MINISTRY OF EDUCATION AND TRAINING
THE UNIVERSITY OF DANANG

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RESEARCH ON EXTRACTED HYDROXYCITRIC ACID COLLECTION FROM THE *GARCINIA COWA* ROXB. RINDS TO MAKE DOUBLE SALT HYDROXYCITRATE SALT FOR OBESITY REDUCING APPLICATION

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INTRODUCTION

1. Rationale (Reason for choosing the topic)

The magic development of science and technology at the swift speed is podsing the great goal to progressively improve the natural, bring the perfect value for life as all human being desire. Today, the reality is that the majority of people living in urban areas have a relatively full physical life, especially a helping with eutrophic nutrients but the less approriate things as the main cause of overweight. Obesity is rapidly increasing and widespread in our modern society. This situation is not only bad for human health and aesthetics, but also a burden for the health sector in particular and the economy in general.

According to statistics of World Health Organization (WHO), in 2014, there are 1,9 billion overweight adults (equivalent to 39% of the population), of which 600 million had obesity syndrome . In accordance with WHO data, the number of people with obesity syndrome has increased twice as available as one since 1980. The cost of syndrome management and treatment is estimated to account for from 2% to 7% compared with total cost for public health care in developed countries. According to data from the Department of Preventive Medicine - Ministry of Health in 2014, Vietnam - a country in the Third World has a proportion of overweight and obesity adults accounting for about 25% of the population. Scientists continue to warn that the number of people suffering this syndrome will continue to increase and are likely to multiply into adolescence. Obviously, overweighting and obesity have become problems, trigerring a burden for the public health solution of the whole society, which is also the initial cause for finding a rational and effective anti-obesity solution to prevent this century disease, a disease just behind the HIV pandemic and armed conflict.

One of the solutions is very concerned by the prospective application, both to ensure efficiency and safety for health and especially to use the rich source of available bio-active domestic

ingredients. Including one of them, the hydroxycitric acid extracted from the rind skin - a naturally occurring obesity-reducing compound - was widely used as a hydroxycitrate in salt manner.

From the 50s of the last century to today, many published collections of hydroxycitric acid and hydroxycitrate salts are obtained mainly from the fruit of *Garcinia* (*Garcinia cambogia*, *Garcinia cochinchinensis* (Lour.) Choisy, *Garcinia atro viridis*, *Garciniai indica*, ...). For the fruit from *Garcinia cowa* Roxb. dependent on family of garcinia cambogia in accordance with published data, the content of hydroxycitric acid accounts for 27,1%, so in the near future, the *Garcinia cowa* Roxb. will be used as a source of raw materials for the purpose of extracting hydroxycitric acid (HCA). However, HCA acid is lactosed free in the process of vaporization and concentrates into liquid, which is not conducive to commercialization, so it is usually converted to solid salts. In addition, the hydroxycitrate salts of cations are absorbed easily water, difficult for preservation. HCA's dual salt solution makes solid product that are both commercially viable and limited ability to absorb moisture at the same time and provides many micronutrients in the body, which is a concerned trend.

In Vietnam, there have been several published studies related to hydroxycitric acid as well as hydroxycitrate element. However, almost no work has been done for completion of the research on chemical composition, especially related to the technology of extracting and making salt from the source of *Garcinia cowa* roxb. *Garcinia cowa* Roxb. fruit is grown in almost all mountainous and midland provinces (Vinh Phuc, Phu Tho, Lao Cai, Tuyen Quang, Bac Giang, Yen Bai), many in the garden and in the field, with the expected output of millions of tons of fruit per year.

Inherited the relevant published scientific researches and material areas with sufficiently high yields to implement the application, we chose the topic ***"Research on extracted hydroxycitric acid collection from the Garcinia cowa* Roxb. *rinds to make double salt***

hydroxycitrate salt for obesity reducing application" to carry out the content of doctoral dissertation.

I hope that this thesis will contribute to the improvement of material of the *Garcinia cowa* Roxb. It is possible to open up a positive outlook on the scale of development of medicinal plants available in the country as well as the suitable, effective method of technology application to improve the people's life in the pharmaceutical area.

2. Research purposes

- Determine hydroxycitric acid content (HCA) in the extract from *Garcinia cowa* Roxb.

- The synthesis ofdouble salt hydroxycitrate protocol from hydroxycitric acid extract of *Garcinia cowa* Roxb. rind.

- Use preparation to evaluate the possibility of weight loss and contribute to enrich the research literature on this *Garcinia cowa* Roxb.

3. Research objects and scope

* Object: The rind of *Garcinia cowa* Roxb. collected in July and August in Vinh Phuc province.

* Scope of research: Extracting of *Garcinia cowa* Roxb. rind by water soluble; determining the content of hydroxycitric acid (HCA); Syntheticizing of hydroxycitrate double salt; the usage of biological activation of double salt hydroxycitrate.

4. Methodology

* Theoretical methodology: Synthesize the literature on research method on *Garcinia*, especially *Garcinia cowa* Roxb. Hydroxycitrate double salt synthesis methods.

* Experimental Methodology: Sampling and material process methods. Extract hydroxycitric acid (HCA) and synthesize hydroxycitrate double salt. Determine acid content, metal ion content in double salt, atomic absorption spectroscopy - AAS method, determine the structure of synthetic products and biological application methods of hydroxycitrate double salt.

