

**DETERMINATION OF HYDROXYPROLINE IN KALANCHOE
PINNATA BY HPLC**

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**RESEARCH REPORT SUBMITTED IN FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
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ABSTRACT

Kalanchoe pinnata is an important plant which has many traditional medicinal uses. In this work, hydroxyproline was extracted from *Kalanchoe pinnata*. Determination of hydroxyproline in this plant was done using HPLC. Hydroxyproline is one of the element for the formation of collagen. There are three solvent composition used for extraction; 100% of water, 100% of ethanol and 50% of ethanol. It was found that combination of two solvent, ethanol and water can optimize the extraction as when comparing the concentration of hydroxyproline detected in the same extraction time, this system showed the highest concentration of hydroxyproline detected. Another parameter is the duration of maceration time, one to five hours which the time interval between analysis in one hour. The longer the time taken for extraction, the more hydroxyproline extracted from the sample.

ABSTRAK

Kalanchoe pinnata adalah tumbuhan yang penting yang mempunyai banyak kegunaan perubatan tradisional. Ekstrak *Kalanchoe pinnata* telah dinilai bagi kandungan hidroksiprolinnya. Hidroksiprolin adalah salah satu elemen untuk pembentukan kolagen. Terdapat tiga komposisi pelarut yang digunakan untuk pengeluaran; 100% air, 100% etanol dan 50% etanol. Didapati bahawa gabungan dua pelarut, etanol dan air boleh mengoptimumkan pengekstrakan apabila membandingkan kepekatan hidroksiprolin dikesan dalam masa pengeluaran yang sama, sistem ini menunjukkan kepekatan tertinggi hidroksiprolin dikesan. Parameter lain adalah tempoh masa kehabisan tenaga, satu hingga lima jam yang selang masa antara analisis dalam satu jam. Semakin lama masa yang diambil untuk pengekstrakan, lebih banyak hidroksiprolin diekstrak daripada sampel.

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LIST OF ABBREVIATIONS

g	:	gram
HPLC	:	High Performance Liquid Chromatography
Hyp	:	hydroxyproline
ng/uL	:	nano gram per micro liter
UV	:	ultraviolet
AGP	:	arabinogalactan proteins
EXT	:	extensins
PRP	:	proline-rich proteins
C18	:	column 18

CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Anti-Aging in Natural Way

As the world is moving forward, technology offers several choices for people to maintain their appearance from aging. Even though the technology had been upgraded, human still look up for the natural resources in a way to sustain and maintain their appearance as technology is not always bring benefit due to side effects plus the treatment is somehow expensive. Nowadays, the beautiful and uniqueness of herbs are for health purposes which brings out many purposes of opportunity of research and natural products nowadays focusing on antioxidants. There is an increase of awareness among people nowadays to look good naturally.

Somehow, aging is a natural process and it is unavoidable. Still, people strive to find ways to fight aging and try to look young and feel young. Our skin is first thing that gives away our age because our wrinkles show and increase as we get older. Aside from aging, we are not forever in a good health condition as our health somehow deteriorates too. These create awareness among people that conscious about aging and health condition. It is possible that one person looks younger than his or her biological if one

engages in healthy living life style and eating healthily by increasing the intake of good healthy food such as whole grain, fruits, vegetables, beans and legumes.

Most nutritional experts believed daily intake of supplement is good for us to stay healthy. A healthy individual seeks to repair from the inside out and that is from where true beauty is reflected. Anti-aging means being the most vibrant and healthy person you can be and maintaining a lifestyle that is pain free and comfortable.

1.1.2 Hydroxyproline

Hydroxyproline (Hyp) is a non-essential amino acid that is derived from another amino acid, proline. It is created by the interaction of ascorbic acid, also known as vitamin C, and proline. This causes a hydroxyl group, which is a bonded oxygen-hydrogen molecule, to attach itself to the carbon atom of the proline acid, changing it into hydroxyproline.

Hyp is derived from the amino acid proline and is used in structural proteins including collagen, connective tissue in mammals, and in plant cell walls. It plays role as to maintain the stability of collagen.

Hydroxyproline is also an ingredient found in some cosmetic and personal care products including anti-aging products, moisturizers, eye cream, facial cleansers and facial masks. Cosmetic manufacturers add hydroxyproline to products as an anti-aging ingredient, based on the fact that collagen is the primary support structure for skin and helps to keep it firm and youthful. With age and sun exposure, collagen in the dermis of the skin becomes damaged. This is a factor in the formation of wrinkles. Manufacturers

hope that by adding hydroxyproline to cosmetic products, it will boost the synthesis of collagen, thereby increasing skin firmness and reducing wrinkling. (Retrieved from <http://skinfopedia.com/Hydroxyproline>)

Without vitamin C, the production of hydroxyproline is impossible. This is a serious problem, as without it, the body is unable to manufacture its most important structural protein, collagen. Both proline and hydroxyproline are essential in the formation of this key substance, and a lack of either one can lead to serious collagen instability in the body.

