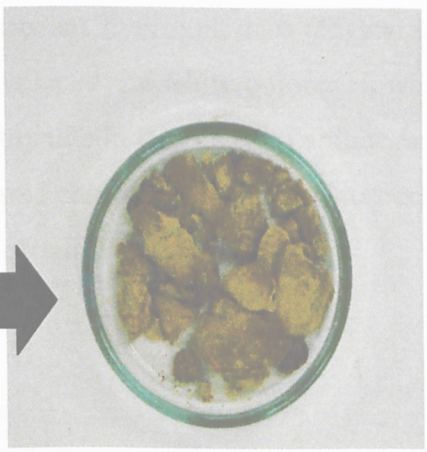
Extract is dark brown in colour

Roselle calyces with lower contents of anthocyanins

Extract is brown in colour



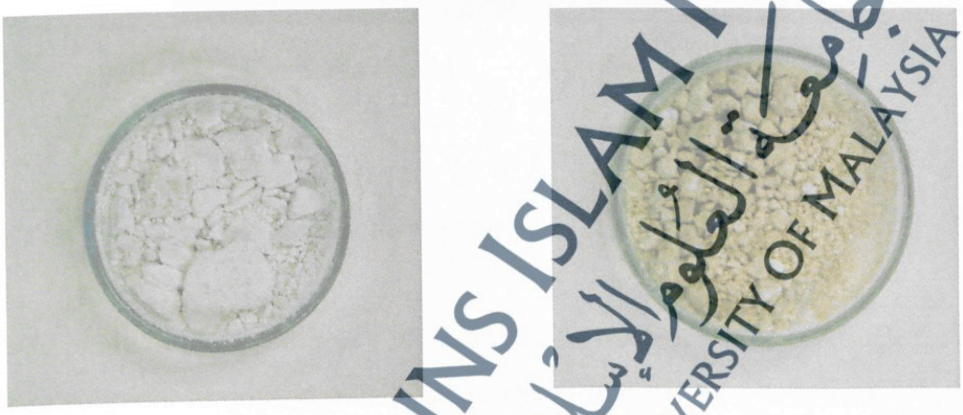
Fruits of *Garcinia cambogia*



Extract is beige in colour

Using the modified extraction method, the colour of extracts ranged from white to offwhite (Figure 4.4). Table 4.4 shows that the colour of extracts of Acc. 2, HS0275-30-4-4-1-1-1, Acc. 3, HS03100-29-7-1-6-3-1, Acc. 12 and HS1250-1-18-1-1-1-1 was white. The white colour was also exhibited by the controls *Garcinia cambogia* and *G. atroviridis*. Only Acc. 6, Acc. 21, HS0275-30-3-3-1-1-1, HS03100-29-2-1-17-15-1, and HS1250-18-18-1-1-1-1 had extracts offwhite in colour.

Figure 4.4: Colours of extracts of 13 samples used in the study using the modified extraction method



The drastic changes in terms of the extract colour from brown to white is caused by the elimination of anthocyanins pigments as a result from the treatment of activated charcoal prior to the formation of potassium hydroxycitrate (Figure 4.5). With the absence of anthocyanins, the extract developed the white colour. However, few extracts which showed offwhite colour probably due to the traces of anthocyanins still present and were not fully captured by activated charcoal treatment. These traces gave effect to the colour of the extracts making it offwhite in colour.

Figure 4.5: Comparison of colours of extracts produced by two extraction methods

4.2.3 Test for pH of 5% Extract Solution

Extracts produced from both extraction methods were prepared to check for their pH value in solution. Majeed et al. (2005) reported that pH of 5% extract solution to be around 7.0 to 9.0. The pH values of extract solutions of samples used in the study is shown in Table 4.5. All samples from both HCA extraction methods showed pH values within the range of 7.11 to 8.60. According to Morris et al. (1984), potassium is able to increase pH in the solution. Since the extract used in the study was in potassium form, it is expected to be alkaline due to the presence of potassium.

Table 4.5: pH value of 5% extracts solution from samples used in the study

Samples	pH 5% solution (Original extraction method)	pH 5% solution (Modified extraction method)
Accession 6	$8.12^{cdef} \pm 0.10$	$7.89^{abc} \pm 0.08$
Accession 21	$8.03^{def} \pm 0.02$	$7.86^{bc} \pm 0.05$
Accession 2	$7.88^{f} \pm 0.03$	$8.09^{a} \pm 0.06$
HS0275-30-4-4-1-1-1 (Red calyx)	$7.96^{ef} \pm 0.02$	$7.75^{cd} \pm 0.06$
HS0275-30-3-3-1-1-1 (White calyx)	$8.06^{def} \pm 0.04$	$7.95^{abc} \pm 0.03$
Accession 3	$8.15^{bcde} \pm 0.05$	$7.99^{ab} \pm 0.11$
HS03100-29-2-1-17-15-1 (Red calyx)	$8.16^{bcde} \pm 0.15$	$7.65^{d} \pm 0.09$
HS03100-29-7-1-6-3-1 (White calyx)	$7.92^{ef} \pm 0.05$	$7.28^{ef} \pm 0.05$
Accession 12	$8.50^{a} \pm 0.10$	$7.35^{e} \pm 0.06$
HS1250-18-18-1-1-1-1 (Red calyx)	$8.40^{ab} \pm 0.10$	$7.10^{f} \pm 0.10$
HS1250-1-18-1-1-1-1 (White calyx)	$8.33^{abc} \pm 0.15$	$7.16^{ef} \pm 0.03$
Asam keping (*Garcinia atroviridis*)	$8.10^{cdef} \pm 0.10$	$7.25^{ef} \pm 0.06$
Garcinia cambogia	$8.27^{abcd} \pm 0.15$	$7.27^{ef} \pm 0.07$

4.2.4 Extract Solubility Test in Solvents

Extracts obtained from both extraction methods were prepared to check for their solubility in solvents such as water, hydrochloric acid, aqueous methanol, benzene, chloroform and methanol. Majeed et al. (2005) reported that potassium hydroxycitrate extract was soluble in water, acids and aqueous alcohol but insoluble in alcohol, benzene and chloroform. Solubility test results are shown in Table 4.6. Extracts obtained were highly soluble in water, aqueous methanol and hydrochloric acid but insoluble in benzene and chloroform. Potassium is an alkali metal, so it reacts rapidly with water. Potassium compounds generally have excellent water solubility due to the high hydration energy of the K ion, thus explaining its high solubility in aqueous-based mediums (De Pena & Caimi, 1967).

Table 4.6: Solubility tests for extracts from 13 samples used in the study using two extraction methods

Samples	Extracts produced through original extraction method – Solvent						Extracts produced through modified extraction method – Solvent					
	Water	HCl	Methanol (aqueous)	Benzene	Chloroform	Methanol	Water	HCl	Methanol (aqueous)	Benzene	Chloroform	Methanol
Accession 6	++	+	++	-	-	-	++	+	++	-	-	-
Accession 21	++	+	++	-	-	-	++	+	++	-	-	-
Accession 2	++	+	++	-	-	-	++	+	++	-	-	-
HS0275-30-4-4-1-1-1 (Red calyx)	++	+	++	-	-	-	++	+	++	-	-	-
HS0275-30-3-3-1-1-1 (White calyx)	++	+	++	-	-	-	++	+	++	-	-	-
Acc. 3	++	+	++	-	-	-	++	+	++	-	-	-
HS03100-29-2-1-17-15-1 (Red calyx)	++	+	++	-	-	-	++	+	++	-	-	-
HS03100-29-7-1-6-3-1 (White calyx)	++	+	++	-	-	-	++	+	++	-	-	-
Acc. 12	++	+	++	-	-	-	++	+	++	-	-	-
HS1250-18-18-1-1-1-1 (Red calyx).	++	+	++	-	-	-	++	+	++	-	-	-
HS1250-1-18-1-1-1-1 (White calyx)	++	+	++	-	-	-	++	+	++	-	-	-
Asam keping (*Garcinia atroviridis*)	++	+	++	-	-	-	++	+	++	-	-	-
Garcinia cambogia	++	+	++	-	-	-	++	+	++	-	-	-

*++ Highly soluble +Soluble -Insoluble

4.3 Determination of Potassium Hydroxycitrate in Extracts Using HPLC

Extracts obtained from both extraction methods had successfully been determined through HPLC analysis. HPLC conditions and parameters used were from Jayaprakasha & Sakariah (1998). Potassium hydroxycitrate was successfully separated on an ODS Hypersil column. One to two major compounds were detected with very few minor compounds. List of retention times of potassium hydroxycitrate recorded for all samples is shown in Table 4.7. Overall retention time recorded for HCA was around 4.500min. There was a slight variation in retention times and this is probably due to the differences in immobile phase composition from batch to batch, changes in temperature of the room and thus the HPLC instrument, which would affect analyte-column interactions or changes in wall integrity giving results to the slight differences in retention times. It is interesting to note the slight differences in each retention time from both extraction methods which suggested that results of HPLC employed in the study were reproducible and that the compound eluted off the column at nearly the same time for each run.

Table 4.7: Retention time of potassium hydroxycitrate determination using HPLC

Sample	Retention time(min) (Original extraction method)	Retention time (min) (Modified extraction method)
Accession 6	4.522±0.003	4.573±0.005
Accession 21	4.538±0.004	4.540±0.03
Accession 2	4.530±0.019	4.583±0.006
HS0275-30-4-4-1-1-1 (Red calyx)	4.555±0.006	4.551±0.001
HS0275-30-3-3-1-1-1 (White calyx)	4.540±0.019	4.530±0.009
Accession 3	4.538±0.005	4.526±0.008
HS03100-29-2-1-17-15-1 (Red calyx)	4.562±0.006	4.562±0.001
HS03100-29-7-1-6-3-1 (White calyx)	4.573±0.004	4.551±0.008
Accession 12	4.540±0.015	4.530±0.01
HS1250-18-18-1-1-1-1 (Red calyx)	4.574±0.003	4.583±0.004
HS1250-1-18-1-1-1-1 (White calyx)	4.551±0.005	4.562±0.002
Asam keping (*Garcinia atroviridis*)	4.522±0.006	4.522±0.003
Garcinia cambogia	4.522±0.007	4.522±0.005

The existing methods for the determination of HCA use an acid-base titration which gives the total acidity of fruit extracts (AOAC, 1970). In this method, the concentration of HCA, HCA lactone and other organic acids cannot be estimated separately. The values obtained by the HPLC method accounted for only HCA because the values correspond to the area of HCA peak. Another method that can be used to determine HCA content is gas-liquid chromatography (GLC). Generally, determination by means of gas-liquid chromatography is lengthy in that the organic acid must be derivatized to volatile silyl derivatives. For silylation, the sample should be dried completely and the HCA has the tendency to undergo cyclization of the γ-lactone during drying (Loweinstein & Brunengraber, 1981). Due to the highly hygroscopic nature of HCA, it is rather difficult to dry the sample completely. Hence, free HCA can not be determined and estimated. Both methods have its own merits and demerits in respect of accuracy and convenience. But using HPLC, free HCA can be quantified without concentrating, drying and derivatization which put it at an advantage compared with previous methods mentioned.

