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Isolation and quantification of hydroxycitric acid from batuan [*Garcinia binucao* (Blanco) Choisy] fruit

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Abstract

Hydroxycitric acid is a compound with anti-obesity property which is predominant in *Garcinia cambogia*. In the Philippines, another species, *Garcinia binucao*, locally known as batuan, is an indigenous fruit used as a souring agent.This study was conducted to determine the most effective method of hydroxycitric acid extraction from batuan *(Garcinia binucao)* fruit. Acid isolation was performed using three methods including water, methanol and acetone as extraction solvents. Isolated hydroxycitric acid was quantified using spectrophotometric analysis while thin layer chromatography was employed to determine its purity. Results revealed that *Garcinia binucao* is a potential source of hydroxycitric acid. The extracted hydroxycitric acid from batuan amounted to 4.81 + 0.12 g/100 g sample using water extraction method. Methanol and acetone extraction methods isolated 2.65 + 0.18 g/100 g sample and 2.76 + 0.08 g/100 g sample, respectively. Thin layer chromatography revealed that collected isolate using water extraction method is pure. Water extraction method was found out to be the most effective and efficient method to isolate hydroxycitric acid from batuan fruit.

Introduction

World Health Organization (1998) defined obesity as "phenotypic manifestation of abnormal or excessive fat accumulation that alters health and increases mortality". According to WHO Fact Sheet (2016), more than 1.9 billion adults are overweight, of these, over 600 million were obese. The worldwide obesity has more than doubled since 1980.

In the Philippines, a survey by the Food and Nutrition Research Institute (2011) showed that 22.3% of Filipino adults are overweight and 6.1% are obese. Based on the gathered data, the prevalence of overweight Filipinos is expected to increase significantly by 2015. There were numerous factors leading to obesity in developing countries like the Philippines. One leading factor is increasing world and domestic food prices, forcing increased purchase of unhealthy processed food over healthy and staple but expensive food (Astrup, 2001).

While the strategy of reducing dietary fat content combined with increased physical activity has been shown to be effective in preventing obesity (Astrup, 2001), numerous studies have shown that this simple message is being ignored and alternative strategies are being sought (Wadden, 1993; Stern *et al.*, 1995; Kruger *et al.*, 2004). With these findings, scientists developed interest in studying and developing potential dietary compounds which can help counteract obesity.

Many compounds have been identified as having anti-obesity properties. In the recent years, hydroxycitric acid has been identified as one with high potent activity in controlling obesity. It was first isolated by Von Lippman on 1883 as a minor constituent of sugar beet. Since then, researches about the possible health benefits from hydroxycitric acid emerged. In 1970, Watson and Lowenstein reported the powerful inhibition by (-) hydroxycitric acid of citrate cleavage enzyme, a key factor in fatty acid synthesis, thus, preventing fat accumulation. Today, hydroxycitric acid is commercially available as a dietary supplement. This compound is commonly extracted from *Garcinia cambogia*, a well-known source of hydroxycitric acid.

In the Philippines, a close relative of *Garcinia cambogia* called *batuan (Garcinia binucao)* is gaining economic significance. It is an indigenous, under-utilized crop that is well-known in the Visayas region as a souring agent (dela Cruz, 2012). In Palawan, the Bureau of Agricultural Research, Department of Agriculture (DA-BAR) is studying the export potential of *batuan*, together with *paratungon* and *dugyan*, to give the indigenous groups in the province

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an alternative source of living.

As a close relative of *Garcinia cambogia,* there is a high possibility that hydroxycitric acid is also present in *batuan* fruit. If this is proven, *batuan* will have a greater value as a crop since it will be a potential source of hydroxycitric acid for the food and pharmaceutics industry. Hence, this study was conducted to isolate and quantify hydroxycitric acid from *batuan*. It also aimed to determine the most effective and efficient extraction solvent and method for hydroxycitric acid isolation from *batuan* fruit.

Materials and Methods

Source of Batuan fruits

Fresh and unripe *batuan* fruits were collected from a farm located at Brgy. La Granja, La Carlota City, Negros Occidental, Philippines. The fruits that were used in the study came from the same batch with characteristic green color, hard covering and diameter of about 3.7-5.5 cm.

Preparation of fresh Batuan fruit pulp

Fresh *batuan* fruits were washed initially with tap water to minimize microbial load and remove adhering contaminants such as soil and leaves. Then, the whole fruits were disinfected by soaking in 10 ppm hypochlorous acid (HOCl) solution for 20 seconds. The fruits were washed again in running potable water to remove excess chlorine in the fruits. After cleaning, the pulp was separated from the seeds and then chopped into small pieces.