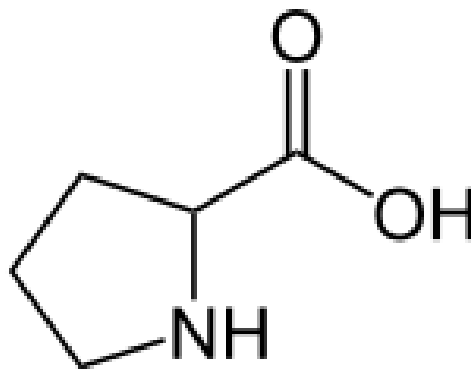


Figure 1.1: Structure of proline

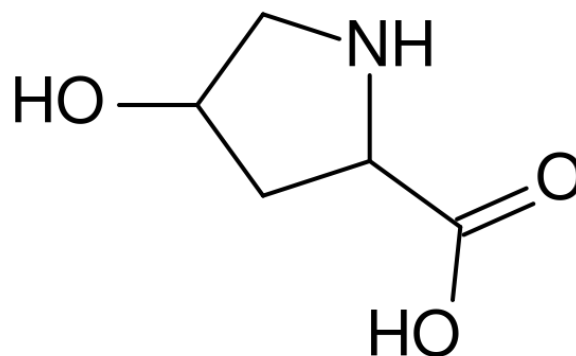


Figure 1.2: Structure of hydroxyproline

A deficiency of this amino acid is often one of the first visible signs of scurvy. The ultimate cause of scurvy is too little vitamin C, but this will first manifest itself as a

collagen deficiency, due to the poor production of hydroxyproline. Without it, any collagen in the body becomes unstable and is often expelled in the urine. The lack of the collagen protein causes easy bruising of the skin, breakdowns in connective tissue, and possibly internal bleeding. Other issues caused by limited amounts of this amino acid include hair loss and receding gums.

1.1.3 *Kalanchoe pinnata*

Kalanchoe pinnata is a traditional medicinal plant which is used for the treatment of several health problems. This plant is quite unique because its roots can grow without any other medium except air. The leaves are thick, succulent and juicy, able to provide food and water to the seedlings that germinated on the leaf edges. These plants reproduce very quickly and in some places it is listed as a weed pest. In Malaysia, setawar is widely grown as an ornamental plant because of its beautiful leaves structure. It has a very simple way of breeding through the leaves and it can also reproduce by seeds and stem cuttings.

Local people believe that this plant can heal several skin problems by simply put the crushed leave on to the affected area. For the treatment of fever, leaves are squeezed and soaked in water before applied it to patient's head.



Figure 1.3: *Kalanchoe pinnata*

1.2 Problem Statement

This study is conducted on *Kalanchoe pinnata* because not much research is done on this species previously especially in terms of hyp content. This species is not popular for medicinal purposes in our country, Malaysia, compared to other country such as Brazil, India, Peru, etc as medicinal purposes.

As reported by Chandra Mohan in 2012, this plant contains significant amount of antioxidant. Alcoholic extract of *Kalanchoe pinnata* showed phytochemicals such as total phenolics, flavonoids, lycophenes and β -Carotenes while the dried extract showed considerable inhibiting activity of *in-vitro* lipid peroxidation. (Chandra Mohan, 2012).

A further study on content of this plant is carried out in order to investigate the potential of *Kalanchoe pinnata* as hyp source. In this report, an optimize condition for the extraction is developed. A suitable solvent and time interval is proposed as to investigate the effect of solvent composition and duration of extraction on total content of hyp from the sample.

1.3 Significance of Study

As reported, not much study had been conducted on this plant in the aspect of hydroxyproline content especially in Malaysia. Therefore, this study will give the insight on the available amount of hydroxyproline content from *Kalanchoe pinnata*. From this finding, the potential of *Kalanchoe pinnata* as anti-aging product can be further discussed for more research work.