Potassium hydroxycitrate showed an absorption maximum at 210nm. Hence 210nm was used for HPLC detection. The chromatograms of every sample used in both extraction methods were illustrated in Figures 4.6 to 4.18. Chromatographic results showed good separation of analytes. ODS Hypersil column used in HPLC had successfully separated the compound studied which is the potassium hydroxycitrate. There was no interference from other compounds, while the existence of one or two major compound can be clearly detected in the chromatograms produced. These results indicate that the method is sufficiently selective. The identity of potassium hydroxycitrate peak was confirmed by determination of retention time and by spiking with standard potassium hydroxycitrate (Figure 4.19). The first major peak appeared in every chromatogram at retention time 2.7min was identified as lactone as confirmed by determination of retention time and spiking with standard of lactone (Figure 4.20). Lactone was still found in samples used in both extraction methods because the extraction methods involved heating process in extracting its compound and this led to lactonization process to occur. In addition, some of the extracts samples were kept for some time before HPLC analysis were done. The exposure of extracts to unstable environment could cause it to degrade to its unstable physical form due to oxidization which would later produced lactone over time as well. Therefore, there were still traces of HCA lactone formed in the extracts produced which could be identified through HPLC analysis.

Figure 4.6: Chromatograms showing separation of analytes for Acc. 6
(a) according to original extraction method
(b) according to modified extraction method

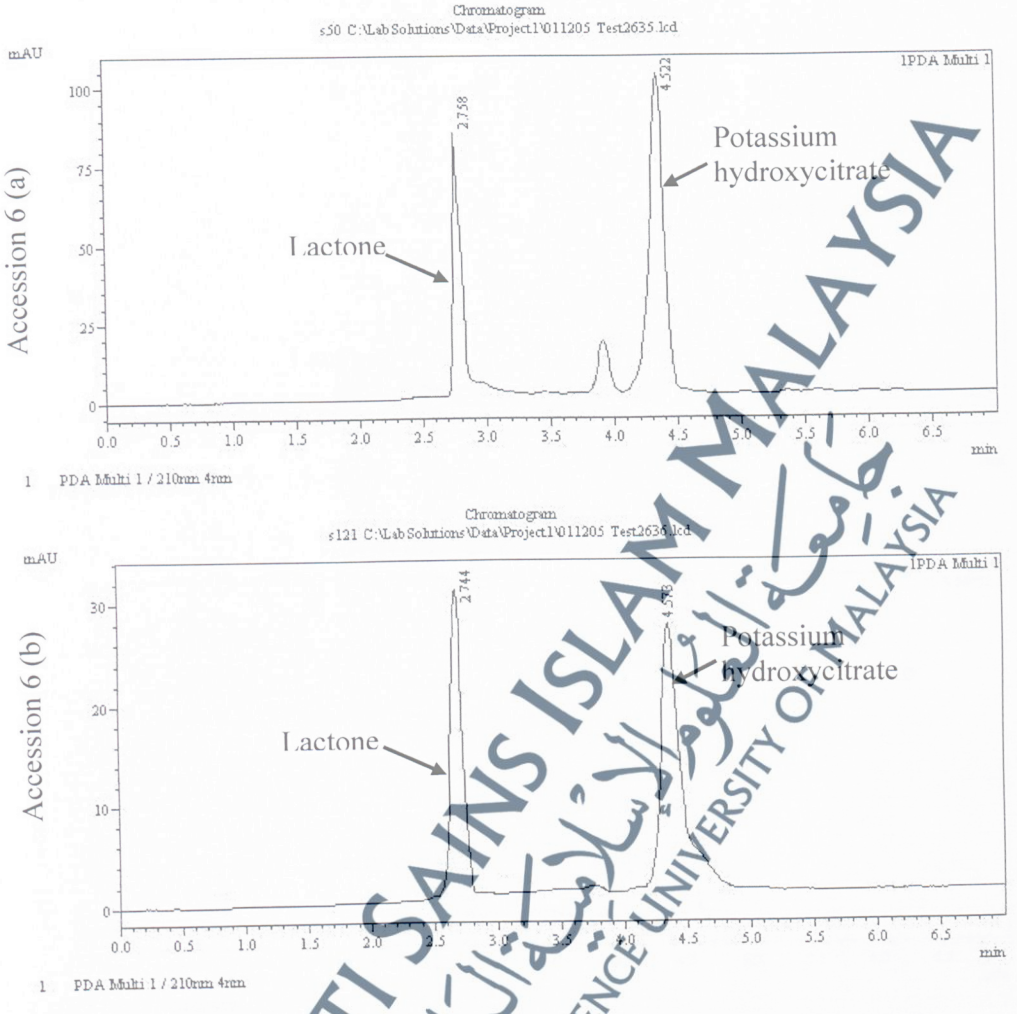


Figure 4.6 presents chromatograms showing separation of analytes for roselle Acc. 6 from extracts produced in both extraction methods. In both chromatograms, there were two major peaks at retention time 2.7min and 4.5min with only one minor peak in between. However, the minor peak was almost unnoticeable in chromatogram resulted in sample from the modified extraction method. Activated charcoal had successfully eliminated the impurities once existed in the extract produced through the original extraction method. The first appearing peak at both chromatograms at retention time 2.7min was recognized as lactone while the second major peak at retention time 4.5min was recognized as potassium hydroxycitrate, the compound sought in the study.

Figure 4.7: Chromatograms showing separation of analytes for Acc. 21
(a) according to original extraction method
(b) according to modified extraction method

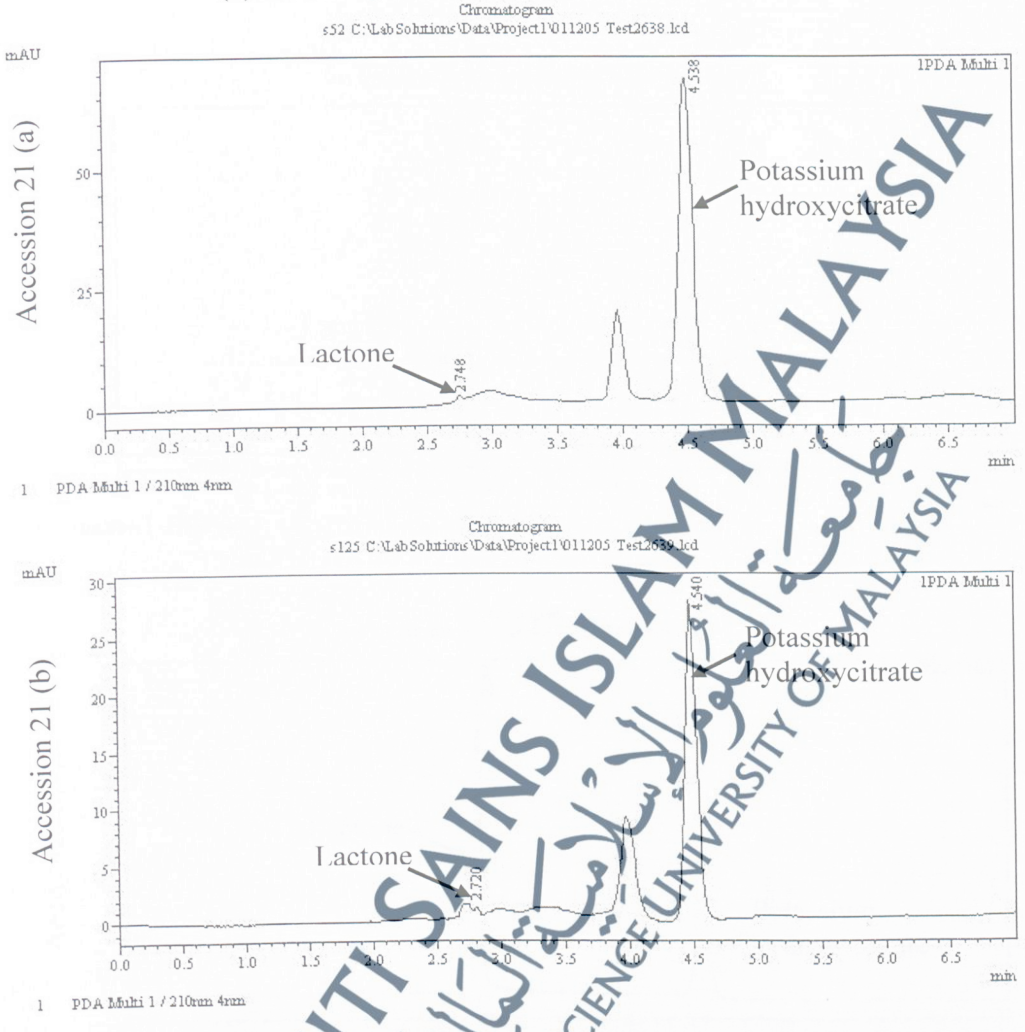


Figure 4.7 illustrates chromatograms showing separation of analytes for roselle Acc. 21 from extracts yielded in both extraction methods. In both chromatograms, there were two major peaks at retention time 2.7min and 4.5min with only one minor peak in between. The minor peak was still sustained in chromatogram resulted using sample from the modified extraction method. Activated charcoal had not fully able to eliminate the impurities through the treatment process, thus can be seen from the offwhite coloured of the extract obtained. The first appearing peak at both chromatograms at retention time 2.7min was recognized as lactone while the second major peak at retention time 4.5min was recognized as potassium hydroxycitrate, the compound sought in the study.

