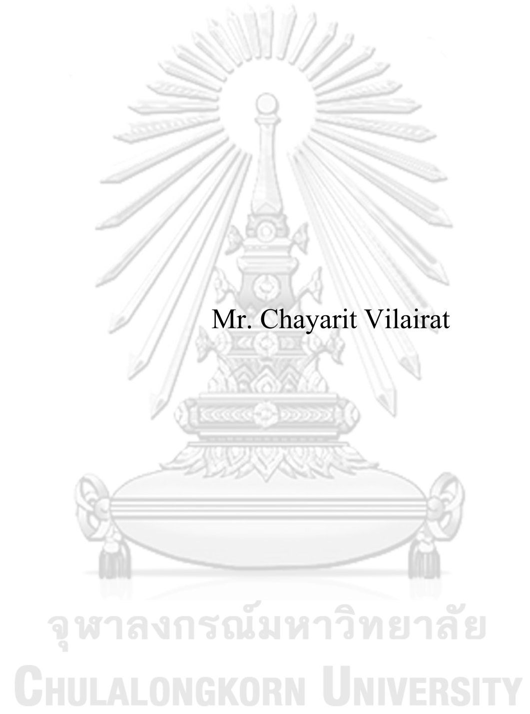
DEVELOPMENT OF AN EXTRACTION METHOD OF *MUCUNA PRURIENS* SEEDS FOR A CHEMICALLY AND PHYSICALLY STABLE EXTRACT



Mr. Chayarit Vilairat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmaceutical Sciences and Technology
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การพัฒนาวิธีสกัดเมล็ดของหมามุ่ยเพื่อให้ได้สารสกัดที่มีความคงตัวทางเคมีและกายภาพ

นายชญาฤทธิ์ วิลัยรัตน์

จุฬาลงกรณ์มหาวิทยาลัย
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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ชญาฤทธิ์ วิลัยรัตน์ : การพัฒนาวิธีสกัดเมล็ดของหมามุ่ยเพื่อให้ได้สารสกัดที่มีความคงตัวทางเคมีและกายภาพ. (DEVELOPMENT OF AN EXTRACTION METHOD OF *MUCUNA PRURIENS* SEEDS FOR A CHEMICALLY AND PHYSICALLY STABLE EXTRACT) อ.ที่ปรึกษาหลัก : รศ. ภญ. ดร.สรกนก วิมลมั่งคั่ง

ลีโวโดปาคือยาที่ใช้ในการรักษาผู้ป่วยพาร์กินสัน ปัจจุบันได้มาจากกระบวนการสังเคราะห์ทางเคมีและจากพืชโดยเฉพาะเมล็ดหมามุ่ย จากผลการศึกษาระดับคลินิกพบว่า การใช้ผงเมล็ดหมามุ่ยที่มีลีโวโดปาช่วยลดอาการข้างเคียงในการรักษาได้ดีกว่าการใช้ลีโวโดปาจากเคมีสังเคราะห์ แต่ผู้ป่วยจะต้องรับประทานผงเมล็ดจำนวนมากต่อครั้ง ซึ่งอาจทำให้เกิดความไม่สะดวก ดังนั้นการใช้สารสกัดจึงช่วยลดปริมาณการใช้ต่อครั้งได้ อย่างไรก็ตามสารสกัดน้ำจากเมล็ดหมามุ่ยเสื่อมสลายได้ง่ายทั้งในด้านเคมีและกายภาพ โดยเฉพาะการเปลี่ยนแปลงของลักษณะปรากฏ เช่น ผงสารสกัดลักษณะเหนียวจับตัวเป็นก้อนมีสีที่เข้มขึ้น รวมไปถึงปริมาณลีโวโดปาที่ลดลง จะเห็นว่ากระบวนการสกัดจึงมีความสำคัญที่ต้องพัฒนาเพื่อแก้ปัญหาดังกล่าว การศึกษานี้มุ่งเป้าปรับปรุงกระบวนการสกัดหมามุ่ยด้วยกรดในวิธีดั้งเดิม เพื่อให้ได้สารสกัดที่มีความคงตัวเพิ่มขึ้น โดยทำการเปรียบเทียบประสิทธิภาพการสกัดด้วยชุดของสารละลายกรดที่มีการศึกษาก่อนหน้า ได้แก่ กรดไฮโดรคลอริก กรดซิตริก และ กรดแอสคอร์บิก กับการสกัดด้วยกรดจากน้ำมะขามป้อมซึ่งมีฤทธิ์ในการต้านอนุมูลอิสระและยังมีประโยชน์ต่อการรักษาโรคพาร์กินสันอีกด้วย จากผลการสกัดเปรียบเทียบความแตกต่างของเมล็ดหมามุ่ยสองสายพันธุ์พบว่าปริมาณของสารลีโวโดปาที่ได้ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ในขณะที่การใช้วิธีการสกัดด้วยเครื่องโซนิเคเตอร์สามารถช่วยเพิ่มประสิทธิภาพในการสกัดดีที่สุด เมื่อเปรียบเทียบการนำสารละลายกรดที่แตกต่างกันเพื่อใช้ในการสกัดพบว่าน้ำมะขามป้อมทำให้ได้สิ่งสกัดที่มีปริมาณลีโวโดปาเทียบเท่าการใช้กรดไฮโรครอลิกที่เป็นสารละลายควบคุม และยังช่วยในการคงสภาพสารสกัดได้ดีที่สุด เมื่อจัดเก็บภายใต้สภาวะเร่งเป็นเวลา 12 เดือน นอกจากนี้การวิเคราะห์คุณภาพทางเคมี ของสารสำคัญในมะขามป้อมและสารสกัดหมามุ่ยที่สกัดด้วยน้ำมะขามป้อม ด้วยเทคนิค HPTLC และ HPLC พบว่าสารลีโวโดปาในสารสกัดหมามุ่ยไม่รบกวนกับสารในมะขามป้อม จากผลการศึกษาทั้งหมดนี้แสดงเห็นว่าน้ำมะขามป้อมมีประโยชน์ต่อกระบวนการสกัดลีโวโดปาอีกทั้งยังเป็นตัวทำละลายจากธรรมชาติที่มีความปลอดภัย สารสกัดที่ได้สามารถนำไปใช้งานเพื่อเป็นวัตถุดิบตั้งต้นในผลิตภัณฑ์สมุนไพรต่อไป

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6370003033 : MAJOR PHARMACEUTICAL SCIENCES AND TECHNOLOGY
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Chayarit Vilairat : DEVELOPMENT OF AN EXTRACTION METHOD OF *MUCUNA PRURIENS* SEEDS FOR A CHEMICALLY AND PHYSICALLY STABLE EXTRACT. Advisor: Assoc. Prof. SORNKANOK VIMOLMANGKANG, Ph.D.