5. New contributions of the thesis

a. As for author searching reference materials, this is the first time the hydroxycitric acid extracting has been studied in the *Garcinia cowa* Roxb. In Vietnam, high content (12,40%) is obtained by water stickling method and this acid is first synthesized by dichorionic salt, which was mainly from other species of G*arcinia* such as *Garcinia cambogia*, *Garcinia indica*, *Garcinia atroviridis*.

b. As for author searching reference material, two salt of Na/Mg HCA (sodium magnesium hydroxycitrate) and Na/Zn HCA (zinc sodium hydroxycitrate) of *Garcinia cowa* Roxb. rind have been synthesized for the first time in Viet Nam.

c. The double salts of Na/Mg HCA and Na/Zn HCA preparations were researched and evaluated biological effects and the safety through experiments on mice in research methods of weight loss, blood lipid lowering effects and acute toxicity, semi-chronic toxicity.

6. Scientific meaning and reality of topic

Build a process of synthesizing double salt hydroxycitrate in the form of industrial scale, creating bio-products that support both health and making contribution to increase of the aesthetics and beauty needs that are increasingly "exploding" in The present era. Therefore, planning material areas, transforming plants, developing medicinal plants for economy, raising incomes, improving living standards for people in disadvantaged areas and pharmaceutical regions in the country are necessary.

7. Structure of thesis

The thesis is consist of 132 pages, of which there are 36 tables and 81 figures. 05 page of introduction, conclusion, new contributions and recommendations of 03 pages, published scientific works of 01 pages, reference material of 15 pages. The content of the thesis is divided into 03 chapters:

Chapter 1. Overview of 31 pages.

Chapter 2. Materials and research methods of 14 pages.

Chapter 3. Results and discussion of 63 pages.

CHAPTER1. GENERAL OVERVIEW

1.1. Introduction to *Garcinia*

Family of Garcinia (Clusiaceae or Guttiferae do Antoine Laurent de Jussieu were published in 1789), is a family of flowering plants, including about 27 to 28 genera and 1.050 species or only 14 genera with 595 species up to the classification opinion.

Garcinia (scientofic name: *Garcinia*) is very large genus in the Garcinia family, a heterogeneous species (flowers like males, females, bisexual flowers) or shrubs and is widely distributed in southern Africa and tropical regions in Asia, Australia and Polynesia. In Vietnam, this species is widely distributed from the northern provinces such as Tuyen Quang, Vinh Phuc, Hoa Binh, ... to the central provinces like Ha Tinh, Thua Thien Hue, Quang Nam and Da Nang. In particular, species of *Garcinia cowa* Roxb. are mainly concentrated in the North and North Central provinces.

1.2. Description and distribution of *Garcinia cowa* Roxb.

1.2.1. Plant description

- *Garcinia cowa* Roxb. with scientific name - *Garcinia cowa* Roxb., is a species of quince in the family of Mangosteen (Clusiaceae).

- Stem is medium, about 16 - 18m high, with a lot of branches, straight stem, slightly dowm top. Leaves is single leaf, opposite, 7-17 cm long, 2,5-7 cm wide. Flowers are heterogenious tree with 3-8 flowers of male flowers, arranged in 1 cm long stalks. Fruit is in the sphere with four to eight squares and thick green gold pods, including 6-10 seeds. Blossom season is March and June,fruit harvest is August and September. Figure of tree, leaves and dried rinds of *Garcinia cowa* Roxb. Figure 1.1.

Figure 1.1. Tree, leaves and rinds of *Garcinia cowa* Roxb.

1.2.2. Distribution

Garnicia cowa Roxb. is a tropical plant for edible fruit, widlly growing on the edge of Southeast Asian forest such as South Thailand, Myanmar, Indonesia, ... and mainly distributed in north eastern India. In Viet Nam, this plant is grown in mountainous and midland areas of Northern and North Central provinces such as Cao Bang, Lang Son, Hoa Binh, Vinh Phuc, Phu Tho, Thanh Hoa, Nghe An, Ha Tinh ..., in secondary forests.

1.3. Chemicals composition

Garcinia cowa Roxb. fruit contains mostly hydroxycitric acid (HCA) (accounting from 23% to 25% in dry fruit shells), with small and unremarkable amounts of compounds such as lactone of hydroxycitric acid, oxalic acid, flavonoid (in rind), xanthone compound (isoxanthochymol and xanthochymol), glucide compound ...

1.4. Situation of hydroxycitric acid (HCA) research of *Garcinia cowa* Roxb.

1.4.1. In the world

a. Structure research of hydroxycitric acid (HCA)

Hydroxycitric acid (acid 1,2 dihydroxypropane-1,2,3-tricarboxylic) found initially in nature as the main ingredient in the highly acidic fruit, *Garcinia cambogia*. Lewis and Neelakantan (2001), found that the structural isomer of hydroxycitric acid is not stable; Thus, it converted acid into more stable potassium and calcium hydroxycitrate salts (Figure 1.2).

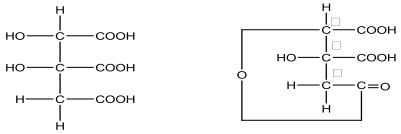

Figure 1.2. Structure of (-)-HCA and lactone form

b. Content of hydroxycitric acid (HCA)

For dry fruit *Garcinia cowa* Roxb. rind: There are two methods of extracting organic acid extraction. For method No.1, the acid content was determined by acid-base titration with 0,1 N NaOH solution and

phenolphthalein indicator, which was 27% in comparison to the dry shell mass. In method No.02, the amount of HCA was about 26,3% and 25,4% according to acid-base titration with 0,1 N NaOH solution and phenolphthalein indicator. Results of analysis on the high performance liquid chromatography system (HPLC), the retention time value of HCA in G*arcinia cowa* Roxb. rind (5,842 min) was also close to that of HCA analysis in *Garcinia cambogia* (5,3 min).