1.4 Objectives of Study

- To extract and determine hyp content from *Kalanchoe Pinnata*
- To study the effect of solvent composition on extraction
- To study the effect of extraction time on hyp content extracted from the sample

CHAPTER 2

LITERATURE REVIEW

2.1 Hyp, the component of collagen

Hyp is a nonessential amino acid, which means that it is manufactured from other amino acids in the liver; it does not have to be obtained directly through the diet. According to Springboard (2004), a deficiency of this amino acid is often one of the first visible signs of scurvy. The main reason why a person can suffer from scurvy is too little vitamin C, but this will first manifest itself as a collagen deficiency, due to the poor production of hydroxyproline. If the body cannot produce hydroxyproline, any collagen in the body becomes unstable and is often expelled in the urine. The lack of the collagen protein causes easy bruising of the skin, breakdowns in connective tissue, and possibly internal bleeding. Other issues caused by limited amounts of this amino acid include hair loss and receding gums. (Retrieved from <http://www.proventus.org.uk/Amino%20Acids.pdf>)

Aside from collagen, the only mammalian protein that is created using hydroxyproline is elastin. As its name implies, this protein has elastic qualities, and is responsible for allowing skin to retain its shape. It is also stored, in mammals, at sites on the body where a great deal of weight is borne, or where there are large transfers of mechanical energy. This allows the body to endure movement and pressure without

significant deformity. A lack of hydroxyproline makes this protein harder to create, but the results are not as dramatic or immediate as when collagen is not being produced (Farage et al., 2010).

In 1969, the origin of this elastin hydroxyproline was studied by comparing the rate of incorporation of [¹⁴C]proline and hydroxy-[¹⁴C]proline into hydroxyproline of dermal and aortic collagen and aortic elastin. As has been repeatedly demonstrated for collagen, it was found that proline is a far better precursor of elastin hydroxyproline than is hydroxyproline itself. Both proteins incorporated a small amount of labeled hydroxyproline directly. No loss of hydroxyproline was seen, and it is concluded that hydroxyproline is indeed a constituent of elastin in which it occurs in a collagenase-resistant sequence (Bentley J. P., 1969).

There is no need for humans to have a dietary source for this amino acid. Its precursor, proline, is also a non-essential amino acid and is produced in the body. Vitamin C, however, is an essential nutrient for the human body, and must be consumed which the source most often come from fruits and vegetables — for the body to function properly.

2.2 Source of Hyp

As reported by Showalter (1993) in his study, 4-hydroxyproline is found in many plant cell-wall glycoproteins such as extensins, the proline-rich proteins, the solanaceous lectins and the arabinogalactan proteins even though plants not express the collagen. Hydroxyproline-rich glycoproteins (HRGPs) are a superfamily of plant cell wall proteins that function in diverse aspects of plant growth and development. This

superfamily consists of three members: hyperglycosylated arabinogalactan proteins (AGPs), moderately glycosylated extensins (EXTs), and lightly glycosylated proline-rich proteins (PRPs) (Showalter, 2011).

Hyp is further found in the hypoxia-inducible factor (HIF) in which the hydroxylation of Pro564, and in some cases also of Pro402, in the HIF-1 α plays a critical role in the adaptation to hypoxia at the cellular and systemic level (for reviews, see Semenza 2000, 2001, Fedele *et al.* 2002).

The phytochemical screening of the ethanolic and aqueous extracts of stem of plant *Kalanchoe pinnata* yielded showed the presence of alkaloids, flavonoids, carbohydrates, saponins, triterpenes, phytosterols, tannins, glycosides, protein, and amino acid and phenolic compounds. The dried stem of *Kalanchoe pinnata* showed more successive extraction with alcoholic solvent as compare to extraction by aqueous (Matthew, 2013).

4-Hydroxy-L-proline is not synthesized as the free imino acid. Rather, it is produced by hydroxylation of the third-position proline in the prevalent Gly-Pro-Pro tripeptide of the procollagen polypeptide chain. Free hydroxyproline is derived from endogenous collagen turnover and from breakdown of dietary collagen. The hydroxyproline degradation pathway resembles that of proline. 1-pyrroline-3-hydroxy-5-carboxylate, the oxidation product of hydroxyproline, is dehydrogenated to 4-erythro-hydroxy-L-glutamate. Transamination with oxaloacetate results in 4-hydroxy-2-ketoglutarate, which is then cleaved to glyoxalate and pyruvate in an aldolase reaction. The enzymes catalyzing these reactions are distinct from those for the degradation of

proline, with one exception: dehydrogenation of P5C and hydroxy-P5C is catalyzed by the same enzyme, P5C dehydrogenase.