Levodopa (L-DOPA) is a drug for the treatment of Parkinson's disease (PD). Currently, it derives from chemical synthesis and plants, especially *Mucuna Pruriens* (MP) seeds. The results of clinical studies found using the L-DOPA-containing MP for the treatment of PD could reduce side effects more than the synthetic one but patients must take MP powder in high amounts per a single dose which may cause inconvenience. Therefore, using the extract can help in the reduction of the dose. Unfortunately, the water extract from the MP seeds can be easily degraded physically and chemically; especially the physical appearance e.g., darkening color, melting, or a solid lump. Moreover, the reduction of L-DOPA content in the extract was commonly observed. Therefore, it is necessary to develop an extraction procedure to solve these problems. This study aimed to modify the extraction of MP using the traditional acidification approach to obtain a higher stable MP extract by comparing the extraction efficiency of a set of previously studied acid solutions (hydrochloric acid, citric acid, and ascorbic acid) and acid extraction from *Phyllanthus emblica* water (PE), which has antioxidant effects and is also useful in treating PD as well. From the comparative extraction results of the two strains of MP seeds, it was found that the amount of levodopa was not statistically different. While using the sonication-assisted extraction method can improve the extraction efficiency. When comparing different acid solutions used for extraction, it was found that PE yielded an extract with a similar amount of levodopa as using hydrochloric acid as the control solution, and also helps to maintain the quality of the extract when stored under the accelerated condition for 12 months. In addition, qualitative analysis of chemicals in PEW and MP extracted with PE by HPTLC and HPLC techniques revealed that L-DOPA in the MP extract did not interfere with the chemicals in PEW. All these studies showed that PEW is beneficial for the L-DOPA extraction process and is a safe natural solvent. The obtained extract can be used as a starting material for herbal products.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature ....
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Rationale, Significant, and Hypothesis

1.1 Literature review

1.1.1 *Mucuna pruriens* (L.) DC. (MP)

M. pruriens (velvet bean) is an annual vine. It has a long tendril and a dense pubescence. As a legume, it is native to scattered tropical and subtropical zones in Africa and Asia. In Asia, Mucuna is found in Thailand, India, China, Myanmar, Laos, Cambodia, etc. There are hundreds of species. MP have different names for each country, but use the same scientific name, *M. pruriens*. In Thailand, two main varieties are found: Thai Ma Mui (*Mucuna pruriens var. pruriens*) and Indian Ma Mui *(Mucuna pruriens var. utilis*) (Figure1) Thai Ma Mui (TMM) has sheaths and seeds size small than Indian Ma Mui (Figure2), and long stingy hairs that cause itching when touched. While Indian Ma Mui (IMM) has two seed colors, white and black, large sheaths, short and soft hairs. There are about 24 species of *M. pruriens* in India and they are widely cultivated in Uttar Pradesh, Madhya Pradesh. Andaman and Nicobar Islands, etc.(1)

Figure 1: Sheaths of *M. pruriens*. A. *M. pruriens var. pruriens.* B. *M. pruriens var. utilis*
(https://ummio.blogspot.com/2015/07/blog-post.html)
(https://www.thailandnatureproject.com/mucuna-pruriens.html)

Figure 2: Comparison of the size of the seed between left *Mucuna pruriens var. pruriens* and right *Mucuna pruriens var. utilis*

1.1.2 Medicinal properties of MP

In India, MP is used for medicinal purposes. According to the writings of the Indian Ayurvedics, medicines are prepared from the seeds of MP for the treatment of sexual dysfunction, Parkinson's disease (PD), diabetes, antiseizure, and microbial infection. According to ancient Thai medical texts, various parts of the MP were used medically. Especially seeds are the source of important bioactive substances. The main substance in the seeds is L-DOPA (4-7%) which was first discovered in 1973[(2)]. It is a precursor to the production of dopamine, a substance involved of the nervous system in the brain. Other important substances have also been found, such as oleic acid, glutathione, gallic acid, linolenic acid, beta-sitosterol, and lecithin[(3)]. MP contains tannins, alkaloids, kaempferol, phenolic compound, saponin, and cardiac glycoside[(4)]. Many species are useful in medicine as an antioxidant[(5)], an antimicrobial, neuroprotective agent, etc. (Table 1). Additionally, the number of people with PD is growing exponentially worldwide[(6)]. As society and patients' personal lives are currently affected, it has been studied in the past to use MP seeds instead of synthetic L-DOPA in the treatment of PD. 45 grammes of MP powder per day (equivalent to 1500 mg of L-DOPA) was found to improve symptoms of PD over a 12–20-week period when compared to the effects of the standard drug carbidopa, a drug used to treat PD[(7)]. Moreover, it was found to be fast-acting, has a longer duration of action, and is more effective than the standard drug.

Table 1: Bioactive substances of *M. pruriens*

Biological / Pharmacological activity	Plant part	Bioactive compound	Ref.
Antioxidant activity	All parts	5-hydroxytryptophan, glutathione	(8), (9)
Antimicrobial activity	leaves, seed	gallic acid, glyceollin 1, L-DOPA	(10),(11),(12)
Antivenoms	seed	protein (gpMuc)	(13),(14), (15)
Antidiabetic effect	seed	cyclitols, oligosaccharides	(16), (17)
Neuroprotective effect	seed	L-DOPA, gallic acid, β-carboline,	(18), (19), (20), (21)
Inhibit dopa decarboxylase	seed	Genistein	(22)
Reduces dyskinesia in vivo	seed	N, N-dimethyl tryptamine (DMT), Nicotine, bufotenine, 5-Methoxy DMT	(23), (24), (25), (26)

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Previous research has shown that MP has many biological functions. It can also help PD, so MP is a new herb that has begun to be cultivated both domestically and abroad. Currently, the market has a high demand for MP and tends to expand. Based on data on the use and export of herbs in groups of traditional medicine, it is worth about 10 billion baht according to traditional Thai medicine. It means that Thai herbs have great potential and can bring a lot of income to the country. Many companies are taking advantage of the opportunity and continuously researching and developing products. As a result, there are many forms of MP nowadays, such as capsules, tablets, coffee, etc.