Figure 4.8: Chromatograms showing separation of analytes for Acc. 2
(a) according to original extraction method
(b) according to modified extraction method

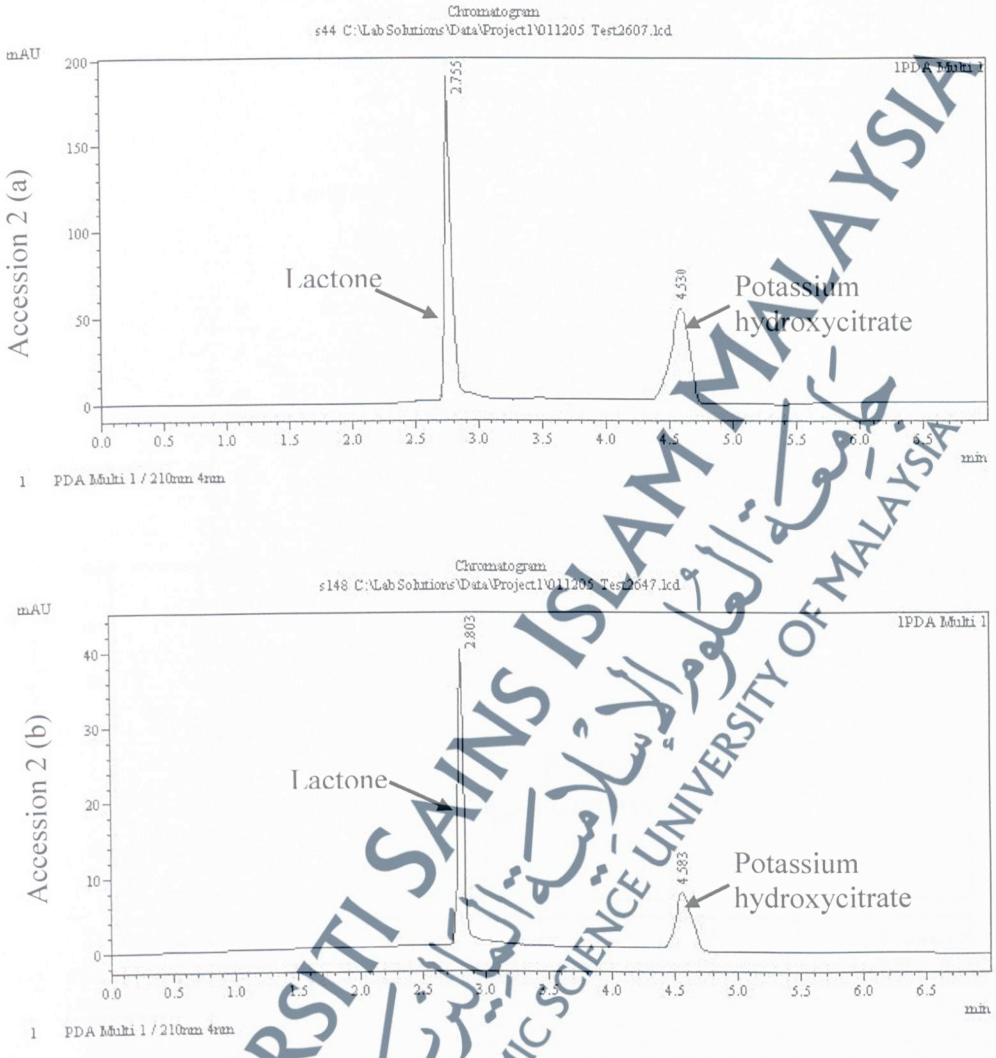


Figure 4.8 shows chromatograms showing separation of analytes for roselle Acc. 2 from extracts obtained in both extraction methods. In both chromatograms, there were only showing two major peaks at retention time 2.7min and 4.5min. Potassium hydroxycitrate obtained from both extraction methods possessed no impurities through their processes as can be seen from the chromatographic results as showed. The first appearing peak at both chromatograms at retention time 2.7min was recognized as lactone while the second major peak at retention time 4.5min was recognized as potassium hydroxycitrate, the compound looked in the study.

Figure 4.9: Chromatograms showing separation of analytes for HS0275-30-4-4-1-1-1
(a) according to original extraction method
(b) according to modified extraction method

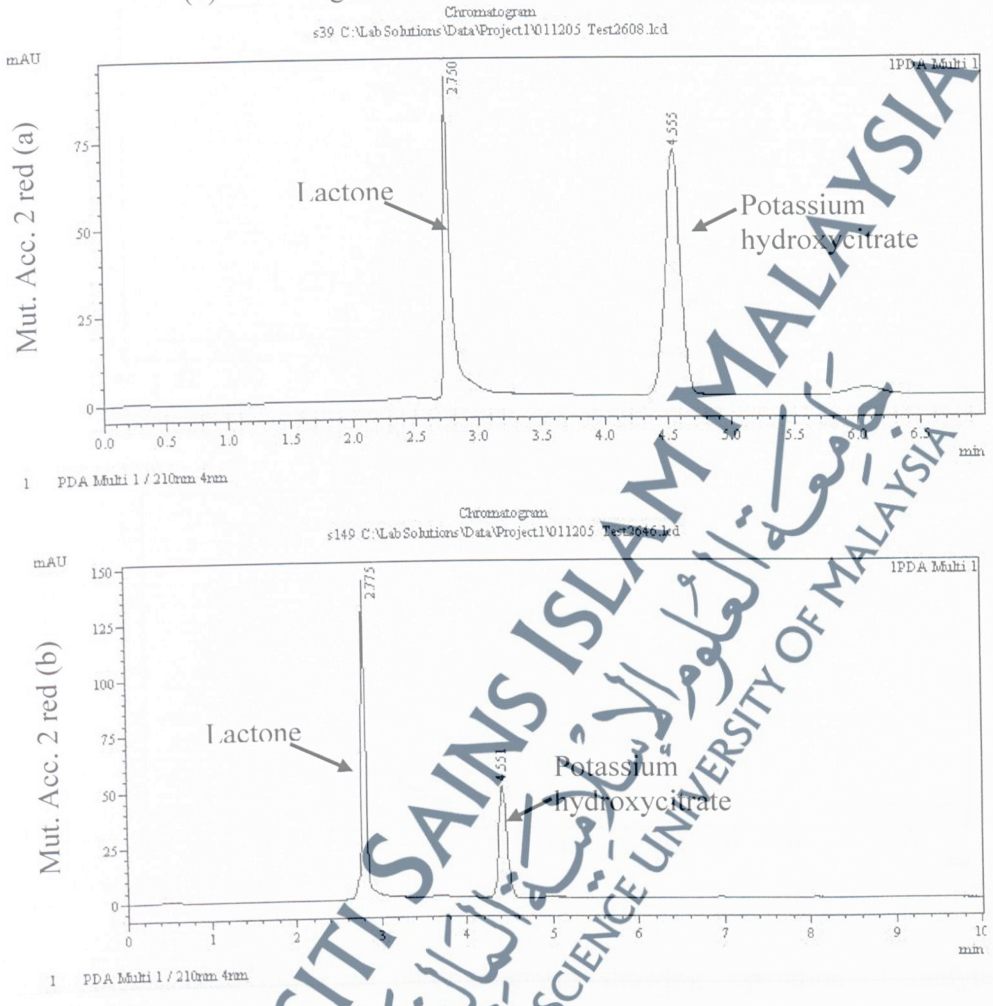


Figure 4.9 shows chromatograms showing separation of analytes for roselle HS0275-30-4-4-1-1-1 from extracts produced in both extraction methods. In both chromatograms, there showed two major peaks at retention time 2.7min and 4.5min. However, after the treatment of activated charcoal, this peak was seen absent suggesting the success of activated charcoal in eliminating impurities. The first appearing peak at both chromatograms at retention time 2.7min was recognized as lactone while the second major peak at retention time 4.5min was recognized as potassium hydroxycitrate, the compound looked in the study.

Figure 4.10: Chromatograms showing separation of analytes for HS0275-30-3-3-1-1-1
(a) according to original extraction method
(b) according to modified extraction method