1.4.2. In Vietnam

In 2013, researcher, Dang Quang Vinh, reported his doctoral thesis on "Research on the extraction, hydroxycitric acid metabolism in *Garcinia cambogia* leaves and rinds and application of fat reducing bio-products"; The author determined the hydroxycitric acid content by high performance liquid chromatography (HPLC) (15,28%) from the *Garcinia oblongifolia* Champ. Ex Benth.

1.5. Results of hydroxycitrate salt preparation formation from hydroxycitric acid (HCA)

1.5.1. Research in the world

a. Mono salt

Scientists, B.S.Jena G.K Jayaprakasha, R.P.Singh, discovered the structure of unsustainable, non-preservative acid during use, should be converted to a monochorionic stable and more convenient potassium, calcium hydroxycitrate salt during use and preservation.

b. Double salt

In 2000, Balasubramanyam, K., Chandrasekhar B., Rama-doss, C. S., Rao; publicly showed the formation of a pair of metal salts of soluble metal in groups of IA and IIA of HCA (Figure 1.3).

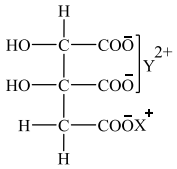

Figure 1.3. Struture formula of double metal salt in groups of IA and IIA

Gokaraju, together with his colleagues, presented the results of a study on "Preparation of new salt formers creation of hydroxycitric acid," in which authors gave their structure as defined in Figure 1.4.

Including, X and Y are dependent on metal selective from metal in groups of II (IIA và IIB) under periodic table classic such as: Be, Mg, Ca, Sr, Ba và Ra (group IIA), Zn, Cd .

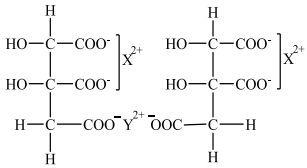

Figure 1.4. Structure of double salt from metal in groups of IIA and (-)-HCA

1.5.2. Domestic research

a. Mono salt

Now, in accordance with our reference, only author, Dang Quang Vinh, publicly shows the doctoral thesis on hydroxycitric acid and hydroxycitric acid metabolism from the pods and leaves of *Garcinia oblongifolia* Champ. and obesity reducing application. The author then synthesized the hydroxycitrate mono salt of metal in groups of IA and IIA, such as HCK , HCCa.

b. Double salt

According to the reference documents in Vietnam, there are almost no scientific works published on the double salt of HCA. In particular, for the double salts of hydroxycitric acid extracted from *Garcinia cowa* Roxb., none of them was mentioned.

1.6. Role of hydroxycitrate salt from HCA in public health care

1.6.1. Acute toxicity activity

In 2002, author Sunny E. Ohia and collaborators have shown that salt interacts with the impact of HCA on the albilos rat's cerebral cortex, through 5-hydroxy-xytryptamine or serotonin ([3H] -5-HT) compound involved in the sensory control of appetite through a neuro-

transmitter system.

1.6.2. Activion of body reducing obesity

In 2003, Michael Shara, Sunny E. Ohia, published the results of "New discoveries of the chemistry-physics effects of extracted hydroxy-citric acid on body weight, selecting organs like fatty liver, DNA fragmentation, hematology, pathologic histology within 90 days, so calcium, potassium salts are soluble with 60% HCA and are extracted from *Garcinia cambogia*, sample are dried and stored at room with temperature of 18-25^{0}C.

CHAPTER2. MATERIAL AND RESEARCH METHODOLOGY

2.1. Materials

2.2. Research method

2.2.1. Sample taking method

2.2.2. Physicla method

2.2.3. Extracting method

2.2.4. Impact of several technology factors on selection efficiency of total content of acid extracted by water under high pressure

a. Survey of period factor

b. Survey of solid/liquid factor

c. Precipitation of pectin elimination

2.2.5. Titration method *(TCVN 4589-88)*

2.2.6. Hydroxycitrate double salt synthesis

2.2.7. Analysis, evaluation and inspection of hydrocycitrate double salt

2.2.8. Research method of obesity reducing role and safe impact evaluation

CHAPTER3. RESULTS AND DISCUSSIONS

3.1. Material

Garcinia cowa Roxb. is havested in July and August in Vinh Phuc Province, in the North of Vietnam, identified by Dr. Nguyen The Cuong and the specimens were kept at the Institute of Ecology and Biological Resources, Academy of Science and Technology of Vietnam.

3.2. Survey of some physical factors affecting extract from *Garcinia cowa* Roxb.

3.2.1. Determination of moisture

* We conducted five tests and obtained an average humidity of 14,250%. The results showed that the moisture content of *Garcinia cowa* Roxb. rind material is quite high, needs to be dried during storage.

3.2.2. Investigation of incineration content

Incineration contentin *Garcinia cowa* Roxb. rinds is very low (5,435 %), which means that its organic content is high.

3.2.3. Determination of heavy metal components

The obtained results of the analysis of the amount of metals, namely: Sn: 0,43/14 mg/l; Pb: 0,021/0,1 mg/l; Cd: 0,073/1,0 mg/l; Hg: 0,0003/0,005 mg/l; MeHg (methyl mercury) and is not detected. Heavy metal content in *Garcinia cowa* Roxb. rind is much lower than "Limit of Heavy Metal Pollution in Food the according to National Technical Regulation".