1.1.3 Levodopa

Levodopa (L-DOPA) is an amino acid that naturally has the isomeric structure of 3,4-dihydroxyphenylalanine (Figure 3). It was first discovered by Marcus Guggenheim from a *Vicia faba* seed in 1913 [27] Thenin 1911, Casimir Funk had successfully synthesised D, L-DOPA at Welcome Laboratories in London, England, and in 1951 Dale discovered a neurotransmitter

synthesized from L-DOPA. Dopamine (DA), for the synthetic chemical compound discovered by Funk and Guggenheim remains unclear, maybe the primary compound, possibly adrenaline[28]. L-DOPA has a melting point at 284–285 °C and a solubility of 5 g/L in water at 20 ° C. L-DOPA is soluble in acidic solutions such as hydrochloric acid and formic acid but insoluble in benzene, ethanol, and chloroform[29].

Figure 3: Chemical structure of L-DOPA

L-DOPA, a direct precursor to DA synthesis (Figure. 4), was used as the main therapeutic product, as L-DOPA has a more easily transmissible CNS-mediated property to deliver DA. However, long-term treatment with L-DOPA results in motor complications that occur in the most common patients. The symptoms are called dyskinesias and are caused by an excessive dopaminergic tone. [30],[31] L-DOPA is a very effective treatment of PD. It will be used in conjunction with an inhibitor. decarboxylase, such as carbidopa and benserazide[32]. Then it contributes to the peripheral decarboxylase inhibitor and cannot cross the blood-brain barrier. Therefore, it can reduce peripheral breakdown of L-DOPA and thus largely avoid the systemic side effects of the drug[33].

Figure 4 : Synthesis of dopamine from its precursors.

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L-DOPA is found in many herbs, especially in seeds (Table 2). Natural products and herbs play a role in the treatment of PD. Some herbs have been found to be more effective and reliable than conventional synthetic drugs[34]. The MP extract contains a small amount of L-DOPA, serotonin, and nicotine, among other known ingredients. In the treatment of PD, the extract can be more effective and less toxic than synthetic drugs, making it an alternative treatment[35].

Table 2: Seed plants reported to contain L-DOPA %[36]

Plant	L-DOPA %
Alysicarpus rugosus.	0.65
Bauhinia purpurea.	2.20
Bauhinia racemosa.	0.73
Canavalia ensiformis.	2.46
Cassia hirsute.	2.37-2.82
Canavalia gladiata.	2.13
Cassia floribunda.	1.10 -1.90
Dalbergia retusa.	2.20
Glycine wightii.	0.20
M. aterrima.	3.31
M. pruriens.	1.25-9.16
M. pruriens var. utilizes	6.08
M. andreana	6.30-8.90
M. birdwoodina tutcher	9.10
M. cochinchinensis	0.96
M. cochinensis.	3.0 - 4.0
Parkinsonia aculeate.	0.64
Phanera vahlii.	2.35
Prosopis chilensis.	1.25

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1.1.4 Extraction method for MP

Extraction is the process of separation of active substances in the medicinal parts of a plant by using an appropriate solvent to formulate it as a standard procedure. The goal of the extraction pane is to separate the soluble plant matter in the extract and to separate the insoluble residue. The primary crude extracts obtained by these methods contain a variety of plant metabolites such as organic acids, glycoside compound, phenols compound, flavonoids compound. Some extracts are available medicinally as liquid extracts. However, some species must undergo repeated extraction processes with other solvents to keep the substance pure[37]. Types of extraction can be subdivided depending on the state of the solvents used in extraction, including Liquid-liquid extraction, Solid phase extraction, leaching or solid liquid, Supercritical Fluid[38].

The widespread use of herbal medicines throughout the world makes it very important to standardise the formulations of herbal medicines. One of the most important steps in this process is extraction to obtain the vital substance. Changing the extraction method and different extraction solvents can change the amount of bioactive ingredients. Therefore, a suitable extraction method is important for the number of vital substances that can be obtained in the further development of a formulation. The high amount of L-DOPA found in seeds is not suitable for feeding because of its antinutritional composition[39]. Attempts have been made to reduce the L-DOPA content for the purpose of food and feed. There are reports on various methods such as boiling (cooking), soaking (soaking), boiling (boiling), baking, roasting (toasting), heat (roasting), high pressure steaming (autoclaving), and incubation (germination). Each method yields different amounts of important substances. Extraction by heat treatment (roasting) by mixing the samples with heated sand in a 100 ° C oven for 60 min and extracted with 0.1 N HCl at room temperature resulted in an increase in L-DOPA content of 4.96% compared to the raw bean (untreated) and increased by 0.07% when processed in a water bath regulated at 60 °C and the pH was adjusted to 3.2 From the information in the report It is a by-product of extracted L-DOPA that is not needed in animal feed. and can be used as a guideline for the benefit of research[40]. The L-DOPA content was determined in the MP seed for broiler chicken after different preparing methods. When the seeds of white variety and the black variety were soaked with acidic solutions such as tamarind juice, citric acid, and 0.1 N HCl, the L-DOPA content was reduced to 8.72%, 6.81% and 8.72% and 17.3%, 5.14% and 6.10%, respectively. The level of L-DOPA was reduced by 45-70% when MP