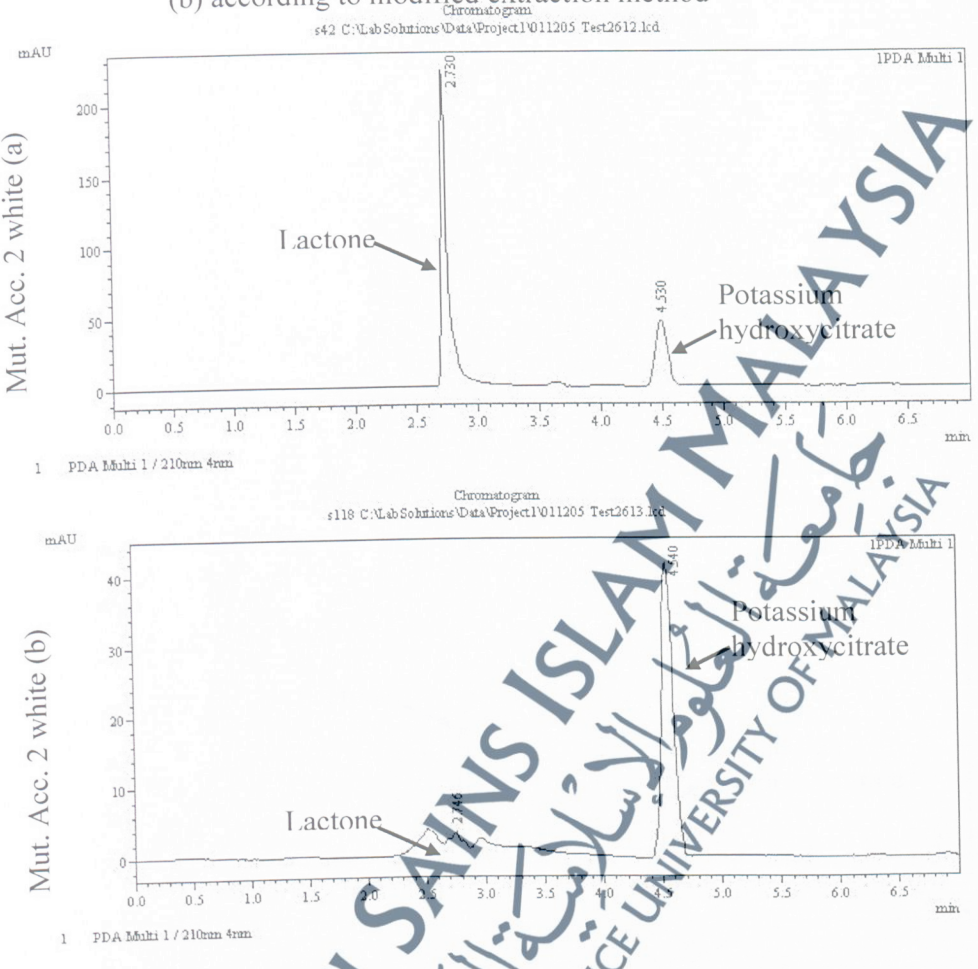


Figure 4.10 illustrates chromatograms showing separation of analytes for roselle HS0275-30-3-3-1-1-1 from extracts obtained in both extraction methods. In both chromatograms, there showed two major peaks at retention time 2.7min and 4.5min only with a presence of very minor peak in between at chromatogram in original extraction method. This peak was however seen absent in chromatogram after modified extraction method proving the success of activated charcoal in eliminating impurities. Separation of analytes in extract sample after using charcoal was not so good initially due to problems in handling processes during the extraction method. First appearing peak at both histograms at retention time 2.7min were recognized as lactone while the second major peak at retention time 4.5min was recognized as potassium hydroxycitrate, the compound sought in the study

Figure 4.11: Chromatograms showing separation of analytes for Acc. 3
(a) according to original extraction method
(b) according to modified extraction method

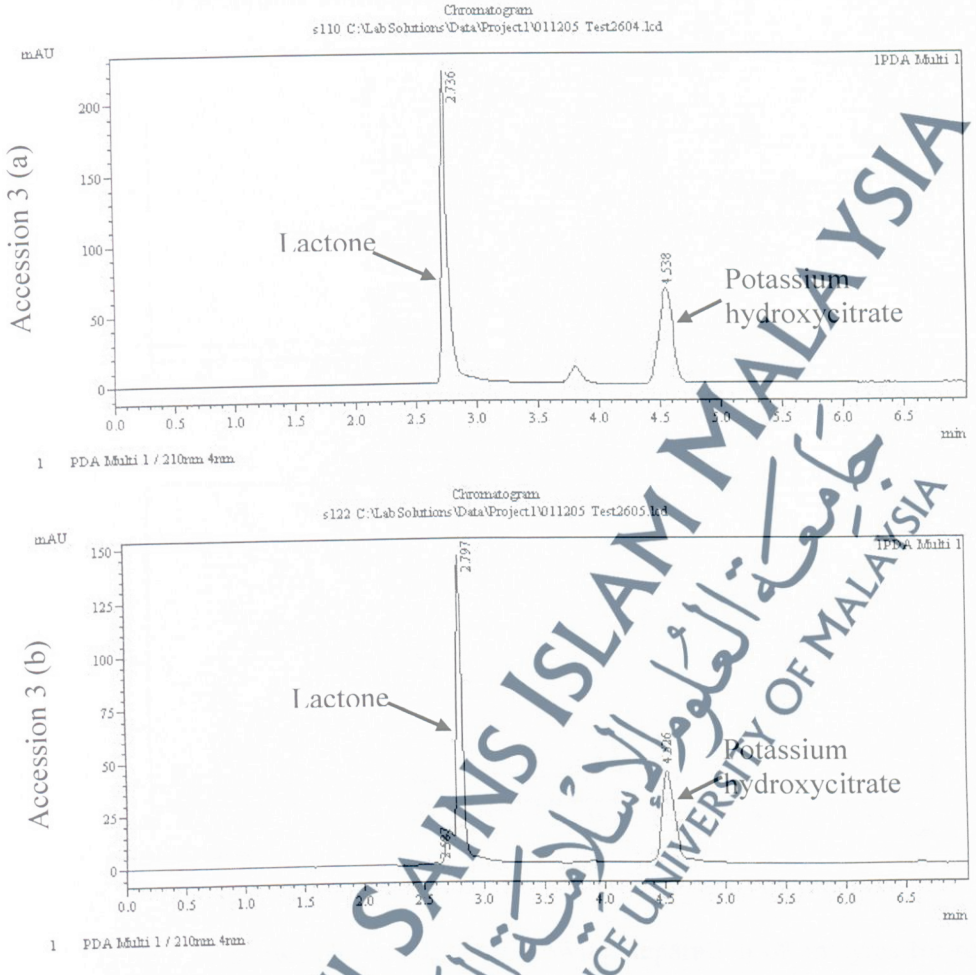


Figure 4.11 presents chromatograms showing separation of analytes for roselle Acc. 3 from KHCA yielded in both extraction methods. Both chromatograms showed two major peaks at retention time 2.7min and 4.5min with only one minor peak in between. However, the minor peak was almost unnoticeable in chromatogram resulted after extract sample from the modified extraction method implying the effectiveness of activated charcoal in eliminating impurities through its treatment. Initially, the separation of analytes in extract sample after using charcoal was not so satisfactory which was be due to handling problems in processes during the extraction method. Lactone was recognized in the first appearing peak at both chromatograms at retention time 2.7min while potassium hydroxycitrate, the compound looked in the study was recognized in the second major peak at retention time 4.5min.

Figure 4.12: Chromatograms showing separation of analytes for HS03100-29-2-1-17-15-1
(a) according to original extraction method
(b) according to modified extraction method

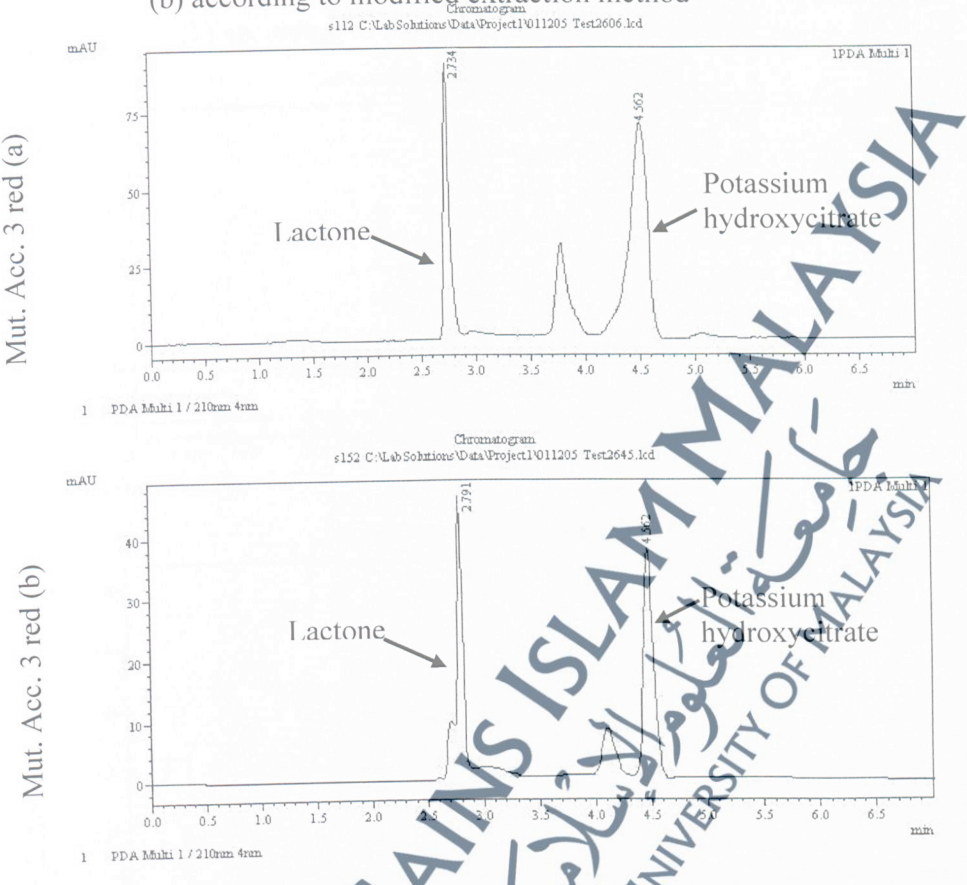


Figure 4.12 shows chromatograms showing separation of analytes for roselle HS03100-29-2-1-17-15-1 from extracts produced in both extraction methods. There were two major peaks at retention time 2.7min and 4.5min with only one minor peak in between in both chromatograms. The minor peak was still sustained in chromatogram resulted in extract sample from the modified extraction method. Activated charcoal had not fully able to eliminate impurities through the treatment process, thus can be seen from the offwhite colour of the extract obtained. The separation of analytes in extract sample after using charcoal was not so clear initially which was caused by the handling problems in processes during the extraction method First appearing peak at both histograms at retention time 2.7min were recognized as lactone while the second major peak at retention time 4.5min was recognized as potassium hydroxycitrate, the compound sought in the study.