Isolation of hydroxycitric acid from fresh Batuan fruit pulp

Water extraction

The water extraction method was based on the protocol by Krishnamurthy *et al*. (1982). Six hundred millimeters (600 mL) distilled water was added to 200 g of *batuan* fruit pulp. It was autoclaved at 115°C for 15 min. The cooled extract was decanted through several folds of cheesecloth and filtered on a Buchner funnel (Whatman No.1 paper).The residue was washed with distilled water and then the filtrate was collected. The dark brown filtrate was concentrated to about 100 mL on a water bath and then treated with 200 mL of ethanol with stirring. The resulting precipitate is a pectinous material that was removed by centrifugation and filtration. The acidic filtrate was neutralized by cautious addition of 40% KOH, with careful stirring. The heavy oily liquid, which was formed, was allowed to settle for a few minutes and the supernatant was decanted and discarded. The oily liquid was washed with 60% ethanol (five portions of 100 mL). After that, it was washed again with absolute alcohol (two portions of 100 mL), the suspension was left to stand for 60 - 90 minutes each time. A further portion of 100 mL of absolute ethanol was added and then it was allowed to stand overnight. Ethanol was decanted, and the yellow hygroscopic semisolid was obtained. To recover the dried sample, minimum amount of water was added to dissolve it. The thick, concentrated liquid was then transferred to a vial and stored in freezer.

Methanol extraction

The methanol extraction method that was used in the study is based on US PATENT 6770782 B1 used in production of CITRIN®-K. The hydroxycitric acid that was isolated using this protocol is in the form of potassium salt. Six hundred milliliter (600 mL) of methanol was used to extract hydroxycitric acid from 200 g of *batuan* fruit pulp at about reflux temperature for 3 hours. The first extract was collected through filtration using cloth. Another 600 mL of methanol was added to the original *batuan* fruit pulp for second extraction. It was filtered again and third extraction using another 600 mL of methanol followed.

All three extracts were collected and combined. It was then treated with methanolic potassium hydroxide at pH 10. The extract was refluxed again for about 3 hours to attain constant pH 10 in order to precipitate potassium hydroxycitrate. The precipitate was filtered and washed with 200 mL methanol and then dried at about 70°C. The dried sample was dissolved in minimum amount of distilled water and then it was transferred to a small vial. The thick, concentrated liquid was stored in the freezer.

Acetone extraction

The acetone extraction method is based on the published protocol by Lewis (1969). Two hundred grams (200 g) of *batuan* fruit pulp was kept in 300 mL of acetone overnight in a shaker. The same sample was re-extracted in the same manner after the first extract was collected. The second extract was also collected and combined with the first. The acetone was removed from the solution of combined extracts by distillation. The viscous solution that remained after distillation was added with 100 mL of water with constant stirring at 45-50°C. The mixture was filtered through cheesecloth and then the insoluble residue was removed. The extract was concentrated to about 40 mL using hot water bath.

Evaluation of isolation methods

To determine the most efficient isolation method,

the obtained extracts using water extraction, methanol extraction and acetone extraction were collected and quantified using spectrophotometric assay. Thin layer chromatography (TLC) was also performed.

Quantification of hydroxycitric acid by spectrophotometry

The following procedure is based on protocol by Antony *et al.* (1999). Exactly 0.2 g of isolated extract using water extraction method was weighed. On the other hand, about 0.4 g of extracts collected using methanol and acetone was prepared in separate containers. Five milliliters (5 mL) of 1N H_2SO_4 was added to dissolve the sample and then it was diluted to 25 mL using distilled water. The solution was filtered into a 50 mL volumetric flask. The residue was washed and then diluted to volume.

Food grade *Garcinia cambogia* extract (80% hydroxycitric acid) was used as standard. Exactly 0.4 g of the standard was weighed and then dissolved in 10 mL 1N H_2SO_4. Fifty milliliters (50 mL) of distilled water was added. The solution was transferred into 100 mL volumetric flask and then diluted to volume. It served as the stock solution for standard.

In the analysis using spectrophotometry, 1% ammonium monovanadate (NH_4VO_3) was used. Preparation of standards and analysis of sample were done using the following procedure. From the stock solution, exactly 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL and 0.6 mL was dispensed in individual 50 mL volumetric flasks and then diluted to volume. Exactly 4.5 mL 1% ammonium monovanadate in water was added to allow color development. The color of the solution became yellow. As time advanced, the yellow color was slightly changed to orange red. Absorbance was noted at 467 nm after 20 minutes. Calibration graph was derived from the absorbance data.