3.3. Extraction of HCA from *Garcinia cowa* Roxb. with water at high pressure

We extracted hydroxycitric acid (HCA) from *Garcinia cowa* Roxb. rind by distillating in pressure cooker as shown in Figure 3.1 below:

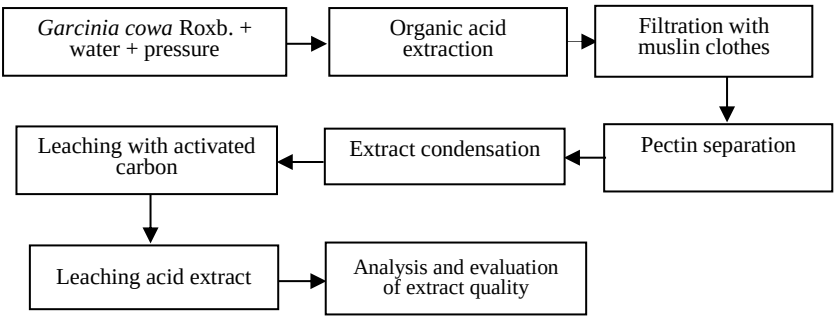

Figure 3.1. Research diagram on HCA extraction from *Garcinia cowa* Roxb. with water at high pressure

3.4. Influence of some technological factors on the efficiency of gaining total acid content extracted by water at high pressure

3.4.1. Periodic factor

The result gained at time t = 90 minutes of distillating period with the highest organic acid content is 23,055 g/100 g.

3.4.2. Survey of solid/liquid ratio

With a solid/liquid ratio of 10 g material/200 mL of water, the highest organic acid gained content is 23,715 g/100 g.

3.4.3. Effect of extract volume/alcohol 96^0

At the ratio of 50 mL extracted /150 mL of ethanol 96^0, the average crude pectine content is 12,863%.

3.5. Determination of HCA content obtained from*Garcinia cowa* Roxb. rind

3.5.1. Acidtitration

Total acid content obtained during distillation in a pressure cooker then titrated to obtain average organic acid content in *Garcinia cowa* Roxb. is about 23,784 g/100 g, with efficiency is 23,8% and this value also not much different compared to reference (27,1 ± 0,760/100 g, efficiency 27,1%). This content is very high compared with (-)-HCA content in some other *Garcinia* such as *Garcinia cambogia* (17-19,2%), *Garcinia indica* (12,48-15,1%), ...

3.5.2. High performance liquid chromatography – HPLC

a. Calibration curve construction results

According to results in Figure 3.2, we found that HCA concentration has linear correlation between concentration and absorbance, from which we obtain equation Y = 0,59746413.X - 0,5499125; correlation is 0,99996.

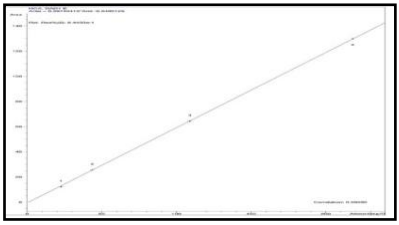

Figure 3.2. HCA calibration curve on HPLC analyzer

b. HCA content in the extract from *Garcinia cowa* Roxb. rind

We obtained the peak signal at a retention time of R = 5,974 minutes with a very high content (12,40%) and this value is insignificantly diffenrent from the study result of the world on hydroxycitric acid of *Garcinia cowa* Roxb.

3.5.3. Infrared spectrum - IR

In accordance with the IR spectrum, we find that υ = 3403,4 cm^{-1} is the signal of the valance oscillation of the group -OH; similarly at υ = 1605,2 cm^{-1} it is defined as the location of group -C(= O)-; it shows that *Garcinia cowa* Roxb. rind contains organic acid.

3.6. K/Ca hydroxycitrate double salt synthesis (K/Ca HCA)

3.6.1. Experimental procedure

3.6.2. Determination of content and structure of K/Ca HCA double salt

a. High-performance liquid chromatography (HPLC)

At the peak retention time of 5,976 minutes, the content of pure double salt is 96,87%, which is not significantly different with the retention time of extracted HCA acid (R = 5,974 min), which demonstrates that K/Ca HCA double salt contains HCA acid base.

b. Infrared spectrum – IR

The results show that the wave-length of the group -COO has values respectively of υ = 1599,04 cm^{-1}; υ = 1592,23 cm^{-1}; υ = 1614,78 cm^{-1}.

c. Structure inspection by nuclear magnetic resonance spectrum - NMR

* ^{13}C-NMR spectrum of K/Ca HCA double salt

On ^{13}C-NMR spectrum, it appears spectrum with values of δ = 42,452 ppm corresponding to methylene carbon; δ = 76,581 ppm corresponding to methane carbon and δ = 79,687 ppm corresponding to quaternary carbons.

* ^{1}H-NMR spectrum of K/Ca HCA double salt

Spectrum signal^{1}H-NMR of proton methylene (H_A-4 and H_B-4) appears in δ = 2,999 ppm and δ = 3,054 ppm. Spectrum in δ = 4,360 ppm, with a single peakshows that it is proton of methine group (H-2).

Protons of -OH and -COOH groups are not shown on the spectrum ^{1}H-NMR (D_2O, 300MHz).

d. Mass spectrometry K/Ca HCA double salt

The obtained result is m/z = 206,6; this is a mass of hydroxycitric acid.

e. Ion K^+ and Ca^{2+} content in double salt

Result of analysis by ion exchange chromatography shows that there is no significant difference between the theoretical and experimental values in the double salt (14,02%/13,70% = 1,023 and 14,08%/13,73% = 1,025).