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was boiled in regular water[41]. The result implied that co-extract with acidic solutions can inhibit the degradation of L-DOPA. Furthermore, the 3-day germination process showed a reduction of 20-22.9% when soaked in water for 20 hours[42]. After drying at 55 ° C for 6 h, the L-DOPA content was reduced by 79% [43]. Consistent with the study, extraction using autoclaving at 15 bars and 121 ° C for 30 min in different solvents was found to contribute to the reduction of L-DOPA by 59.4-70.3 %w/w in seed powder[44].Another report mentioned the use of sonication. The results showed that the L-DOPA content in the seeds was reduced from 2.8 to less than 0.2 g/100g[45]. All of the above reports focused on the way to remove L-DOPA from the seed powder. This suggested that the reduced L-DOPA in the seeds may be extracted and dissolved in the solvent. The method that shows a higher reduction in the L-DOPA content in the seed could be used to develop a suitable method to obtain a high amount of L-DOPA extract considering factors that impact the L-DOPA level of L-DOPA, such as solvents and extraction methods. In this study choose between two extraction methods: (autoclave extraction and ultrasonic extraction). They were also compared with the same heat extraction and holding times. The difference of the machine used in extraction is Autoclave uses pressure to assist in extraction, but Sonicate uses high frequency for extraction.

Another extraction method was tested to increase L-DOPA in the extract. It was carried out by defatting with acetone and followed by extraction in water: ethanol (1:1) containing 0.1% ascorbic acid as an oxidative protectant or in water under SO_2, then shaking 3 times. The result showed that extraction with the ascorbic-added solvent gave a higher yielded crude L-DOPA, which upon further recrystallization in hot water gave pure crystals (1.78%) than water extraction under the oxidative protectant SO_2 protection oxidative protectant (0.98%)[46].Even MP extract containing L-DOPA shows promise for the treatment of PD, the clinical effect of the drug is however attenuated by motor complications with prolonged treatment with MP extract. This may be due to the neurotoxic effect of L-DOPA. Therefore, the combination of L-DOPA therapy with vitamin C in an experimental model in healthy mice was used to investigate the possibility of reducing oxidative stress induced by L-DOPA. For some indicators of oxidative stress measured as malondialdehyde levels, protein carbonyl content, and advanced glycation end products in blood plasma, the results showed that all decreased in the vitamin C pretreated group compared to the same controls [47]. From the above research, the idea of extracting *Phyllanthus emblica,* which

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is an herb rich in vitamin C (1.28%, w/w),[48] along with MP seeds emerged. It would not only stabilise L-DOPA in acidic condition but it can also reduce side effects and provide symptomatic relief. It is also a good option for poor patients with Parkinson's disease.

1.1.5 Phyllanthus emblica and chemical constituents

Phyllanthus emblica (PE) (synonym: *Emblica officinalis*), colloquially known as Indian gooseberry (English), Amalaka (Sanskrit), and amla (Hindi), is an important deciduous tree. The plant belongs to the Euphorbiaceae family. It was originally native to India, but now grows throughout Asia. It is a small-medium perennial plant, 8-12 m tall, the trunk is often bent, and the bark is greyish brown. Smooth or relatively smooth surface the bark is bright pink. Single leaves are like compound leaves like tamarind leaves. Oblong-elliptic, alternately arranged, 0.25-0.5 cm wide, 0.8-12 cm long, light green, closely arranged. Leaves very short, small leaf flowers tree, 3-5 flower spurs, tightly packed at the end of the branch, 6 sepals, white or white flowers. Round fruit with a thickness of 1.2-2 cm. The young fruit is light green. The mature fruit is light green and relatively clear, with 6 observable longitudinal streaks. The flesh is edible, sour, bitter, and sweet. The seed coat has 6 hard strands, 6 seeds[49].

Figure 5 : Leaves and fruits of *Phyllanthus emblica*[50].

PE is rich in vitamin C. It also contains several phenols as constituents: proanthocyanidins, gallotanin, ellagic acid, quercetin, korilagin, geraniin, gallic acid furoxin,. Emblicanin, flavonoids, glycosides, and camphorol. The root of PE contains many tannins and glycosides. PE is a fruit with high antioxidant activity. The content of ascorbic acid in fruits is

mainly responsible for antioxidant activity, 45-70 percent. Other bioactive compounds that express antioxidant properties are emblicanins, gallic acid, geraniin, corilagin, furosin, and methyl gallate. The following table 3 shows the type and chemical constituents of PE [51].

Table 3 : Type and Phytochemical compound of *Phyllanthus emblic.*

Type	**Phytochemical compound**
Hydrolysable Tannin	Corilagin (Ellagitannin), Ellagotannin, Punigluconin, Pedunculagin, Chebulinic acid (Ellagitannin), Geraniin (Dehydroellagitannin), Emblicanin A and B, Chebulagic acid (Benzopyran tannin)
Amino acids	Cystine, Lysine, Aspartic acid, Alanine, Proline, Glutamic acid
Phenolic compound	Methyl gallate, trigallayl glucose, Gallic acid, Ellagic acid
Alkaloids	Phyllantidine, Phyllembein, Phyllantine
Flavonoid	Kaempferol, Quercetin
Organic acid	Citric acid
Vitamins	Vitamin C (Ascorbic acid)
Carbohydrate	polysaccharide (Pectin)

The antioxidant effect of PE was evaluated compared to that of vitamins by different antioxidant assays. The data obtained showed that PE fruit contains ascorbic acid 0.40%(w/w) and that ayurveda improves the fruit's healthy properties by increasing its antioxidant activity and the ascorbic acid content 1.28 % (w/w). Vitamin C has also been found to account for approximately 45-70% of its antioxidant activity. [48]. According to studies of traditional Chinese medicine, the active ingredient of ripe dried fruits of PE has been found to be active on the target protein in ferroptosis Iron metabolism. Processes are associated with the cause of PD by important substances as compounds of the flavonoid group[52].

1.1.6 Drying Method

Drying methods are often used to concentrate extracts to reduce or remove water or other liquids. There are several methods such as spray drying, hot air drying, vacuum with pump drying, microwave drying and freeze drying. The product after drying can be in solid form or dry powder[53, 54].