2.3 Application of Hyp

According to a small study published in *Inside Cosmeceuticals*, hydroxyproline has the potential to decrease fine lines and wrinkles when used topically and also helps to increase penetration into the skin of other anti-aging skin care ingredients. In addition, hydroxyproline binds water, making it an effective moisturizer and skin conditioner. Another study showed that participants that took hydroxyproline as an oral supplement experienced less skin dryness and better skin texture. Whether hydroxyproline penetrates deeply enough to increase collagen synthesis is still unclear, but, at the very least, it appears to be an efficient moisturizer.

According to Aksnes (2007), an increase in intake of hydroxyproline in diet can enhance growth of tissue. The sample used in this study is salmon fish. The fish meal is included with hydroxyproline in their diet. In this study, it is shown that those fishes fed diets supplemented have a significant hyp content and high hyp to proline ratio. Other amino acid and protein content of vertebrae were however not much different among fish has fed the other experimental diets. The observed effects in vertebrae composition of fish fed dietary hyp supplementation were relatively small and can easily be evaluated as insignificant in a biological sense.

A recent study by Zhang et al. (2013) was conducted to evaluate the effects of dietary hydroxyproline (Hyp) on survival, growth, feed utilization, body composition, Hyp and collagen concentrations in tissues, and prolyl 4-hydroxylase $\alpha(I)$ (P4H $\alpha(I)$)

gene expression of juvenile turbot fed high plant protein diets with initial body weight of 8.11 ± 0.01 g.

Seven isoproteic (50% crude protein) and isolipidic (12% crude lipid) experimental diets were formulated to contain 0.12, 0.33, 0.51, 0.60, 0.80, 1.03, and 1.23% Hyp, respectively. From the study, it can be concluded that the supplementation of crystalline L-Hyp in high plant protein diets did not indicate positive effects on growth performance of juvenile turbot whereas the expression of P4H α (I) gene in muscle was decreased significantly as dietary Hyp increased. Free Hyp in plasma, total Hyp contents in liver and muscle and total collagen concentration in muscles were increased significantly as dietary Hyp increased (Zhang et al., 2013).

2.4 *Kalanchoe pinnata* extract

Previous studies have reported this plant is useful for preventing alcoholic, viral and toxic liver damages. Fresh leaf juice or infusion is applied externally and taken internally for various bacterial, viral, and fungal infections. It is biologically active as the aqueous extract of this plant has shown anti-inflammatory, anti-diabetic, anti-tumor and cutaneous leishmanicidal activities (Biswas *et al.*, 2011).

As reported by Mahmood *et al.*, *Kalachoe pinnata* has the potential as a wound healing compound. It is because it contains hydroxyproline which promote the growth of tissue. This experiment was done on excision wound model of albino rats. There are three different medium of extraction; petroleum ether, alcohol and aqueous. All three extracts showed significant increase in the breaking strength of incision wound. When comparing the cotton pellet dry weight and hyp content on granulation tissue with

control groups showed significant increase in wound contraction and formation of scars on 17th post wounding day. The result discovered that aqueous extract accelerate the healing process in open wounds (Mahmood et al., 2002).

Patil *et al.* (2008) investigated the diuretic and antiurolithic activity of *Kalanchoe pinnata*. The ethanolic extract of leaves was administered to male wistar rat orally and injection, intraperitoneally. The dosages are 100, 300, 500 and 800 mg/kg. the effect of urine output was determined by comparing the urine volume collected by keeping individual animal in metabolic cages. Calcium oxalate urolithiasis was induced in rats by giving ethylene glycol orally for 7 days and the effect of the extract was observed by its concurrent administration. The extract was found to have significant diuretic and anti-urolithiatic activity and the intraperitoneal administration of the extract gave more potent diuretic effect.

Another study conducted by Vinit *et al.* shows that this plant is antimicrobial active as it can also inhibit the growth of microorganism. The findings of the study showed that the aqueous (water) and chloroform extracts of *kalanchoe pinnata* had successfully inhibit the zones of microorganism. The present study results clearly indicate that aqueous water and chloroform extract of possesses the antibacterial activity of the plant leaves evaluated against microbial flora organisms in *E. coli*, *Condid Albican*, *Rhodococcus Rhodochrous* and *Arthrobacter Protosphormial*. (Vinit *et al.*, 2012).

CHAPTER 3

METHODOLOGY

3.1 Materials and apparatus

The materials used in this experiment were divided into four parts; materials, reagents, apparatus and instrumentation.

3.1.1 *Kalanchoe pinnata* leaves

The plants were collected from Subang, Selangor.

3.1.2 Reagents

A standard of L-hydroxyproline was used. For the extraction process, the solvents used were ethanol and distilled water. For chromatographic analysis, acetonitrile was used.