Figure 4.13: Chromatograms showing separation of analytes for HS03100-29-7-1-6-3-1
(a) according to original extraction method
(b) according to modified extraction method

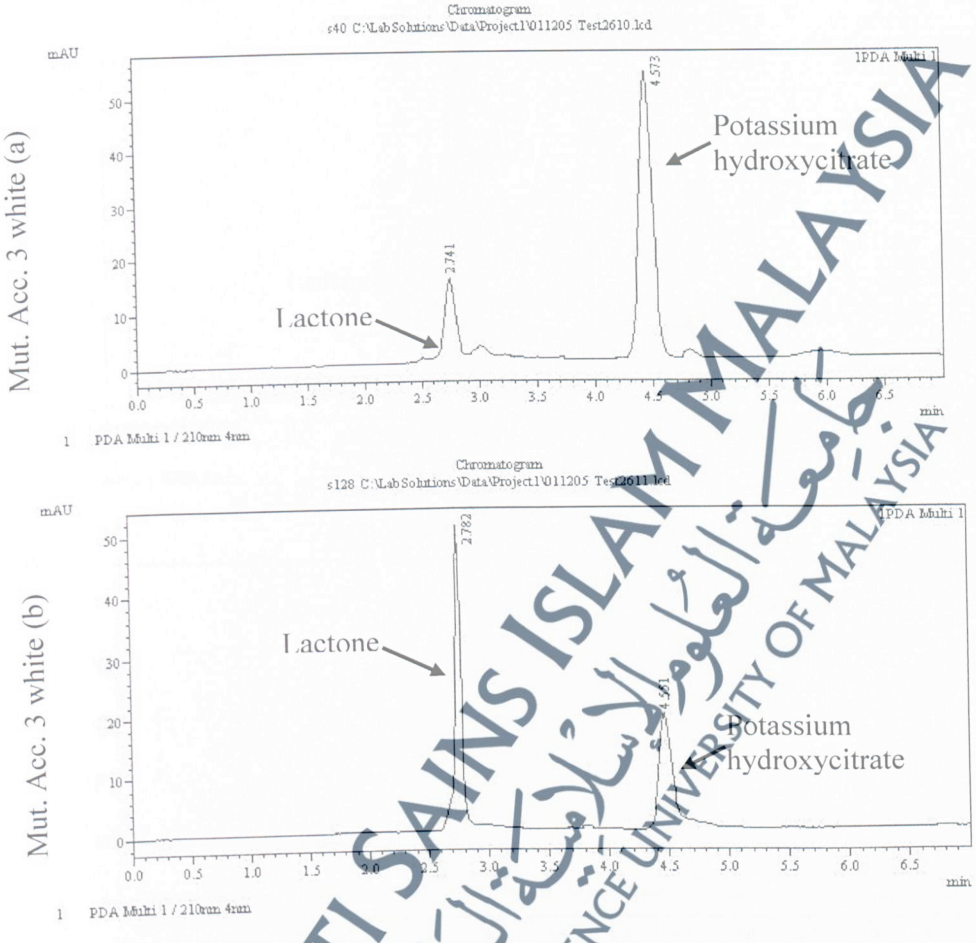


Figure 4.13 illustrates chromatograms showing separation of analytes for roselle HS03100-29-7-1-6-3-1 from extracts obtained in both extraction methods. Two major peaks at retention time 2.7min and 4.5min appeared in both chromatograms but only with one additional very minor peak in chromatogram from the original extraction method. In chromatogram from modified extraction method, the minor peak was almost oblivious demonstrating the effectiveness in the treatment of activated charcoal in eliminating impurities which can be clearly seen from the white-coloured extract obtained. Lactone was recognized in the first appearing peak at both histograms at retention time 2.7min while the compound looked in the study, potassium hydroxycitrate was recognized in the second major peak at retention time 4.5min.

Figure 4.14: Chromatograms showing separation of analytes for Acc. 12
(a) according to original extraction method
(b) according to modified extraction method

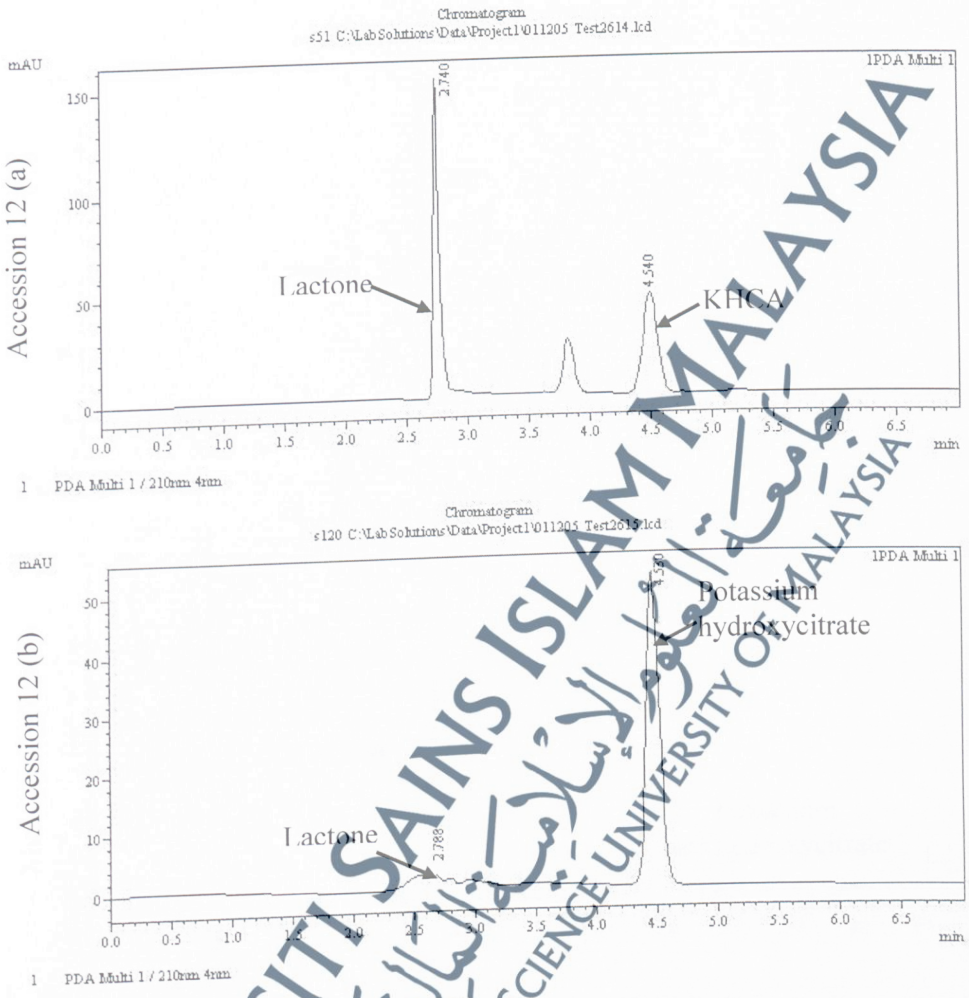


Figure 4.14 shows chromatograms showing separation of analytes for roselle Acc. 12 from extracts produced in both extraction methods. In both chromatograms, there were two major peaks at retention time 2.7min and 4.5min with only one very minor peak in between in the original extraction method. The minor peak can be disregard in chromatogram from the modified extraction method. Activated charcoal had successfully eliminated the impurities which existed in extracts produced through the original extraction method as can be proven from the white-coloured of extract yielded. The first appearing peak at both histograms at retention time 2.7min were recognized as lactone while the second major peak at retention time 4.5min was recognized as potassium hydroxycitrate, the compound sought in the study.

Figure 4.15: Chromatograms showing separation of analytes for HS1250-18-18-1-1-1- 1 (a) according to original extraction method
(b) according to modified extraction method

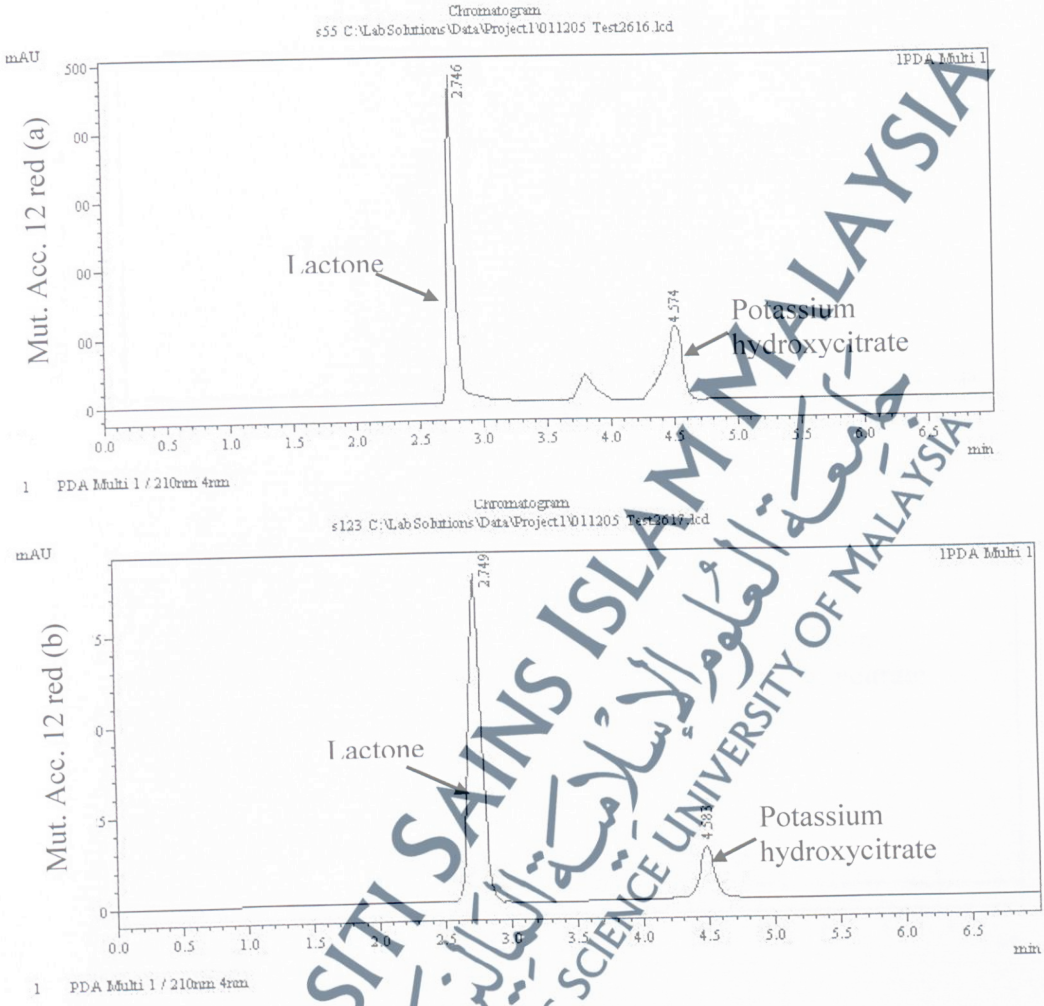


Figure 4.15 presents chromatograms showing separation of analytes for roselle HS1250-18-18-1-1-1- 1 from extracts yielded in both extraction methods. Two major peaks at retention time 2.7min and 4.5min existed in both chromatograms, but in chromatogram from the original extraction method there was one very minor peak in between the two peaks appeared. After the treatment of activated charcoal, this peak was seen absent implying the success of activated charcoal in eliminating the impurities. The first appearing peak at retention time 2.7min in both histograms was recognized as lactone while the second major peak at retention time 4.5min representing the compound looked in the study was recognized as potassium hydroxycitrate.