3.6.3. Inspection of hygroscopic capability of K/Ca HCA double salt

Examining doube salt K/Ca HCA with the time marker 30 minutes, 60 minutes, 120 minutes, 180 minutes and 360 minutes respectively, we found the mass of double salt aproximately unchanged (5,417 g/5,417 g).

*Conclusion 1: From the above analysis data, we can confirm that double salt K/Ca hydroxycitrate has successfully synthesized. Double salt is white, odorless, almost tasteless, and convenient for use as food. The structure double salt of K/Ca HCA is as shown in Figure 3.3

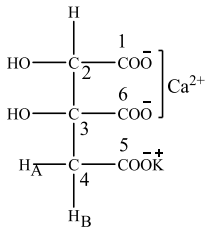

Figure 3.3. Structure of K/Ca HCA double salt

3.7. Synthesis of K/Mg hydroxycitrate double salt (K/Mg HCA)

3.7.1. Experimental procedure

3.7.2. Determination of content and structure of K/Mg HCA double salt

a. High-performance liquid chromatography – HPLC

At the peak retention time of 5,980 minutes, the content of pure double salt is 95,71%, which is not significantly different with the retention time of extracted acid HCA (R = 5,974 min), which demonstrates that K/Mg HCA double salt contains HCA acid base. .

b. Infrared spectrum – IR

From results gained, we have a basic conclusion that infrared spectrum – IR structures of K/Ca HCA, HCCa and synthesized double salt K/Mg hydroxycitrate are similar (Figure 3.4).

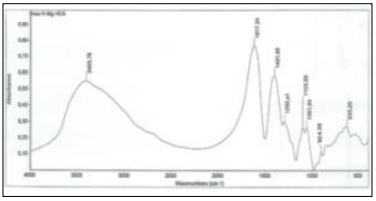

Figure 3.4. IR spectrum of K/Mg hydroxycitrate double salt

c. ^{13}C-NMR spectrum

Fromspectrum ^{13}C-NMR, we find that the peak at value of δ = 41,892 ppm is the result equivalent with group carbon methylene (-CH_2-), while peaks at values δ = 75,640 ppm and 78,512 ppm are of group carbons methine (=CH-) and quaternary carbons (C-2,2$^{'}$ và C-3,3$^{'}$).

d. ^{1}H-NMR spectrum

We find that the ^{1}H-NMR spectra signal of proton methylene (-CH_2-) occurs at peak of 2,76 ppm and 3,60 ppm. The single signal peak at 4,055 ppm represents the proton of the methine group (=CH-).

e. Mass spectrometry of synthetic K/Mg HCA double salt

The analysis result obtained is that m/z = 206,0 and m/z = 411,3 are respectively the values of mono and double hydroxycitric acid.

g. Metal ion content by ion chromatography (IC)

Metal ion content in double saltdetermined by ion chromatography are respectively: K^+ (26,41%) and Mg^{2+} (4,04%).

From theabove values, it is predicted that the structure of the double salt K/Mg hydroxycitrate is compatible with the structure in Figure 3.5.

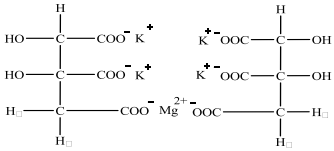

Figure 3.5. Structure of K/Mg HCA

3.7.3. Inspection of hygroscopic capability of K/Mg HCA double salt

Based on evaluation timelines: 30 minutes, 60 minutes, 120 minutes, 180 minutes and 360 minutes, we find that the mass of double salt nearly unchanged (5,135 g/5,135 g).

*** <u>Conclusion 2</u>:** From the above analysis data, we can confirm that K/Mg hydroxycitrate has been successfully synthesized from acid (-)-HCA extracted from *Garcinia cowa* Roxb.

3.8. Na/Mg hydroxycitrate double salt synthesis (Na/Mg HCA) – new double salt product

3.8.1. Experimental procedure

3.8.2. Determination of Na/Mg HCA double salt by high-performance liquid chromatography – HPLC

At the peak signal of the retention time of 5,977 minutes, sample volume of 101,26 mg, dilution volume of 100 mL, sample quantification value in HPLC chromatogram of 98,77766 mg/l, the content of double salt gained is 97,54%. This value is not significantly different with retention time of acid HCA extracted (R = 5,974 min), which proves that double salts contain HCA acid base.

3.8.3. Determination of double salt structure

a. Infrared spectrum - IR

Based on the IR spectrum of Na/Mg HCA, we find that the peak signal of wavelength υ = 3411,4 cm^{-1} has a value which is nearly identical to that of the standard spectrum (υ = 3400,11 cm^{-1}) and the double salt of K/Ca hydroxycitrate that we have published (υ = 3352,08 cm^{-1}).

b. Nuclear magnetic resonance spectrum – NMR of Na/Mg HCA double salt

** ^{13}C-NMR spectrum of Na/Mg HCA*

On the ^{13}C-NMR spectra, spectrum value of δ = 42,238 ppm is for methylene carbons group, δ = 75,979 ppm is for carbons methyl and δ = 78,594 ppm is for the quaternary carbons. In addition 3 spectrum with values as follows δ = 178,271 ppm, δ = 177,768 ppm and δ = 177,584 ppm respectivly are of carbonyl carbons (C-1, C-5 and C-6) of carboxylate group.