3.1.3 Apparatus

Apparatus used in this experiment were listed as below;

- filter papers
- vacuum pump
- beakers
- filter funnel
- mortar and pestle
- parafilm

3.1.4 Instrumentation

The analysis was performed on Agilent 1220 Infinity LC 1220. The system was equipped with C18 column, Agilent ZORBAX LC column of 250 mm x 4.0 mm, 5 µm. The wavelength is set at 254 nm and the temperature was set up to 25°C. The system used water and acetonitrile as a mobile phase. The flow rate of the mobile phase was set up at 1 ml/min. It used UV detector and the detection was occurred at 254 nm.

3.2 Method

In this experiment, the methods were divided into three parts which consisted of extraction of plant material, preparation of sample and preparation of standard.

3.2.1 Extraction of Plant Material

3.2.1.1 Solid Liquid Extraction Method

Leaves were washed thoroughly with tap water followed by distilled water for the removal of dust and soil particles. The leaves were dried at room temperature for one day. By using mortar and pestle, 14 g of the leaves were crushed into pieces for each analysis.

The solvent system consists of two solvents; ethanol and distilled water. It is then set up to three types which consist of 100% ethanol, 50% ethanol and 100% water. Each analysis used total volume of 100 ml for solvent. The crushed leaves are then extracted with these three types of solvent system for five intervals of time.

The extraction process was done at room temperature. The filtrates were then filtered off by using Whatman filter paper. All the extracts were stored in a closed bottle and kept in refrigerator.

3.2.2 Preparation of Standard

Standard solutions were prepared in three concentrations, 50 ng/uL, 100 ng/uL and 450 ng/uL. As to prepare the standard solution, hyp is available as a solid compound and it is soluble with water.

3.2.3 Preparation of Sample

All samples were directly used from extraction for analysis. After the extraction, the sample was vacuum-filtered through vacuum pump and kept in refrigerator. For analysis, 20 uL of sample was taken to be injected into the column.

3.3 Analysis

The analysis was done by using a standard solution of L-hydroxyproline. The liquid chromatographic analysis of the sample used an isocratic system of mobile phase. The composition of the mobile phase consisted of 70:30 of acetonitrile and water respectively at the flow rate of 1 mL/min. HPLC introduced the sample in a small volume, 20 μ L.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Optimization of Extraction Condition

In this chapter, the finding from the analysis will be discussed with more detail. HPLC as an instrument of the analysis which is chosen to identify the presence of hydroxyproline in *Kalanchoe pinnata* extract. The optimizing parameter for this experiment was duration of extraction. So other than identification, the effect of extraction duration was studied as well.

The analysis was carried out by using a mixture of acetonitrile and water as the mobile phase in the composition of 70:30. Previous works showed that the analysis could be done using other types of solvent system such as methanol, sodium acetate buffer.

To optimize the condition of analysis, C18 is chosen as the stationary phase. As reported by Liu *et al.* (2012), the analysis of hydroxyproline in rat tissue was performed on Phenomenex C₁₈ column. It is also possible that the analysis was carried out with C8 column in order to identify the mixture of L-hydroxyproline oligo-peptides (Sun *et al.*, 2007).

The identification of hydroxyproline in the sample was confirmed by comparison of peak of compound of interest with peak of standard solution prepared.

4.2 Extraction

In this experiment, there was three system of solvent used which consisted of 100% water, 100% ethanol and 50% water: 50% ethanol (v/v). The solvent systems were labelled as A, B and C, respectively and the volume for each system was 100 mL. Ethanol was used as the extraction solvent due to its ability to extract phenolic compounds, flavonoid and hydroxyproline. As the analyte contains hydroxyl group, it is most suitable to be extracted by using ethanol and water. Beside that, ethanol is one of a good solvent for polyphenol extraction and it is also safe for human consumption (Shi *et al.*, 2005).

The colour of the extraction solvent turns into greenish from colourless as the crushed leaves are macerated on it. The samples are macerated in the solvent for one to five hours. The time interval for each analysis is one hour. The colour changed was believed to be caused by the extraction of compounds from the sample by the solvent (Vankar *et al.*, 2009). It was found that greenish colour in Solvent B is darker than the rest as it is solely made of ethanol. The greenish colour possibly causes by the content of chlorophyll.