Figure 4.16: Chromatograms showing separation of analytes for HS1250-1-18-1-1-1-1 (a) according to original extraction method
(b) according to modified extraction method

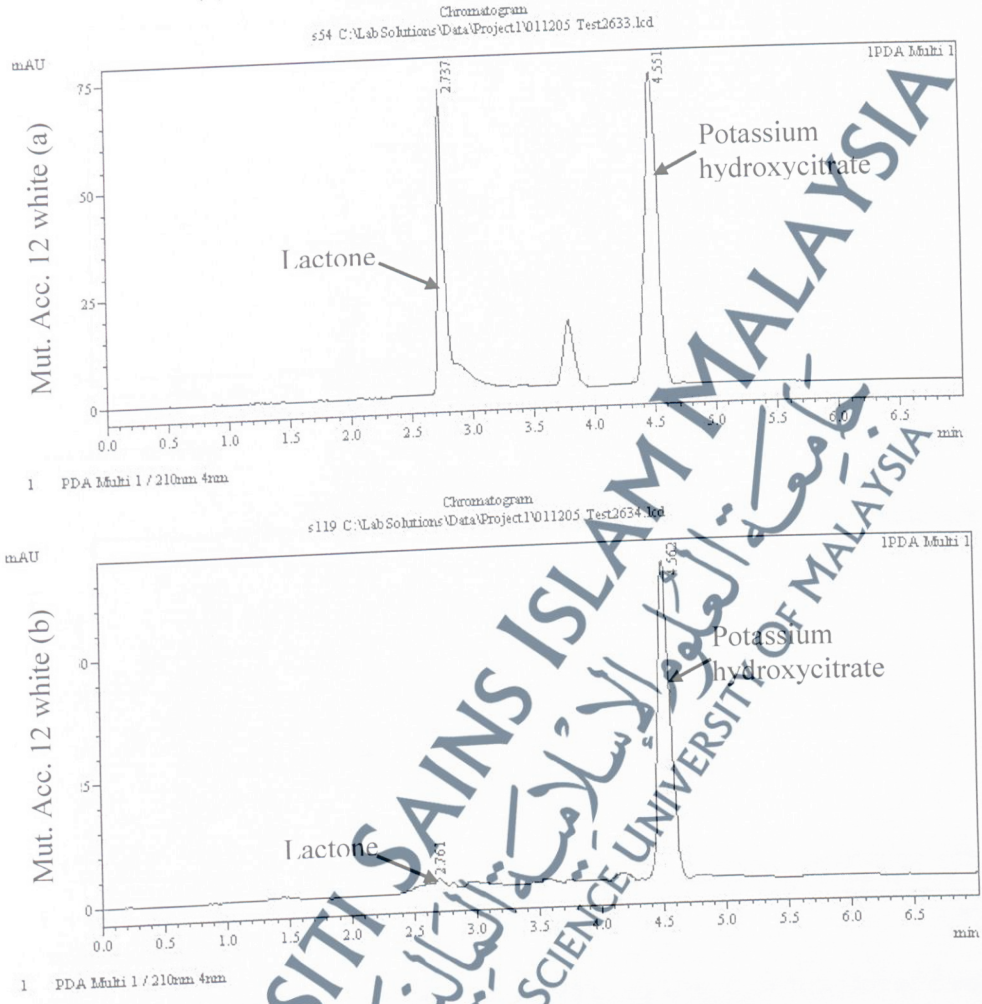


Figure 4.16 shows chromatograms showing separation of analytes for roselle HS1250-1-18-1-1-1-1 from extracts in both extraction methods. Two major peaks at retention time 2.7min and 4.5min appeared in both chromatograms but only with one additional very minor peak in chromatogram from the original extraction method. This minor peak was oblivious in chromatogram from extracts sample in the modified extraction method. The white-coloured of extract produced after the treatment of activated charcoal stated the elimination of impurities. The first appearing peak at both histograms at retention time 2.7min was recognized as lactone while the second major peak at retention time 4.5min was recognized as potassium hydroxycitrate, the compound sought in the study.

Figure 4.17: Chromatograms showing separation of analytes for *Garcinia atroviridis*
(a) according to original extraction method
(b) according to modified extraction method

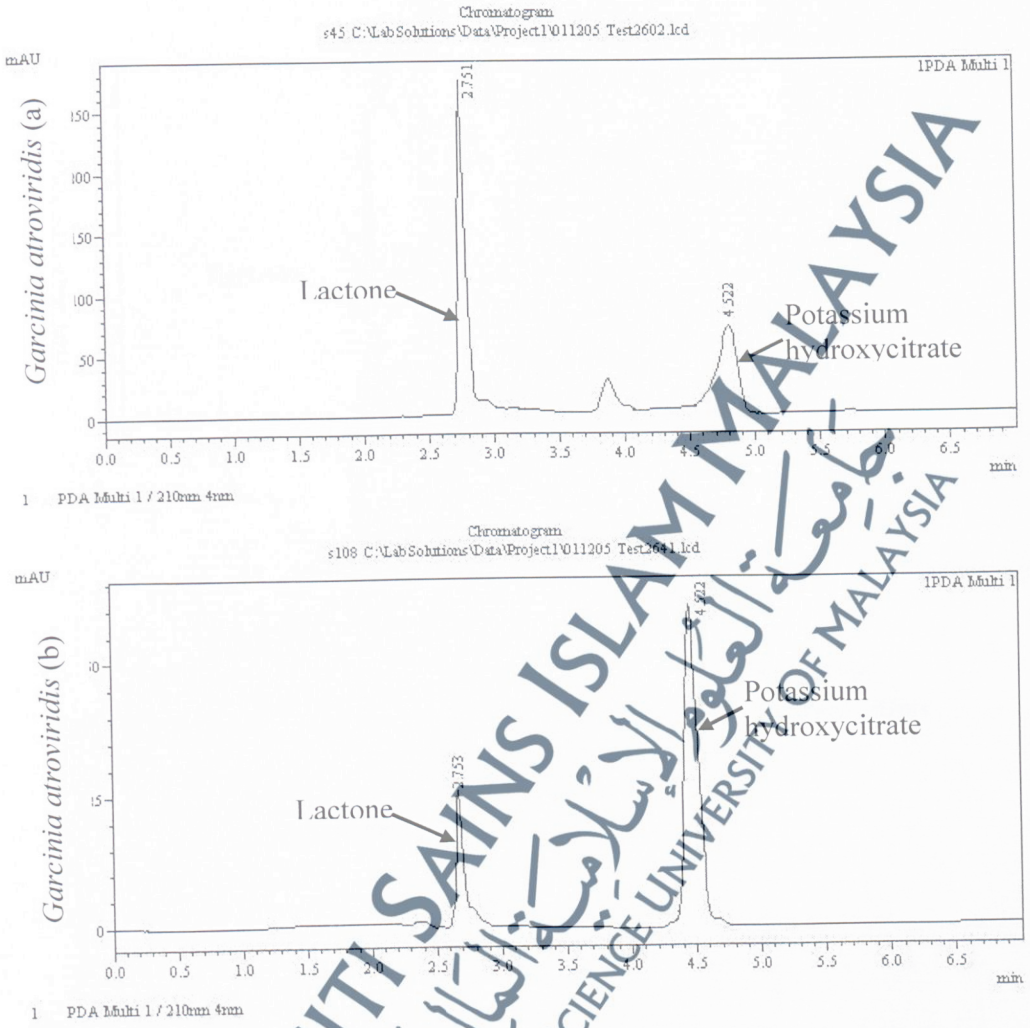


Figure 4.17 shows chromatograms showing separation of analytes of controls used in the study, *Garcinia atroviridis* from extracts in both extraction methods. Both chromatograms showed excellent separation of analytes. In both chromatograms, there were two major peaks at retention time 2.7min and 4.5min with only one very minor peak in between in the original extraction method. However, after the treatment of activated charcoal, this peak was non-existent proving the success of activated charcoal in eliminating impurities thus the white-coloured extract produced. Lactone was recognized in the first appearing peak at both histograms at retention time 2.7min while the compound looked in the study, potassium hydroxycitrate was recognized in the second major peak at retention time 4.5min.

Figure 4.18: Chromatograms showing separation of analytes for *Garcinia cambogia*
(a) according to original extraction method
(b) according to modified extraction method