** ^{1}H-NMR spectrum of Na/Mg HCA*

^{1}H-NMR spectrum signal of methylene proton (H_A-4 và H_B-4) appears at δ = 2,745 ppm and δ = 3,588 ppm. The spectra at δ = 4,061 ppm with only one peak indicates that this is proton of methine (H-2) group.

c. Mass spectrometry – MS of Na/Mg HCAdouble salt

Observing the mass spectrum (MS), we find the values m/z = 206,9 and m/z = 411,6, which are the values of mono and double structures of HCA acid in the double salt.

d. High resolution mass spectrometry – HRMS

We found Na/Mg HCA double salt has experimental mass value (542,9415) and theoretical value (542,9431). Through analysis results, there is no significant difference; specifically, Δ_{mass} = 542,9431 – 542,9415 = 0,0016 Da = 1,6 mDa (milli dalton). Thus the mas difference of less than 5 mDa is satisfactory.

3.8.4. Metal ion content in Na/Mg HCA double salt

a. Ion chromatography method

We find that the ratio between the two metal ions Na^+ and Mg^{2+} shows no significant difference between the experimental and theoretical data (17,37%/4,58% = 3,79 and 17,49%/4,56% = 3,84).

b. Inductively coupled plasma emission mass spectrometry or inductively coupled plasma mass spectrometry

The rate of content of ion in metal from double salt with the equivalent value in comparison with theory (17,49%/4,56% = 3,84 &

92/24 = 3,83) and reality (11,35%/4,13% = 2,75 & 1602,24/529,51 = 3,03).

3.8.5.Inspection of hygroscopic capability of Na/Mg HCA double salt

Moisture absorption of Na/Mg HCA with the time marker within 30 minutes, 60 minutes, 120 minutes, 180 minutes and 360 minutes; we found that the volume of double salts hardly increased (5,263 g/5,263 g)

*Conclusion 3: From data analyzed above, we can confirm that we successfully synthesized Na/Mg hydroxycitrate double salt from (-)-HCA acid extract of *Garcinia cowa* Roxb. (Fig. 3.6).

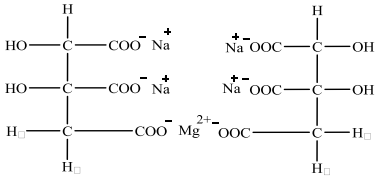

Figure 3.6. Structure double salt of Na/Mg HCA

3.9. Na/Zn hydroxycitrate double salt synthesis (Na/Zn HCA)

3.9.1. Synthesis process

3.9.2. Determination of Na/Zn HCA double salt content

At the peak signal of retention time 5,979 minutes, with a sample volume of 101,15 mg, dilution volume of 50 mL, the quantitative value in the HPLC chromatogram was 192,174 mg/l; The obtained content of double salts is 95,00%. And this value was not significantly different with the retention time of extracted HCA (R = 5,974 minutes). Proving that double salt contains HCA origin.

3.9.3. Inspection of the structure of Na/Zn HCA double salt

a. Analysis of (IR) infrared spectroscopy of Na/Zn HCA double salt

Results are shown in Figure 3.7. The signals of the -OH, -COO, -CO groups at 3423,0 cm^{-1}, 1588,4 cm^{-1}, 1396,7 cm^{-1} wave length and the groups on the IR spectra of the Na/Zn HCA double salt are basic functional groups in Na/Mg HCA, K/Ca HCA double salt formulation.

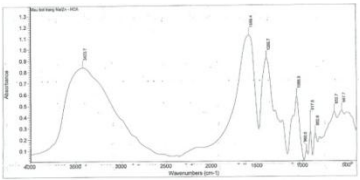

Figure 3.7. IR Spectrum of Na/Zn HCA double salt

b. Structural analysis by nuclear magnetic resonance spectroscopy - NMR

* ^{13}C-NMR spectrum of Na/Zn HCA: peak signal at δ = 42,22135 ppm is the signal of the carbons methylene group (-CH_2-) and at peaks with δ = 75,829 ppm; δ = 76,532 ppm is the signal of the carbons methine groups (=CH-); quaternary carbons expressed at δ = 76,832 ppm.

* ^{1}H-NMR spectrum of Na/Zn HCA: signal at peak with δ = 2,70 ppm and δ = 2,45 ppm is the signal of methylen group (-CH_2-), signal with δ = 4,257 ppm is expressed up to the methine group (=CH-).

c. Analysis of mass spectrometry – MS

The obtained result is m/z = 210,9; this is a mass of hydroxy-citric acid.

d. Analysis of High Resolution Mass spectrometry.

Obtained results from double salt of Na/Zn HCA are the values of testing mass (296,9568) and theoretical mass (296,9559) which did not differ significantly; specifically, Δ_{mass} = 296,9568 - 296,9559 = 0,0009 Da = 0,9 mDa (milli dalton). Thus, the mass difference of volume was much smaller (0,9 mDa) than the allowable value of the measurement (5 mDa) and was meet high demand in the analysis.

3.9.4. Determination of metal content in Na/Zn HCA double salt

a. Atomic Absorption Spectroscopy method - AAS

The content result metal ion of Zn^{2+}: 223 mg/g and Na^{+}: 81,6 mg/g.

b. Atomic emission epectroscopy method – ICP/MS

The results of the calibration curve for each ion metal were obtained by Na^{+} (99,59%) and Zn^{2+} (99,72%) respectively.