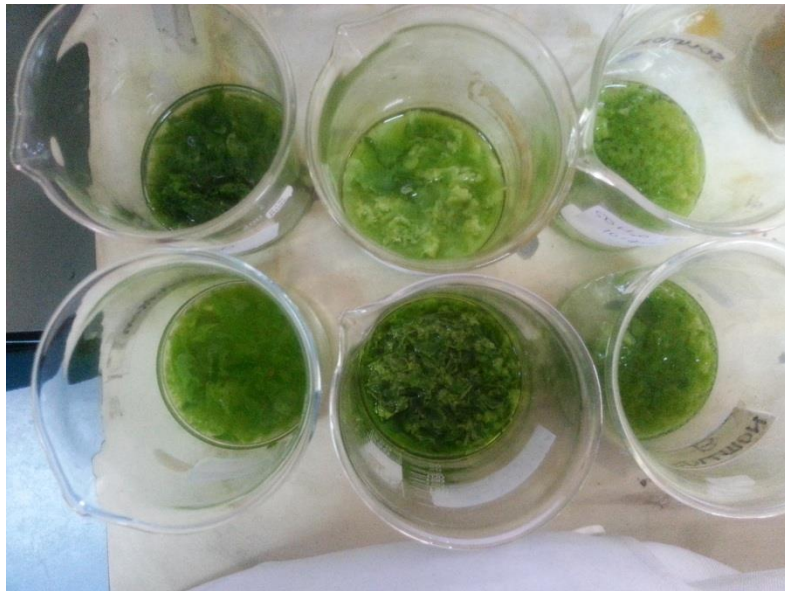


Figure 4.1: Extraction process of sample in solvent

As ethanol is known as easily vaporized and volatile compound, precaution step should be taken in order to avoid the extraction from escape to surrounding. It is best to cover the beaker with parafilm. Once the extraction process was done, the samples were then placed in a closed sample bottle prior to analysis. All bottles are kept in a refrigerator.



Figure 4.2: Conical flask containing sample covered with parafilm

In the study by Majaz (2011), the extracts of *Kalanchoe pinnata* roots were subjected to preliminary phytochemical screening in order to detect the presence of various phytoconstituent. The results show that petroleum ether extract contain steroids, the chloroform extract contain steroids and alkaloids, the alcoholic extract contain steroids, saponins, alkaloids, glycosides, flavonoids tannins carbohydrates and proteins while the aqueous extract contain saponins, glycosides, flavonoids, tannins, carbohydrates and amino acids.

4.3 Standard Solutions

Calibration is such a common and important step in analytical methods, it is essential that a good calibration curve is develop before the analysis is started thus to evaluate the results obtained.

Standard solutions of hydroxyproline of different concentration are prepared. For this experiment, three different concentrations of standard solution of hydroxyproline were prepared; 50 ng/ μ L, 100 ng/ μ L and 450 ng/ μ L.