The concentration of hydroxycitric acid (HCA) in the samples were computed using the following formula:

$$\text{HCA content (g/100 g sample)} = \frac{[HCA] \times Vol.\ extract \times DF}{Weight\ of\ sample} \times 100$$

Determination of hydroxycitric acid purity by thin layer chromatography (TLC)

The purity of the isolated extracts using different isolation methods from *batuan* fruit pulp was determined using thin layer chromatography. Standard solutions of citric and hydroxycitric acids were prepared to serve as the basis for analysis.

Three hundred milligrams (300 mg) of chemically pure citric acid was dissolved in 10 ml 0.1 N HCl. For preparation of HCA standard, 380 mg of food grade *Garcinia cambogia* extract was dissolved in the same solvent. The solutions were filtered to remove the suspended solids.

For *batuan* extracts, 500 mg of crude extracts from water, methanol and acetone extraction methods were dissolved in 10 mL 0.1 N HCl. It was then filtered to remove residues. After filtration, the filtrate was directly used for the thin layer chromatography analysis.

Precoated TLC plates prewashed with methanol were used as the stationary phase while methanol water solution (6:2 v/v) was used as the mobile phase. Three microliters (3 µL) of citric and hydroxycitric acid standards were spotted onto the TLC plates. On the other hand, samples were spotted at 2 µL. After spotting, the spots were allowed to dry.

Ascending, one dimensional development of the plates inside the saturated chamber followed. The solvent was allowed to run up to 8 cm from the point of sample injection. The running time was approximately 30 minutes.

After the development, the plates were heated using hair blower to dry the samples and mobile phase. To visualize the spots, 1% ammonium monovanadate was sprayed in the developed chromatogram. The chromatogram was baked in then oven for 2 minutes at 105 °C. The color of the spots were noted and the R_f values of the standards and samples were computed and compared with one another. The R_f value was computed using the following formula:

$$\text{Retention factor } (R_f) = \frac{Distance\ traveled\ by\ the\ solvent\ (cm)}{Distance\ traveled\ by\ the\ sample\ (cm)}$$

Statistical analysis

All analyses was done in triplicates. The gathered data were analyzed statistically using Analysis of Variance (ANOVA) to determine if the samples significantly differed from one another, followed by Tukey's Honest Significant Difference (HSD) Test to know which among the samples are significantly different. All data were analyzed using STAR software.

Results and Discussion

Comparative analysis of hydroxycitric acid and citric acid

Knowledge and awareness about hydroxycitric acid is relatively new compared to other organic acids present in fruits. Due to its high structural similarity with citric acid, it has been difficult to quantify and study hydroxycitric acid alone. Most of the studies dealing with organic acid profile of fruits do not include hydroxycitric acid. Considering the affinity

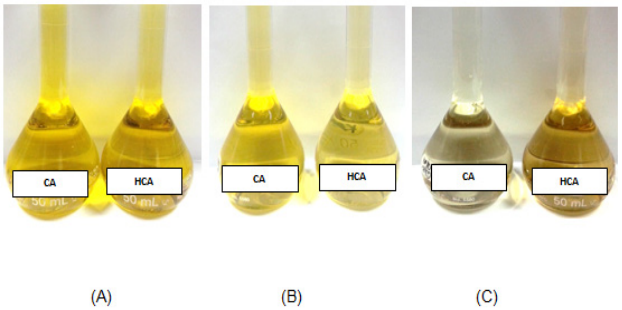

Figure 1. Color formation of hydroxycitric acid (HCA) and citric acid (CA) at different concentrations (A) 31 µg/mL (B) 92 µg/mL (C) 308 µg/mL