Based on the analysis results, content value of the synthestic Na^+ ion is 7,1% (theoretical 7,8%), the actual Zn^{2+} ion is 19,95% (theoretical 22,2%); we found that the ratio of metal ions in the Na/Zn HCA double salt structure was not much difference between theoretical 2,85 (22,2%/7,8%) and the actual 2,81 (19, 95%/7,1%). Formulas of form are shown in Figure 3.8.

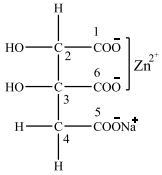

Figure 3.8. Structural formula of Na/Zn HCA double salt

3.9.5. Inspection of hygroscopic capability of Na/Zn HCA double salt

Based on evaluation time marker 30 minutes, 60 minutes, 120 minutes, 180 minutes and 360 minutes respectively; we found that the volume of double salts hardly increased (5,217 g/5,217 g).

* Conclusion 4: From the data analized above, we have successfully synthesized double salt Na/Zn hydroxycitrate from the acid HCA extracted from *Garcinia cowa* Roxb.

3.10. Synthesis of Na/Ca HCA double salt

3.10.1. Experimental procedure

3.10.2. Determination of Na/Ca HCA double salt content by high-performance liquid chromatography - HPLC

At the peak signal of the retention time of 5,981 minutes, with a sample weight of 185,823 mg, a dilution volume of 100 mL, the sample value in the HPLC chromatogram was 179,79888 (mg/l); at the same time, this value was not significantly different with the retention time of extracted HCA (R = 5,974 min).

3.10.3. Determination of the structure of Na/Ca HCA double salt

a. Measurement of Infrared spectrum – IR

IR spectra of double salt Na/Ca HCA is expressed that, the peak -OH signal has a number of waves υ = 3404,9 cm^{-1}, which is nearly identical to the reference signal (υ = 3400,11 cm^{-1}) and the signal of the

double salt K/Ca HCA. Previous publication (υ = 3352,08 cm^{-1}) was up to the group -OH.

b. Nuclear magnetic resonance spectroscopy - NMR

* ^{13}C-NMR spectrum of Na/Ca HCA double salt: peaks at δ = 42,682 ppm and 45,768 ppm are signals of metylen group (-CH_2-); and peaks with δ = 58,051 ppm; 76,882 ppm; 79,377 ppm are signals of methine group (=CH-).

* ^{1}H-NMR spectrum of Na/Ca HCA double salt: signal at peak has δ = 3,03 ppm and δ = 3,18 ppm is of methylen group (-CH_2-), signal of methine group (-CH-), also presents at δ = 4,53 cm^{-1}.

c. Analysis of mass spectrometry - MS of Na/Ca HCA double salt

The mass of spectra obtained result of Na/Ca hydroxycitrate (m/z = 268) are mass m/z = 205,7 and m/z = 223,6. This is a piece of hydroxycitric acid.

3.10.4. Determination of metal content in Na/Ca HCA double salt

Experimental value obtained is 1,76 (14,96%/8,51%) and theoretical value is 1,74 (14,93%/8,58%). Thus, structural formula of Na/Ca HCA double salt synthetized has a form as in Figure 3.9.

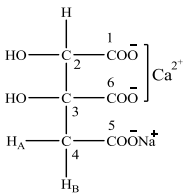

Figure 3.9. Structural formula of Na/Ca HCA double salt

3.10.5. Inspection of hygroscopic capability of Na/Ca HCA double salt

Results of dichorionic Na/Ca HCA salt moisture absorption test with the time makers 30 minutes, 60 minutes, 120 minutes, 180 minutes and 360 minutes respectively, show that its volume is almost unchanged in comparison with the origin (5,312 g/5,312 g).

* <u>Conclusion</u> 5: From all of the data above, we have synthesized successfully double salt of Na/Ca HCA, simultaneously

have proven its ability to absorb moisture, its color, and flavor during synthesis.

3.11. Study evaluating the weight loss effects and safety of Na/Mg HCA and Na/Zn HCA double salt

3.11.1. Research results on weight loss and blood lipid lowering effects

a. Changes in body weight of mice studied

The result of change in weight of rats when drinking Na/Mg HCA and Na/Zn HCA after 7 weeks is presented as below: Na/Mg HCA salt = 140,60/220,50 g; Na/Zn HCA salt = 140,36/220,50 g.

b. The change in total cholesterol, Triglycerid, HDL-Cholesterol, LDL-Cholesterol, VLDL-Cholesterol, Atherogenic index (A.I) contentin blood of the studied rat group.

* Reducing in bad lipid index (mg/dl) (after/before the testing): Triglycerid = 0,76/1,05 & 0,84/1,05; Total cholesterol = 3,98/5,66 & 4,12/5,66; LDL cholesterol in blood = 2,35/3,99 & 2,36/3,99; VLDL cholesterol in blood = 0,34/0,48 & 0,39/0,48.

* Increasing good lipid in blood: HDL cholesterol = 1,28/1,20 & 1,36/1,20.

3.11.2. Acute toxicity

a. Na/Mg HCA double salt

Determining the LD_{50} value of Na/Mg HCA salt by drinking on the albinic rat is: LD_{50} = 1406,443 (1264,787 ÷ 1563,965) mg/kg at P= 0,05.

b. Na/Zn HCA double salt

Do not find the LD_{50} of Na/Zn HCA salt by drinking on the albinic rat with the highest dose that it drinks within 24h is 6000mg/kg of weight.