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The choice of drying method plays an important role that can affect the extract quality, especially in bioactive compounds. In comparing the drying processes of different methods such as hot air drying, spray drying, vacuum drying, microwave drying, and freeze drying to produce plant extract powder, the performance showed that the powder produced by freeze-drying has better quality in terms of appearance colour, flavor, and nutrition in which it contains the highest sugars, soluble proteins, vitamin C, total polyphenol content, and significantly the highest antioxidant capacity compared to other drying methods(55). A study was conducted to study different drying methods in lemon myrtle leaves. Among drying methods including hot air drying, vacuum drying, microwave drying, sun drying, shade drying, and freeze drying, it showed that freeze dried leaves had high content of bioactive compounds and the highest antioxidant properties(56). However, the choice of drying method also has cost and energy factors in the drying process when it comes to industrial application. The cost of production was compared in Table 4, and it would guide the decision on the drying method(57).

Table 4 : Production cost of processes drying. Compare Lyophilization.

Processes drying	**Manufacturing cost (%)**
Lyophilization	100.00
Vacuum drying	51.60
Spray drying	20.00
Drum drying	24.10
Fluid bed drying	17.90
Air drying	17.90

1.1.7 Quality Control of Extract

Quality control in the pharmaceutical aspect is an important issue in ensuring the reliability of a pharmaceutical product. It indicates the characteristics of the extract and evaluates the results for the determination of acceptance criteria. In the quality control process, the type of additives, substitutes and the purity of the extract were identified. Therefore, chemical composition analysis is very important in the accuracy analysis of medicinal plants. In the pharmaceutical field, the quality control process is carried out both quality and quantity where the measurement of a

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substance marker of herbs is compared with the authentic samples. Quality control of an extract may be a general assessment to control physical and chemical properties[58].

1.1.7.1 Physical analysis by Munsell color chart system

The Munsell colour chart system identifies colours in the properties of the color scale: hue (basic color), chroma (hue), and value (brightness). The Munsell system was invented by Professor Albert H. Munsell in the first decade of the century. It is certified by the United States Department of Agriculture (USDA) and has the official application of the Munsell colour system for soil colour surveys and research[59].

The previous colour system was defined in one of three dimensions, but Munsell's colour system had a separate shading designation. and colour values are dimensional patterns that are consistent and independent of each other. and this is the first time the color systematically in the form of three dimensions. Munsell's colour system provides a description to illustrate and identify the types of colours. The basic colour response of the human eye is introduced by scientific principles in processing. This makes Munsell's colour system popular and applied in many research[60].

Munsell colour systems are used in the food industry to inspect raw materials and research, such as to check the color of Pecan Kernel Color[61]. For the colour of MP seed powder, the colour may range from white to brown depending on the process and the colour of the extract may have a colour in the range of yellow to brown. Thus, basic colours can be applied in the range of 10YR (Figure 6).

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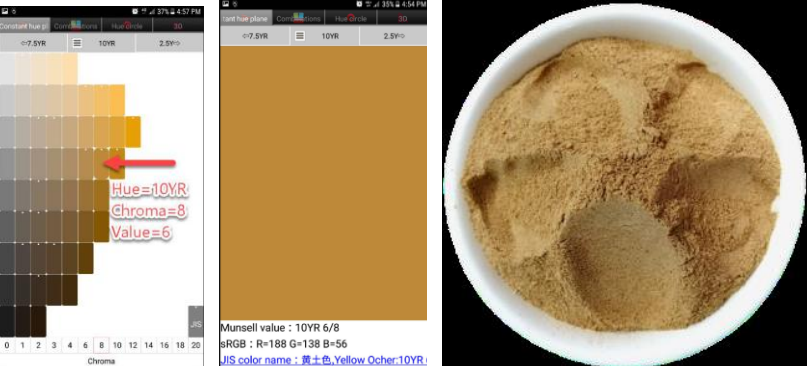

Figure 6: Munsell colour system in the application smart phone sample of yellow ocher: Hue=10YR, Chroma=8, Value =6

1.1.7.2 Chemical analysis of L-DOPA

Chemical analysis is used for qualitative and quantitative quality control. To monitor and evaluate the results of the extract in terms of the the quality of specific substance. and the quantity obtained after extraction and stability test which can be tested in several ways such as liquid chromatography in tandem with mass spectrometry (LC-MS)[62], high performance liquid chromatography (HPLC)[63], HPLC-MS/MS[64], high performance thin layer chromatography (HPTLC)[65]etc.

To determine the amount of L-DOPA that is an active ingredient in a herbal drug, the selection of the measurement procedure should be considered. Studies on the content of L-DOPA in the seeds, drug formulations, or MP products of MP are usually carried out by HPTLC and HPLC[66]. In the seeds of MP, the content of L-DOPA was investigated using both techniques (HPTLC and HPLC). A standardised method was established for HPTLC analysis[67]. The amount of L-DOPA from MP seeds in dry weight was 5.60%[68], 4.83%(70), 2.23-5.36%[66] and 3.29-5.44%[69]. By HPLC analysis, MP was found that MP contained 4.0-6.0%[70], *Stizolobium pruriens* var. *utilis* contained 3.9-10.6%[71] and 4.39-5.21% in MP var. *utilize*[72].

In addition, a research team established a standard method for the determination of the L-DOPA content in commercial nectarine products or capsules using HPTLC[65, 67], which demonstrated a L-DOPA content of 2.11-2.19%.[73], 3.80-4.30%[67], and 7.48- 8.44%[74]. L-DOPA content was also found from muscle products drug treatment tumour by HPLC method 3.0-6.0% [75].

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In addition to the analytical methods used for MP and its products, HPLC-MS/MS was used to determine the L-DOPA content in an amount found in rat plasma[76] and micro-dialysis-HPLC was performed in plasma from patients with PD patients[77]. Spectrofluorimetric is another technique to quantify L-DOPA reported in MP (7.20%) and from products (4.20-5.60%)[78].