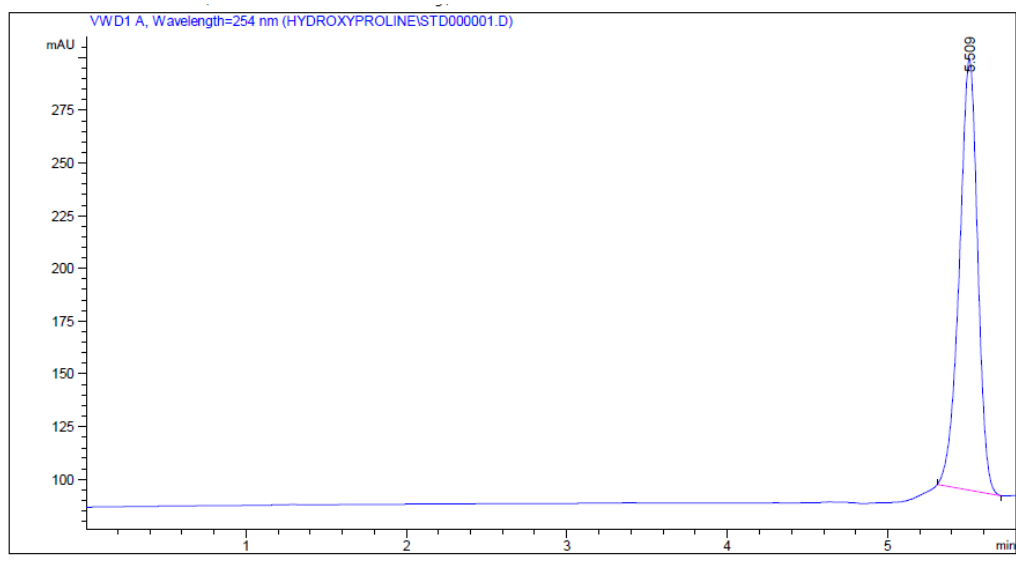


Figure 4.3: Chromatogram of hydroxyproline standard: 50 ng/uL

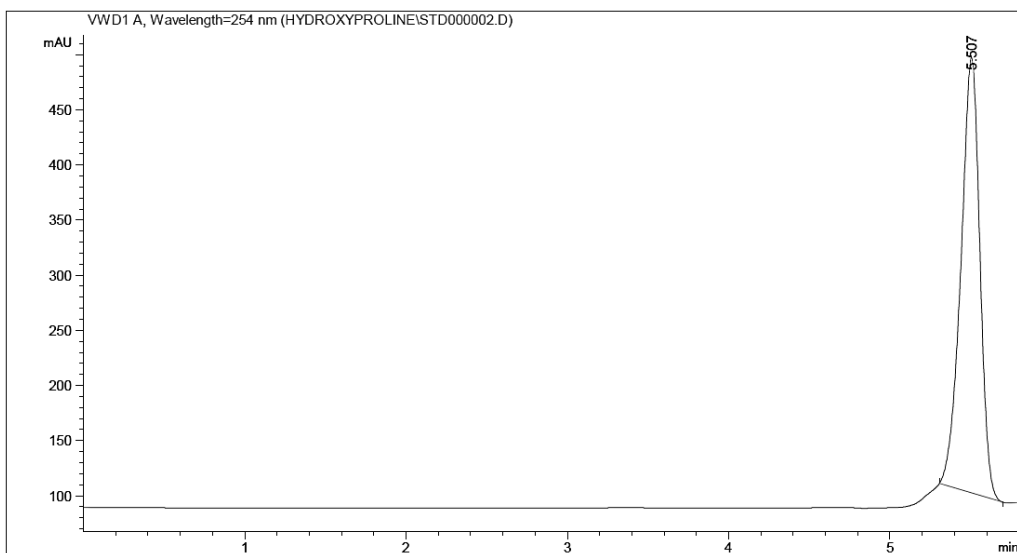


Figure 4.4: Chromatogram of hydroxyproline standard: 100 ng/uL

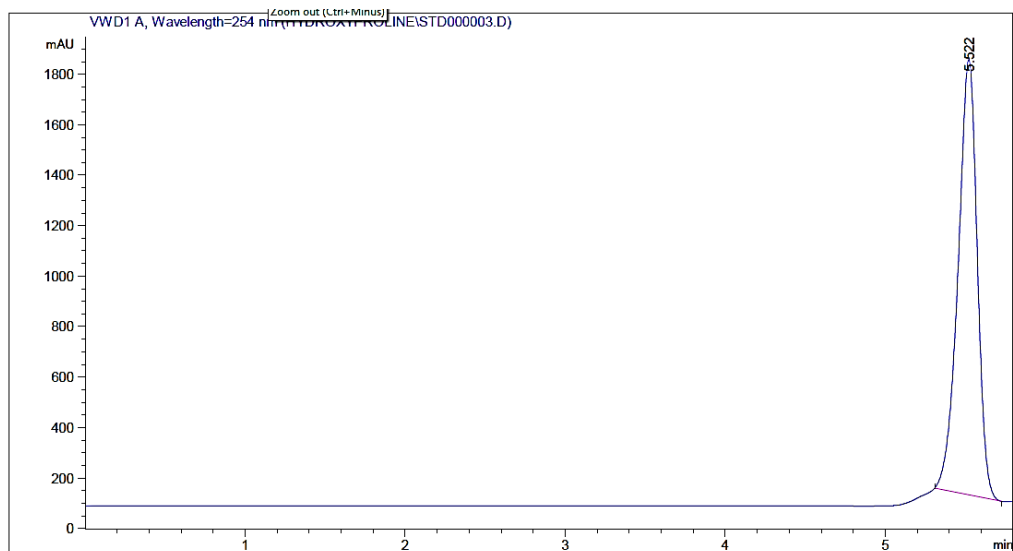


Figure 4.5: Chromatogram of hydroxyproline standard: 450 ng/uL

Table 4.1 : Concentration and area of standard solution of hydroxyproline

Concentration (ng/ μ L)	Area (mAU*s)
50	50.3
100	100.6
450	449.8

The standard solutions were labelled as 1, 2 and 3 in ascending order of concentration accordingly on the calibration curve (Figure 4.6). The standard solution was prepared by solvation of solid hyp with deionized water.