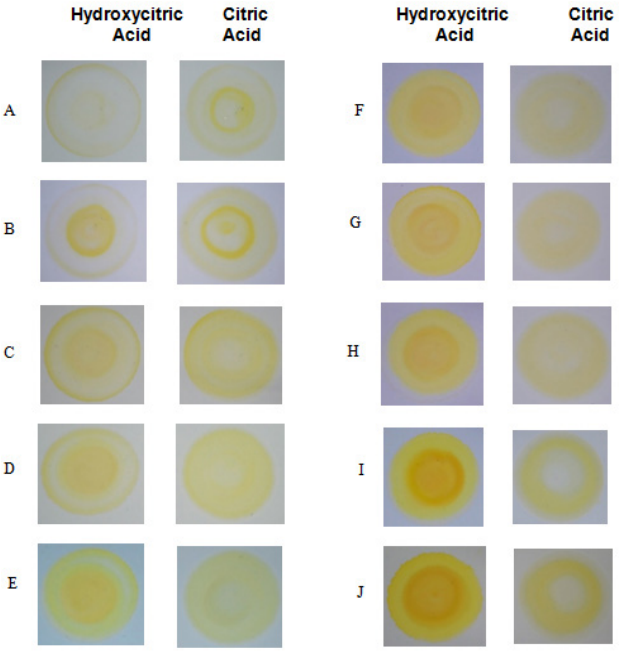

Figure 2. Color formation of hydroxycitric acid and citric acid with 1% ammonium monovanadate at different concentrations on TLC plates (A= 4.0 mg/mL, B= 8.0 mg/mL, C= 12.0 mg/mL, D= 16.0 mg/mL, E= 20.0 mg/mL, F= 24.0 mg/mL, G= 28.0 mg/mL, H= 32.0 mg/mL, I= 36.0 mg/mL, J= 40.0 mg/mL)

of citric and hydroxycitric acid in different solvents which is greatly dependent on their structure, there is high probability that hydroxycitric acid is mistakenly quantified as citric acid. Since this study deals with hydroxycitric acid alone, it is important to be certain that the quantification method measures the amount of hydroxycitric acid alone in the samples.

Differences in color formation of hydroxycitric and citric acids were observed at higher concentration. Citric acid produces light yellow color while hydroxycitric acid produces red orange color (Figures 1 and 2). This could be explained by their difference in absorbance spectra as shown by their different λmax values (Figure 3). Lambda max (λ_{max}) is defined as the wavelength of maximum absorbance (Nielsen, 2010). The λ max of citric and hydroxycitric

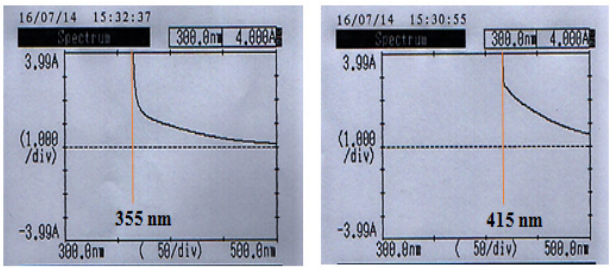

Figure 3. Absorbance spectrum of (A) citric acid (B) hydroxycitric acid

acid is 355 nm and 415 nm, respectively. It indicates that these two acids have different reaction with 1% ammonium monovanadate despite the close similarity in their structure. Reaction of the acids with the reagent lead to formation of two different light absorbing species. Since color differences are detected only at higher concentrations (308 µg/mL), citric acid can be an interference and might be accounted for as hydroxycitric acid. Therefore, a method for the isolation of the hydroxycitric acid from citric acid is necessary.

Spectrophotometric quanitification of hydroxycitric acid extracted from Batuan fruit using various solvents

Quantification of hydroxycitric acid was done using spectrophotometry. The assay involves formation of a red orange complex when hydroxycitric acid reacted with vanadate (Antony *et al.,* 1999). The intensity of the color complex formed is directly proportional to quantity of hydroxycitric acid present in fruit.

Table 1 shows that water extraction method was able to isolate the highest amount of hydroxycitric acid (4.05 ± 0.02 g/100 g *batuan* pulp), followed by acetone extraction method (2.76 ± 0.08 g/100 g *batuan* pulp) and the least is methanol extraction with 2.65±0.18 g/100 g *batuan* pulp. The water extraction method yielded significantly higher amounts of hydroxycitric acid compared with the other extraction methods. On the other hand, acetone and methanol extraction methods were not significantly from each other statistically.

The difference of the isolation methods mainly depends on the polarity of the extraction solvent that was used. Among the three extraction solvents, the most polar is water followed by methanol and then acetone. According to Barwick (1997), the polarity index of water is 9.0 while methanol and acetone have polarity index equal to 6.6 and 5.4, respectively. Based on the structure of hydroxycitric acid, it is relatively polar because of two hydroxyl and three

Table 1. Computed hydroxycitric acid content of batuan fruits using different extraction methods.