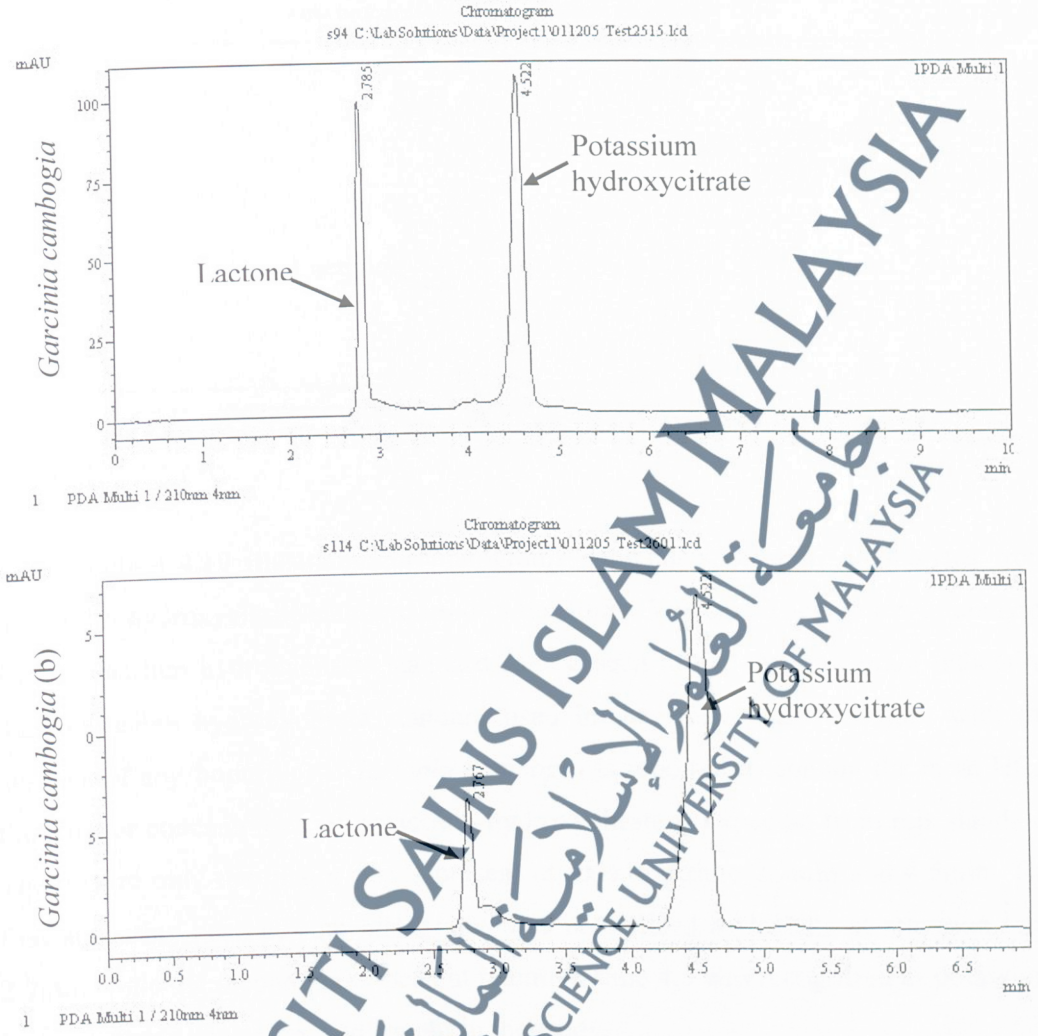


Figure 4.18 presents chromatograms showing separation of analytes of controls used in the study, *Garcinia cambogia* from extracts in both extraction methods. Both chromatograms showed very clear separation of analytes from extracts obtained in both extraction methods. HCA is reported to be the most in *Garcinia cambogia* thus higher concentration of potassium hydroxycitrate is expected from this control compared from roselle samples. In both chromatograms, there were only two major peaks present at retention time 2.7min and 4.5min. The first appearing peak at both histograms at retention time 2.7min were recognized as HCA lactone while the second major peak at retention time 4.5min was recognized as KHCA, the compound looked in the study.

Figure 4.19: Chromatograms showing separation of analytes for potassium hydroxycitrate standard

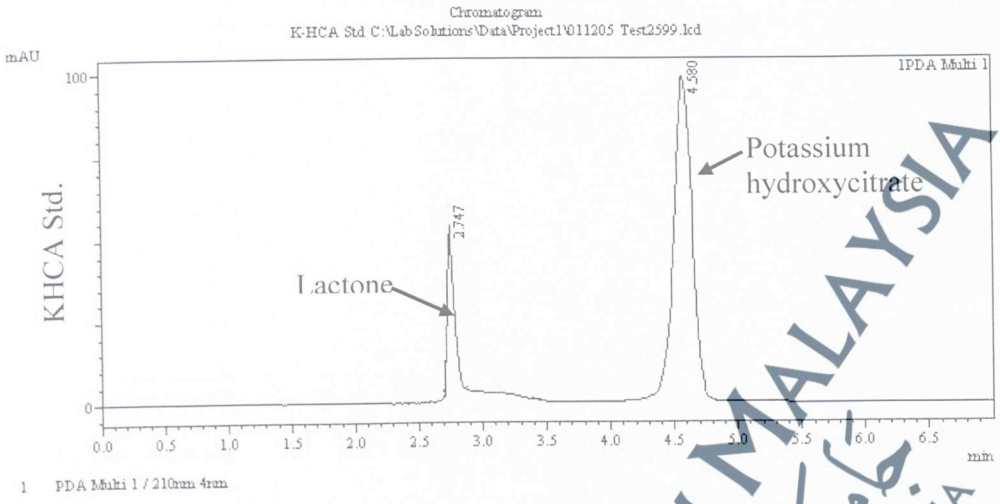


Figure 4.19 illustrates chromatograms showing separation of analytes from potassium hydroxycitrate standard used in the study. Very good separation of analytes from potassium hydroxycitrate standard can be seen in the chromatogram indicating that potassium hydroxycitrate standard used in the study was very pure with the absence of any impurities. *Garcinia cambogia* is reported to contain the most HCA thus higher concentration of potassium hydroxycitrate is expected from this standard. There were only two major peaks present at retention time 2.7min and 4.5min. The first appearing peak at both histograms was recognized as lactone at retention time 2.7min while the second major peak at retention time 4.5 was recognized as potassium hydroxycitrate, the compound sought in the study.

Figure 4.20: Chromatograms showing separation of analytes for lactone standard

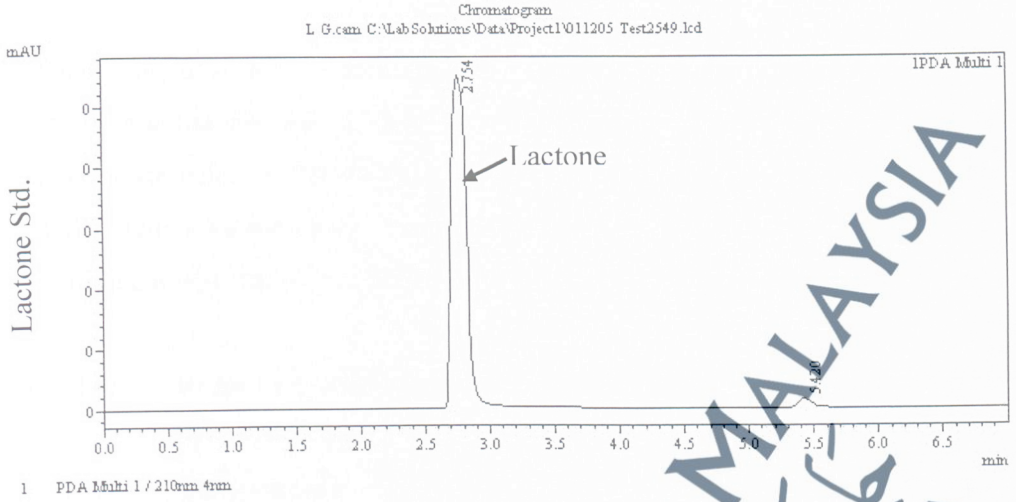


Figure 4.20 illustrates chromatograms showing separation of analytes from lactone standard used in the study. This lactone standard was used as another controls besides potassium hydroxycitrate standard in conducting the HPLC analysis to make sure the identity peak between potassium hydroxycitrate and lactone. As can be seen, at every chromatographic result, the presence of two major peaks was obvious. Therefore, it is very important to make sure which of these two peaks represent potassium hydroxycitrate, the compound studied. Then, it is only able to determine the concentration of potassium hydroxycitrate in every extract samples studied. Excellent separation of analytes from lactone standard can be seen in the chromatogram indicating that the lactone standard used in the study was very pure and free from any impurities. There were only one major peak appeared at retention time 2.7min which was recognized as lactone.

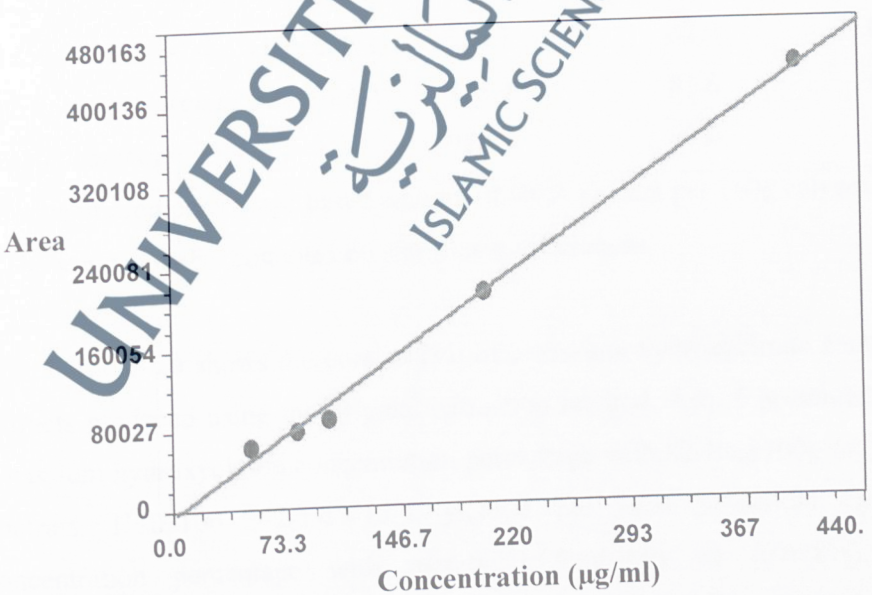
4.3.1 Quantification of Potassium Hydroxycitrate

In order to quantify potassium hydroxycitrate content in all extracts samples used in the study, a calibration curve was established from five concentrations of potassium hydroxycitrate using two replications. Table 4.8 shows the respective areas obtained from different concentrations of potassium hydroxycitrate. Figure 4.21 shows the calibration curve of potassium hydroxycitrate plotted using Curve Expert software.

Table 4.8: Respective areas obtained from different concentrations of potassium hydroxycitrate

Concentration (µg/ml)	Retention Time	Rep I	Rep II	Mean Area
50	4.538	44961	81257	63109±25665
80	4.562	76656	80431	78544±2669
100	4.522	88157	91638	89898±2461
200	4.526	193344	232095	212720±27401
400	4.568	432856	440167	436512±5169

Figure 4.21: The linear relationship between the respective areas and concentrations of potassium hydroxycitrate



The linearity has a good reproducibility and accuracy. The following regression equation was obtained: $y = 10558 - 4365.1x$, where y is the peak area and x is the concentration of potassium hydroxycitrate. The coefficient of determination (R^2) of the calibration graph was ≥ 0.998.