1.1.8 Shelf-Life Stability

Stability studies in herbal products should be carried out in at least three batches of samples to monitor the shelf life and persistence of herbal active ingredients. commonly referred to as long-term stability under natural atmospheric conditions. Using modern analytical techniques such as spectrophotometry, HPLC, HPTLC and applying appropriate guidelines according to International Council on Harmonization (ICH) is a committee that provides the

pharmaceutical stability guidelines for industries[79]. another reference pharmaceutical stability The United States Pharmacopeia (USP) is an independent, scientific non-profit organisation focused on building trust in the supply of safe, quality medicines[80] , Able to generate stability data of herbal products and predict shelf life. This process improves the global acceptance of herbal products.

The determination of the shelf life of an herbal medicinal product is the same as that of chemically defined active ingredients, but the nature of the bioactive in the example of an herbal product considering packaging and storage in a cool dry place should be considered. In the case of herbal medicinal products containing natural products or herbal preparations containing known therapeutic active ingredients. The variation of components during the proposed shelf life should not exceed ± 5 % of the initial value. Unless it is reasonable to extend the range to ± 10 % or more. The low marker concentration in the final product justifies the wider range[81].

Accelerated stability is a method of controlling high temperature stability, and the decomposition of products is configured faster. Data are used to predict shelf life or to compare relative stability of the drug formulation or extract stabilisation studies reduce testing time. In addition to temperature, other conditions catalyse the reaction, such as humidity, light, and pH[82]. The ICH specifies accelerated storage conditions at 40± 2°C and75± 5% RH.

1.2 Rationale and significance

Parkinson's disease (PD) is a common and serious disease that occurs among older people around the world, including Thailand. PD is caused by degeneration of the brain and nervous system and is the second most common after Alzheimer's disease[83]. PD is not curable and affects personal life in everyday life, where the patient cannot help himself and be independent of the help of others. The patient is likely to suffer from anxiety, lack of self-confidence, and feelings of embarrassment that can be caused by loss of self-esteem in social interactions. It also leads to caregivers being overwhelmed with the long-term care and support of the patient. Therefore, PD affects both the patient and caregivers physically and psychologically.

One of the treatments for PD is by increasing the dopamine level in the brain. Dopamine is an essential neurotransmitter in the brain that is found in very low amount in patients with

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PD[84]. Currently, the main drug used to treat PD is levodopa (L-DOPA), which is a precursor to the synthesis of dopamine. It is often combined with a group of medications that are decarboxylase inhibitors to reduce conversion that can cause side effects such as nausea, vomiting, and low blood pressure[85] and help L-DOPA to pass through the brain better. Recently, research has found high concentrations of L-DOPA in the bloodstream or long-term treatment of synthetic L-DOPA. It increases the risk of dyskinesia, which is a twisted back and forth movement of the limbs and trunk in which movements cannot be controlled[86]. However, aqueous extracts of MP have been found to reduce side effects in animal studies (dyskinesia). [87] and reduce adverse events (psychiatric, nausea, somnolence, dizziness) in human[88]. Therefore, phytomedicines are of interest as an alternative treatment option to substitute synthetic drugs.

L-DOPA is naturally found in the seeds of MP. In traditional Indian medicine (Ayurveda), MP has long been used to treat PD. Due to its high content of L-DOPA, MP is currently used as a precursor to produce PD Drug[2]. The powder of MP has been clinically studied for the treatment of PD and has been found to be more effective and has side effects than synthetic drugs. [89]. Interestingly, the dyskinesia rate decreases when using MP powder. It is suspected that the MP seed may contain some active ingredient that acts as a decarboxylase inhibitor. [7]. However, with prolonged treatment with PD, clinical outcomes are reduced by motor complications. Due to the therapeutic neurotoxicity of L-DOPA, L-DOPA administration should be delayed, if possible, to avoid adverse effects. Therefore, treatment of L-DOPA with antioxidants was studied to reduce oxidative stress induced by L-DOPA and found that the oxidative toxicity of L-dopa was significantly reduced in rats.[47]

Attempts have been made to extract L-DOPA from MP seeds, but it was found that L-DOPA readily separate extract by various extraction methods such as soaking, steaming, steaming, roasting, sprouting and alkali fermentation.[40] Among all processing extract methods, only extraction by acidic pH roasting can increase the L-DOPA content. Therefore, it is necessary to find an efficient extraction method. On the basis of the above research on antioxidant supplementation therapy. The idea was to extract MP seeds with a solution of *Phyllanthus emblica*, an acidic herb that contributes to antioxidant activity.

In some reports, the seed coat of MP was removed before extraction[40, 66]. So far, there has been no report of L-DOPA in the seed coat. It was thought to accumulate in the seed. Also,

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the seed coat is not easy to remove. Therefore, it should be observed that if the seed coat is not removed, how will it affect the amount of L-DOPA. This will help decide whether the seed coat should be removed before extraction. If there was no significant L-DOPA content in the extract, the seed coat was preserved. and reduce the time spent in the production process. As a result, it can reduce overall production costs. In addition, the seed coat is made up of phenolic groups that may prevent oxidative damage during extraction[90].

The extraction process should be considered for not only the stability of the drying extracts but also the cost because it is one of the costs that affect the price of the extract. Generally, pharmaceutical products use freeze drying which provides good stable[91]. But the drawbacks of freeze drying include cost of energy, equipment, and maintenance[92]. Therefore, an alternative drying method with acceptable stability and low production cost will be considered in this study. Because L-DOPA is heat stable, tests vacuum dry will be performed to reduce the production cost. It was reported in one study that vacuum drying can reduce cost down 48% compared to freeze dry[57]. It may be an alternative to the production of MP extract.

This study aims to develop a simple water extraction method to produce a high concentration of L-DOPA MP extract, and the process could be further adapted to the industrial production scale by which it would be later used to develop a drug formulation in the treatment of PD.

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1.3 Hypothesis

Co-extraction of Mucuna seed with *Phyllanthus emblica* extract can increase levodopa content and the overall chemical and physical quality of the Mucuna extract.