EXTRACTION METHOD	HYDROXYCITRIC ACID CONTENT (g/100 g fresh *batuan* pulp)
Water	4.05 ± 0.02[a]
Methanol	2.65 ± 0.18[b]
Acetone	2.76 ± 0.08[b]

*Means followed by the same superscript are not significantly different from one another at 0.05 level of significance.

carboxyl groups present in its structure. Thus, it is more soluble in water compared to methanol and acetone. The obtained values from the experiment agrees with the stated properties of extraction solvents.

Determination of hydroxycitric acid purity by thin layer chromatography

Thin layer chromatography was employed to confirm the identity and purity of the isolates from fresh *batuan* fruit pulp obtained using different extraction methods. Chemically pure citric acid (CA) standard and food grade hydroxycitric acid (HCA) standards were used as a basis for the identification of the compounds.

Visualization of spots was done by spraying the developed chromatogram with 1% ammonium monovanadate solution. The visualizing agent produce red orange color when it reacts with hydroxycitric acid while yellow color was produced when citric acid is present. Figure 4 shows the appearance of the chromatogram during visualization.

Spots with different colors were observed from the chromatogram. Hydroxycitric acid produces red orange spots while citric acid produces yellow spots (Antony *et al*., 1999). Samples collected using different extraction methods also produces red orange spots. It means that all extraction procedures were able to isolate hydroxycitric acid. However, it is evident that samples collected using water extraction was of higher purity compared with samples collected using methanol and acetone extraction. The spots observed in water extraction samples were smaller and has minimum amount of smears that could be caused by contaminants.

The retention factor (R_f) of the spots were also computed relative to the solvent front of the chromatogram. Based on the result, citric acid has relatively higher R_f value compared with hydroxycitric acid. TLC plates used in the experiment consist of silica gel. Silica gel which serves as the stationary phase is highly polar. The mobile phase consists of methanol and water mixture (6:2). This

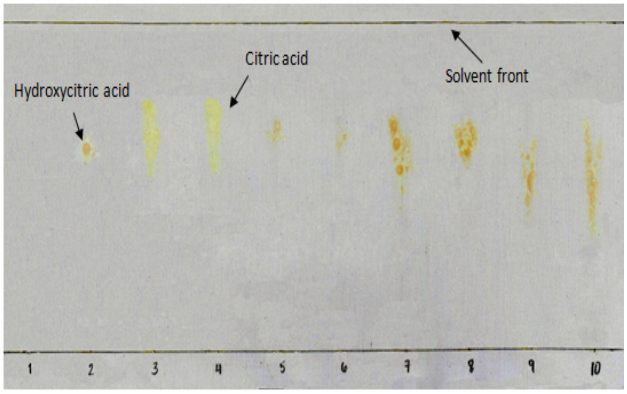

Figure 4. Thin layer chromatogram. Lane 2 HCA standard (30.7 mg/mL), 3-4 CA standards (30.4 mg/mL), 5-6 water extraction (50.1 mg/mL), 7-8 methanol extraction (51.8 mg/mL), 9-10 acetone extraction (52.3 mg/mL)

solvent is considered as nonpolar due to high amount of methanol. Comparing the structure of citric and hydroxycitric acid, hydroxycitric acid is more polar than citric acid due to additional hydroxyl group in its structure. Thus, hydroxycitric acid has higher affinity to the polar stationary phase while citric acid tend to have higher affinity to the mobile phase.

Even though citric acid has higher Rf value (0.60-0.70) compared with hydroxycitric acid (0.55-0.65), the observed difference was not significant due to the overlap of the computed values. Therefore, color difference is a more reliable parameter to consider in identifying the presence and purity of hydroxycitric acid in the chromatogram. This observation is an important evidence proving that hydroxycitric acid and citric acid react differently with 1% ammonium monovanadate. It strengthens the validity of quantification of hydroxycitric acid using spectrophotometry.

Conclusion

Results revealed that *batuan* fruit is a potential source of hydroxycitric acid. Isolation of hydroxycitric acid was necessary for its quantification as citric acid can be an interference at low concentrations. Water extraction method isolated the highest amount of hydroxycitric acid from the fruit pulp followed by acetone extraction and methanol extraction. Analysis using thin layer chromatography revealed that all three extraction methods were able to isolate hydroxycitric acid. However, extracts from acetone and methanol have low purity. Based on the result of spectrophotometric analysis and thin layer chromatography, it can be concluded that water extraction method is the most effective method for hydroxycitric acid isolation from *batuan* samples.

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