3.11.3. Research results on semi-chronic toxicity

a. Effects of Na/Mg HCA and Na/Zn HCA on general condition and body weight changes of white rats when being used in a long term: the results show that hair, skin, mucosa, normal secretions; the preparations

significantly reduced the weight gain of rats compared to the physiological balance.

b. The effect of double salts Na/Mg HCA and Na/Zn HCA on rat's electrocardiography: rat's electrocardiography is normal; skin, mucous membrane, endocrine are normal.

c. The effect of Na/Mg HCA and Na/Zn HCA salt on rat'shematology index: the result shows that the product does not change the hematology index (the number of red cell, hemoglobin content, hematocrit, average volume of red cell, the number of white cell, the number of platelet)

d. Evaluate the effect of Na/Mg HCA and Na/Zn HCA salt on blood biochemical indicators: the product does not change the biochemical indicator including content of ATS liver enzyme, ATS in blood, total bilirubin in blood, creatinin in blood, albumin in plasma.

e. The result of histopathologyof internal organs of the lab rats to Na/Mg HCA and Na/Zn HCA double salt: Observe by using magnifying glass with 25 times of magnification, we find that colour, form of liver, spleen and kidney at the two group using the double salt are not different from that at the goup with physiological symtom.

3.11.4. Conclusions about evaluation of the safety and weight loss effects on animals

From Results obtained in the experiments, we conclude:

- The rat group using studied product with the dose of 94 mg/kg/24 h & 282 mg/kg/24 h (Na/Mg HCA salt); 250 mg/kg/24 h & 750 mg/kg/24 h (Na/Zn HCA salt), after 60 days: the lab rat is normal; the condition of rat's fur, skin, mucous membrane, endocrine are normal; the rat's electrocardiography is not affected.

- Do not change hematology index(the number of red cell, hemoglobin content, hematocrit, average volume of red cell, the number of white cell, the number of platelet). Do not change biochemical indicators including content of ATS liver enzyme, ATS in blood, total bilirubin in blood, creatining in blood, albumin in plasma. Do not affect histopathology of liver, spleen and kidney.

The weight of rat group using the product does not vary, while the weight of rat group with physiological symtom stably increases ($p < 0,01$). The total cholesterol in blood in the group using the product decreases more than that in the group with physiological symtom and before using the product.

Consequently, the product with doses and using time as in controlled trial on the white rat is highly safe. Besides, it also has the effect of cholesterol and weight loss on the normal white rat (the rat does not get obesity and dyslipidemia)

CONCLUSIONS AND RECOMMENDATIONS

I. CONCLUSIONS

1. Correctly determining scientific name of the sample used for the research in order to obtain *Garcinia cowa* Roxb. extracted, up to Clusiaceae or Guttiferae family, originating from Vinh Phuc province.

2. Recommending the total procedure to create double salts of hydroxycitrat from the extract containing HCA associated to two metal ions. As a result, there was the creation of 5 double salts including K/Ca HCA, K/Mg HCA, Na/Mg HCA, Na/Zn HCA and Na/Ca HCA.

3. All self-created double salts have the ability to improve the shortcomings of mono salt hydroxycitrate in term of moisture, colour, and taste absorption

4. Research ofthe weight loss effect on 02 double salts: Na/Mg HCA with the dose of 94 mg/kg/day và 282 mg/kg/day; Na/Zn HCA with the dose of 250 mg/kg/day và 750 mg/kg/day.

5. Determine the LD_{50} value of Na/Mg HCA (1406,443 mg/kg) double salt. In contrast, double salt Na/Mg HCA (1406,443 mg/kg) can not show the LD_{50} algebraic value.

6. The dose is the same as the dose for treatment of semi – chronicity of 02 self-created double salts, used within 60 days. As the result, hematology indexand biochemical indicator do not change; the

images of rat's histopathology do not vary. Therefore, it can be affirmed that the studied product has high safety controlled trials on the animals.

II. RECOMMENDATIONS

1. Continue researching to synthetize many other double salts, especially double salts with 03 metal elements to create weight loss products with higher efficiency.

2. Research and develop a process to extract HCA and manufacture double salts with industrial scale as a basis for the commercialization of the product.

3. Research the growing and developing conditions of *Garcinia cowa* Roxb. cultivation in order to take this plant to be a material for production of active element HCA.

4. Search for cooperation and investment possibilities to put results of the subject into practice.

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[2]. Le Xuan Van, Dao Hung Cuong, Nguyen Thanh Ha Tuan, Nguyen Hoang Ngan (2017), "Research on the safety of Na/Zn HCA salt on experimental animals", *Journal of Military Medicine - Department of Military Medicine*, No.1-2.

[3]. Le Xuan Van, Dao Hung Cuong, Nguyen Hoang Ngan, Dang Van Diep, Nguyen Thanh Ha Tuan (2017), "Research on acute toxicity and effects of Na/Mg HCA on general condition and hematological indicators on experimental animals”, *Journal of Military Traditional Medicine and Pharmacy – Department of Military Traditional Medicine*, Vol.7, No.1

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[6]. Le Xuan Van, Dao Hung Cuong, Tran Van Thang (2015), "Study on synthesis of sodium-calcium double salt of hydroxycitric acid extracted from rind of Garcinia”- Garcinia

cowa Roxb., *Journal of Science and Technology*", *53(4C)*, pg. 43-48.

[7]. Le Xuan Van, Dao Hung Cuong (2013), "Synthesis of double salt of (-)-hydroxy-citric acid extracted from Garcinia rind for obesity control", *Actes de conférence scientifique Franco-Vietnamienne chimie et matériaux pour un environnement durable*, p.97-104.

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