Table 4.9: Potassium hydroxycitrate concentrations from the original extraction method

Sample	Concentration (µg/ml) from calibration curve	Concentration (mg/100g calyces)	Concentration percentage (%)*
Accession 6	821.2	82.1	0.082^{c}±0.001
Accession 21	578.9	57.8	0.058^{f}±0.002
Accession 2	581.7	58.1	0.058^{e}±0.003
HS0275-30-4-4-1-1-1 (Red calyx)	596.7	59.6	0.060^{d}±0.007
HS0275-30-3-3-1-1-1 (White calyx)	309.5	30.9	0.031^{k}±0.006
Accession 3	553.3	55.3	0.055^{g}±0.004
HS03100-29-2-1-17-15-1 (Red calyx)	179.5	17.9	0.018^{m}±0.004
HS03100-29-7-1-6-3-1 (White calyx)	410.3	41.0	0.041^{i}±0.027
Accession 12	386.6	38.6	0.039^{j}±0.006
HS1250-18-18-1-1-1-1 (Red calyx)	295.	29.5	0.030^{l}±0.003
HS1250-1-18-1-1-1-1 (White calyx)	520.1	52.0	0.052^{h}±0.003
Asam keping (*Garcinia atroviridis*)	855.9	85.6	0.086^{b}±0.002
Garcinia cambogia	970.3	97.0	0.097^{a}±0.005

*Concentration percentage based amount of HCA present per 100g calyces

* The same alphabet connotes no significant differences

Table 4.9 shows the content (%) of potassium hydroxycitrate contained in the extracts produced using the original extraction method. Acc. 6 presented the highest potassium hydroxycitrate concentration percentage with 82.1mg/100g or (0.082%). In contrast, HS03100-29-2-1-17-15-1 yielded the least potassium hydroxycitrate concentration percentage with merely 17.9mg/100g or (0.018%). Potassium hydroxycitrate concentration percentage ranged from 0.03% to 0.050%. The list of

potassium hydroxycitrate concentration percentages were as followed: Acc. 21 57.8mg/100g or (0.058%), Acc. 2 58.1mg/100g or (0.058%), HS0275-30-4-4-1-1-1 59.6mg/100g or (0.06%), HS0275-30-3-3-1-1-1 30.9mg/100g or (0.031%), Acc. 3 55.3mg/100g or (0.055%), HS03100-2-1-17-15 17.9mg/100g or (0.018%), HS03100-29-7-1-6-3-1 41mg/100g or (0.041%), Acc. 12 38.6mg/100g or (0.039%), HS1250-18-18-1-1-1-1 29.5mg/100g or (0.030%) and HS1250-1-18-1-1-1-152mg/100g or (0.052%). The controls *Garcinia cambogia* presented with 97mg/100g or (0.097%) and *Garcinia atroviridis* with 85.6mg/100g or (0.086%). Results of the ANOVA showed that the mean squares for methods, samples and interaction between methods and samples were significant in both extraction methods used in the study. From the ANOVA analysis, the samples used in the original extraction method also showed to be significantly different. The different subsets of potassium hydroxycitrate concentration mean from this original extraction method were grouped with different alphabets as shown in (Table 4.9). Different mean subsets were significantly different with different listed alphabets.

Table 4.10 Potassium hydroxycitrate concentrations from modified extraction method

Sample	Concentration (µg/ml) from calibration curve	Concentration (mg/100g calyces)	Concentration percentage (%)*
Accession 6	302.2	30.2	0.030^{f} ±0.034
Accession 21	183.2	18.3	0.018^{i} ±0.021
Accession 2	77.9	7.80	0.008^{k} ±0.009
HS0275-30-4-4-1-1-1 (Red calyx)	340.2	34.0	0.034^{e} ±0.038
HS0275-30-3-3-1-1-1 (White calyx)	290.7	29.0	0.029^{fg} ±0.033
Accession 3	287.7	28.7	0.029^{g} ±0.033
HS03100-29-2-1-17-15-1 (Red calyx)	245.7	24.5	0.025^{h} ±0.164
HS03100-29-7-1-6-3-1 (White calyx)	165.0	16.5	0.017^{i} ±0.019
Accession 12	384.6	38.4	0.038^{d} ±0.044
HS1250-18-18-1-1-1-1 (Red calyx)	100.0	10.0	0.010^{j} ±0.011
HS1250-1-18-1-1-1-1 (White calyx)	425.1	42.5	0.043^{c} ±0.049
Asam keping (*Garcinia atroviridis*)	471.1	47.1	0.047^{b} ±0.053
Garcinia cambogia	911.1	91.1	0.091^{a} ±0.105

*Concentration percentage based amount of HCA present per 100g calyces

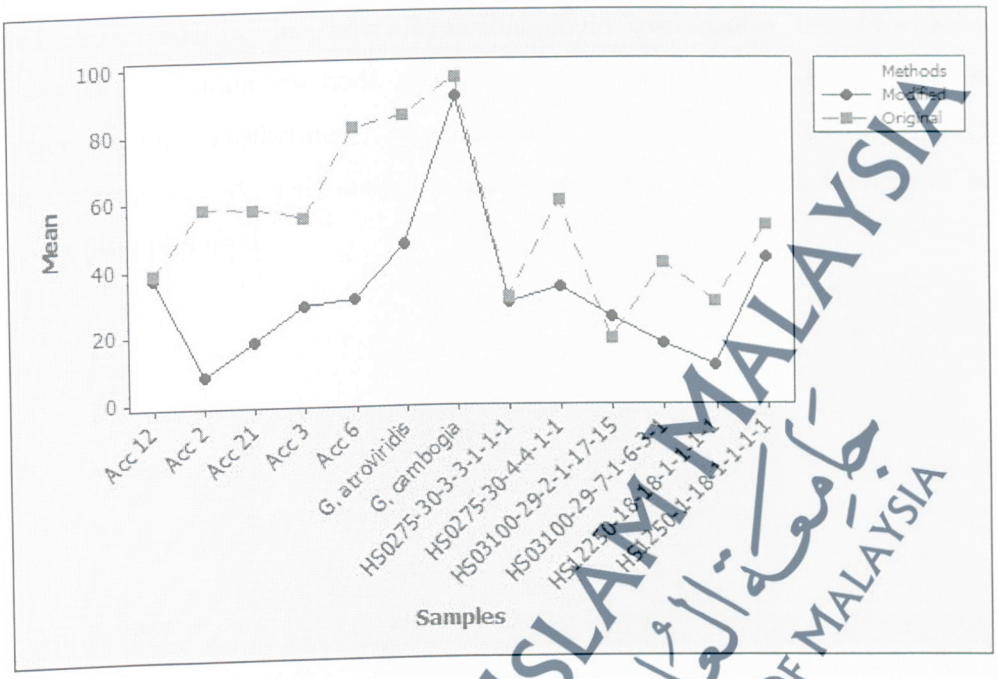
* The same alphabet connotes no significant differences

Table 4.10 shows the percentages of potassium hydroxycitrate contained in the extracts produced using the modified extraction method. The highest potassium hydroxycitrate concentration was in HS1250-1-18-1-1-1-1 42.5mg/100g or (0.043%). In contrast, Acc. 2 gave the lowest potassium hydroxycitrate concentration with only7.8mg/100g or (0.008%). On average, potassium hydroxycitrate concentration in all samples ranged from 0.010% to 0.040%. Acc. 630.2mg/100g or (0.030%), Acc. 21 18.3mg/100g or (0.018%), HS0275-30-4-4-1-1-1 34mg/100g or (0.034%), HS0275-30-3-3-1-1-1 28.7mg/100g or (0.029%), Acc. 3 28.7mg/100g or (0.029%), HS03100-29-2-1-17-15-1 24.6mg/100g or (0.025%), HS03100-29-7-1-6-3-1 16.5mg/100g or (0.017%), Acc. 12 38.4mg/100g or (0.038%) and HS1250-18-18-1-1-1-1 (0.010%).

The controls *Garcinia cambogia* presented with 91.1mg/100g or (0.091%) and *Garcinia atroviridis* with 47.1mg/100g or (0.047%). Results of the ANOVA showed the samples used in the modified extraction method were significantly different. The different subsets of potassium hydroxycitrate concentration mean from this modified extraction method were grouped with different alphabets as shown in Table 4.10. Different mean subsets were significantly different with different representated alphabets.

The potassium hydroxycitrate concentrations obtained from modified extraction method were less compared from the original extraction method. This was due to the treatment of activated charcoal in the process making that affects the overall yield of HCA constituent in the salt. It can be seen that the highest and lowest concentrated potassium hydroxycitrate in roselle samples were not consistent to samples from Majeed et al., (2005) HCA extraction method, probably due to the improper handling technique through the extraction method which require treatments involving activated charcoal treatment and base treatment process that lead to the salt formation that could give effect to the overall yield and concentration of potassium hydroxycitrate obtained finally. The optimal HCA extraction handling technique needs to be established consistently in order to assure the consistency of results obtained.

Figure 4.22: Potassium hydroxycitrate concentrations trend of 13 samples used in the study grouped based on their respective extraction methods



ANOVA analysis done showed that both extraction methods used in the study were significantly different from each other. From Figure 4.22, it can be seen the trend of potassium hydroxycitrate concentrations in both extraction methods. All samples showed significant decrease in potassium hydroxycitrate concentrations from original extraction method to modified extraction method. The reduction rate of extract yield was as high as 75% and more in some samples. It can be concluded that potassium hydroxycitrate concentrations obtained after modified extraction method were reduced compared to potassium hydroxycitrate concentrations obtained in the original extraction methods. Therefore, it is concluded that with the treatment using activated charcoal, traces of HCA may still be adhering to the charcoal, thus decreasing the concentration of HCA overall (Jena et al., 2002). Measures for this condition need to be taken to make sure that the concentration of HCA is maintained although after the treatment of using activated charcoal is conducted. The method could be established with further refinement.

HPLC method employed from Jayaprakasha & Sakariah (1998) used in determination of KHCA content in roselle sample in the study is simple and accurate.

This method can be used as an excellent alternate to other determination methods such as gas-liquid chromatography and titration method for the estimation of HCA in diluted extracts. HPLC has advantages that could overshadow the drawbacks of previous determination methods available which would give better and more consistent results. Furthermore, reproducibility, accuracy and sensitivity of this method are satisfactory. This method can be considered for routine analysis of large number of samples used.