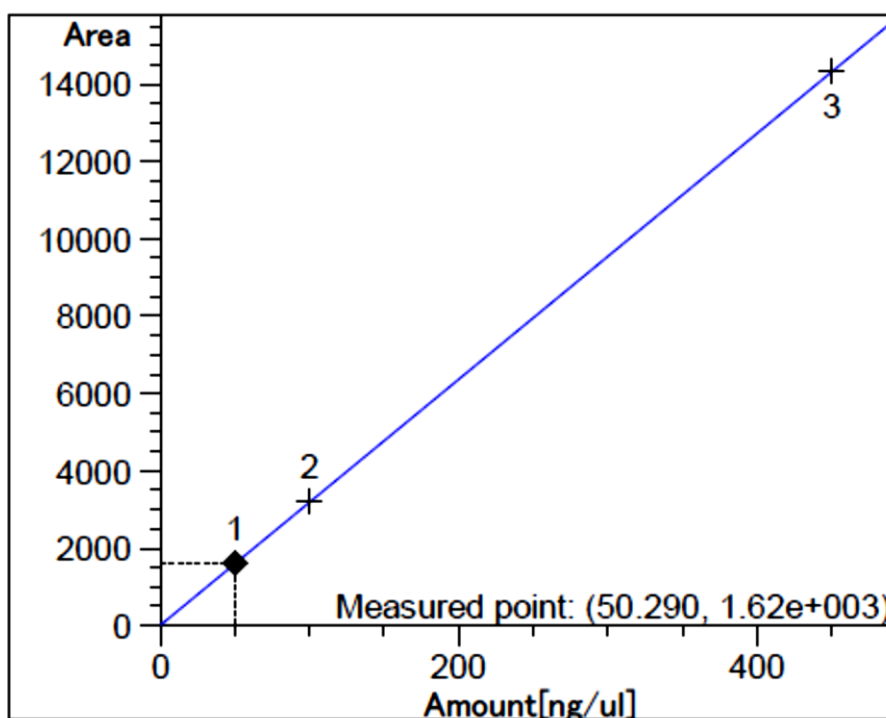


Figure 4.6: Calibration Curve of Standard Solution of Hydroxyproline

4.4 Analysis

In this study, the results of a simple and isocratic HPLC method with photodiode array UV detection of hydroxyproline in *Kalanchoe pinnata* samples. It was found that the concentration of hydroxyproline in each sample varies, depending on the type of solvent and the duration of extraction. The retention time of hydroxyproline detected was around 5.6 minutes for each analysis. The results were summarized in Table 4.2.

From the table, the concentration of hydroxyproline detected also varies with the time interval. It was found that the longer the time taken for extraction, the higher the concentration of hyp extracted from the sample.

Further investigation was focused on the effect of the solvent composition used for the sample analysis. The study on the composition of solvent shows that solvent C can optimize the extraction, thus can extract the sample more efficient. When comparing the amount of hyp detected by different solvent in the same time interval, solvent c shows the highest concentration of hyp.

Table 4.2: Summarization of Results from HPLC analysis of Kalanchoe pinnata extract

Solvent system	duration of extraction (hour)	Concentration (ng/ul)
a	1	2.80
	2	7.48
	3	8.56
	4	8.90
	5	18.54
b	1	2.72
	2	8.74
	3	21.44
	4	21.96
	5	27.55
c	1	not detected
	2	17.99
	3	23.44
	4	25.82
	5	153.2

a:100% water, b:100% ethanol, c:50% water/50%ethanol

CHAPTER 5

CONCLUSION

5.1 Conclusion

From the result obtained, the method adapted from previous researches has successfully extracted the hyp content from *Kalanchoe pinnata* extract. Solid liquid extraction is a suitable method for the extraction of *Kalanchoe pinnata* leaves.

Based on the result, there is a significant increase on the concentration of hyp detected by HPLC as increased in extraction time applied. This is regardless of the type of solvent used for the extraction. The trend in the data showed that as the extraction duration increased, the concentration of hyp extracted increased.

Another optimizing parameter of this experiment is the composition of solvent. According to the result, combination of the two solvents, water and ethanol gave better result. This is because when comparing three system of maceration of the same duration of extraction, mixture of ethanol and water showed the highest amount of hyp from the extraction.

5.2 Future work

This research can be improved in the future by increasing the amount of sample as to increase the amount of hyp detected by the analysis. Furthermore, it is important to determine the optimal extraction time for this analysis. In the future, the duration of extraction can be increased to few more hours as to identify the best time duration for optimization of the extraction.

Another thing that can be improved in the future is to study another peak appear on the chromatogram. The potential and the significant of finding the compound can be further discussed.

As hyp is the component of the collagen, methods for the extraction can be further improved so that the extract can be applied topically or orally, and safe for human consumption.