Research Methodology

2.1 Research plan and experimental detail

2.1.1 Samples

Mucuna seed (Thai Mucuna; *M. pruriens* var. *pruriens*; TMM and India Mucuna; *M. pruriens* var. *utilis*; IMM)

2.1.2 Chemicals

1) Acetic acid (Mw 60.052 g/mol, CAS No.64-19-7)

2) Ascorbic acid (Mw176.124 g/mol, CAS No.50-81-7)

3) Butanol (Mw 74.121 g/mol, CAS No.71-36-3)

4) Ethanol (Mw 46.07 g/mol, CAS No.64-17-5)

5) Formic acid (Mw 46.03 g/mol, CAS No.64-18-6)

6) 3,4-dihydroxyphenylalanine (L-DOPA) (Mw 197.19 g/mol, CAS No.59-92-7)

7) Methanol (Mw32.04 g/mol, CAS No.67-56-1)

8) *Phyllanthus emblica* powder extract (CAS No. 90028-28-7 food grade)

9) Deionised water

2.1.3 Equipment and tools

1) TLC / HPTLC silica gel plates (60 F254, 20 × 10 cm)

2) Ultrasonic water bath (ELMA S30H, Germany)

3) Vortex mixer (Genie 2 /USA)

4) Centrifuge (Hermle refigerator centrifuge,Z 513 K ,Germany)

5) Autoclave (Tomy, INSX700, Japan)

6) Auto spotting (CAMAG Linomat 5 Auto spotting,Switzerland)

7) Automatic Development (CAMAG Automatic Development Chamber2, ADC2, Switzerland)

8) TLC plate heater (CAMAG TLC Plate Heater 3, Switzerland)

9) TLC visualiser (CAMAG TLC Visualizer 2, Switzerland)

11) HPLC (Agilent HPLC 1260 Infinity II, column LiChrosphe r 100 RP-18e 5µm)

12) Grinder (IKA WERKE grinder, M20)

13) pH metre (Seven Compact™ pH/Ion meter S220, Mettler-Toledo AG, Zürich, Switzerland)

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2.1.4 Preparation of MP seeds

The MP seeds will be heated in the microwave for 10 minutes. Therefore, the seeds will be divided into two groups. The SS is the seed with seed coat and Group SN group is the seed without seed coat. Both groups will be further used in the extraction process to compare their L-DOPA content.

2.1.5 Optimisation of extraction process

2.1.5.1 The effect of extraction methods

The heat treated seeds of MP (with seed coat and without seed coat) will be processed by grinding in an IKA WERKE mill (M20). Two extraction methods will be tested, including sonication at 70 ° C for 30 min. and autoclaving at 15 bars, 70 ° C for 30 min. Five grammes of the seeds will be extracted with 50 ml of acidic water (0.1N HCl), centrifuged to retrieve the extract and then brought to dryness by freeze drying. The extract powder will dissolve in 0.1N HCl acidic water for analysis L-DOPA content, physical appearance, time consumption, and cost will be compared. The extraction method which gives a higher L-DOPA content will be selected to carry out in the next experiment.

2.1.5.2 The effect of drying methods

Evaluation of the drying methods will be performed by comparing freeze-drying and vacuum drying. MP seeds (5 g) will be extracted in 0.1N HCl 50 mL with the above selection method (sonication or autoclaving). The extract will be centrifuge at 4000 rpm for 10 minutes. The supernatant solution will be dried by vacuum or freezing drying. The physical appearance will be recorded and the extract powder will be dissolved in acidic water for analysis of the L-DOPA content. The suitable drying method will be selected before being carried out in the next experiment.

2.1.5.3 The effect of difference acid solvents

To evaluate the effect of solvents on the quality of the MP extract, the concentration of *Phyllanthus emblica* extract (PEW) at 0.25%, 0.5%, 1.0%, 2.0%, 5.0%, 10.0%, 20.0% and various acid agents controlling pH condition at 3.0 that were ascorbic acid, citric acid, will be tested to compare with the acidic water served as control (hydrochloric acid). The abbreviation is defined in Table 5. The acidity of each solvent will be recorded as the pH value before and after the extraction process. Weigh 5 grammes of MP powder and add 50 ml of each solvent above. All

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samples will be sonicated for 30 minutes, centrifuged at 4,000 rpm for 10 minutes, and dried by vacuum dry or freeze dry. The physical appearance and L-DOPA content of the extracts will be analysed.

Table 5 Abbreviation acidified solvent codes for the extract.

Acidified Agent	Chemical Structure	Abbreviation Code
Hydrochloric acid	Cl^- H_3O^+	HA
Citric acid	HO, O, OH, OH, O, OH	CA
Ascorbic acid	HO, HO, H, O, O, HO, OH	AA
PE water	N/A	PE

2.1.6 Statistical analysis

A one-way analysis of variance was used. Select the significance of the difference from Duncan's multiple comparison test. In the data analysis ($p < 0.05$) was significant.

2.1.7 Chemical analysis of L-DOPA

2.1.7.1 TLC / HPTLC analysis

MP extract from different processes for analysis on TLC / HPTLC silica gel plates (60 F254, 20 × 10 cm) with a volume of spotting of 4 μl with the Camag Linomat.5 is 8mm width per band, then developments with a mobile phase, ethyl formate: toluene: formic acid: water ratio of 30: 1.5: 4: 3 (*v*/*v*/*v*/*v*), the total distance develops is 85 mm. Next, the product was derived by NP-PEG reagent and analysis of chemical profile under 366 nm UV light with Visualiser II via VisionCATS software.

2.1.7.2 HPLC analysis

The standard L-DOPA prepared by dissolve 0.1% (v/v) formic acid in water (concentration 200-1000 ng/μl) and sample dissolve 0.1% (v/v) formic acid in water as well were then analysed by HPLC Instrument (Agilent HPLC 1260 Infinity II , then using a column LiChrospher 100 RP-18e 5 μm, Merck) and mobile phase A was assigned 0.1% (v/v) formic acid

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in water and B to methanol by gradient programmed separation as follows: 1% B at 0 minutes to 10 minutes and at 1-4% B from 10 to 20 minutes with a flow rate of 0.5 ml/min. And measured at a wavelength of 282 nm.

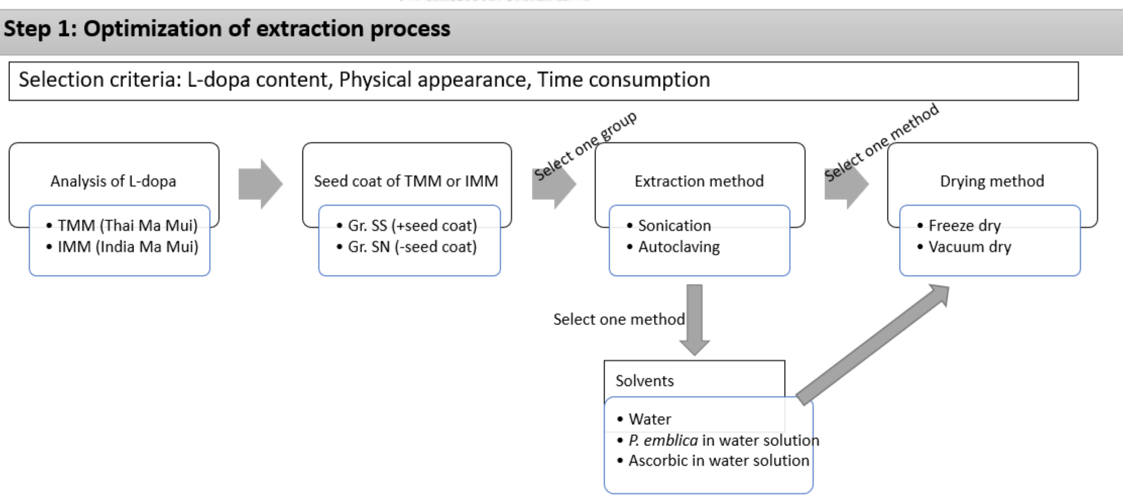
2.1.8 Physical analysis of MP extract

The colour, pH and water contents will be tested as a characteristic of the MP extract. The quality colour of the extracts is determined by comparison. The colour charts range from light brown to fine brown powder. The acidity of the sample extract will be measured as a pH value. (95)

2.1.9 Stability test

The dry extracts of MP seeds from the best extraction process. They were placed in zip-lock bags and stored under accelerated conditions at a temperature of 40±2 °C / relative humidity of 75±5%. All samples were observed at initial, six and twelve months. All samples tested physical and chemical properties including colour, pH, chemical profile fingerprint, and L-DOPA content.

2.2 Research framework (Flow chart)



Result and Discussion

3.1 Result and discussion

3.1.1 The effect of extraction methods

3.1.1.1 selected raw materials (TMM, IMM, SN, SS)

In the production process, raw materials have various factors. The aim of this experiment was to compare 2 varieties. There are main sources of L-DOPA that can be found locally (Thai Mucuna; TMM and Indian Mucuna; IMM) and difference two process of preparing raw material (extracted by removing the seed coat, SN and the whole extract with the seed coat, SS). The results showed that extract from TMM-SN. Its highest L-dopa was 8.34 % (w/w). While for IMM-SN the secondary content was L-dopa equal to 8.02% (w/w) followed by IMM-SS and TMM-SS equal to 7.79 and 7.69 % (w / w), respectively. When considering the value of the extraction yield, IMM-SN was the highest, followed by TMM-SN, TMM-SS, and IMM-SS at 28.5, 28.4, 27.4 and 26.1 %(w/w), respectively. The resulting values were not statistically different between raw materials. (Figure 7) Study in the next order, consider the selected IMM because it has a cheaper cost of raw materials than TMM, and the IMM harvest process is more convenient because it is easier to harvest than the TMM variety and does not have to worry about itching of the pods. In the preparation process, the method of grinding with seed coat (SS) was chosen because it reduces the production process and also reduces cost.

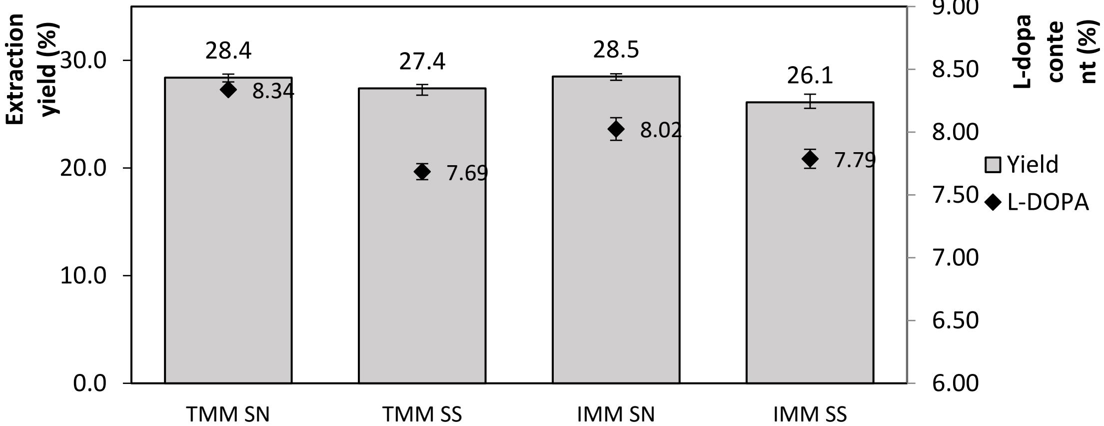


Figure 7. This is compared to raw material from the prepared process extracted without seed coat (SN) and whole with seed coat (SS). The report was shown as a mean value ± standard deviation (SD) (n = 3).

3.1.1.2 selected extraction methods (Autoclaved or Sonicated)

The extraction method was an important factor to consider in selecting extraction for good quality of the desired extract or product. in this study, two methods were selected to compare the efficiency of extraction, such as autoclave extraction and sonicate extraction. The results showed that extract from sonicate. The results showed that the extraction of sonicate produces higher L-dopa than the autoclave method at 8.59 % (w/w) and the yield was 23.6 % (w/w). However, autoclave extraction produced a negative and significant difference, with a L-dopa of 3.73 %(w/w) and a yield of 19.4 %(w/w). Therefore, the decision to choose an extraction method to study in the next step uses sonicate extraction method to help increase the quantity and productivity in the production process. Consistent with the research of Dogan *et al.* (2019), the application of sonicate in the extraction of Sage and mint has been shown to have good physicochemical results as well.[93]

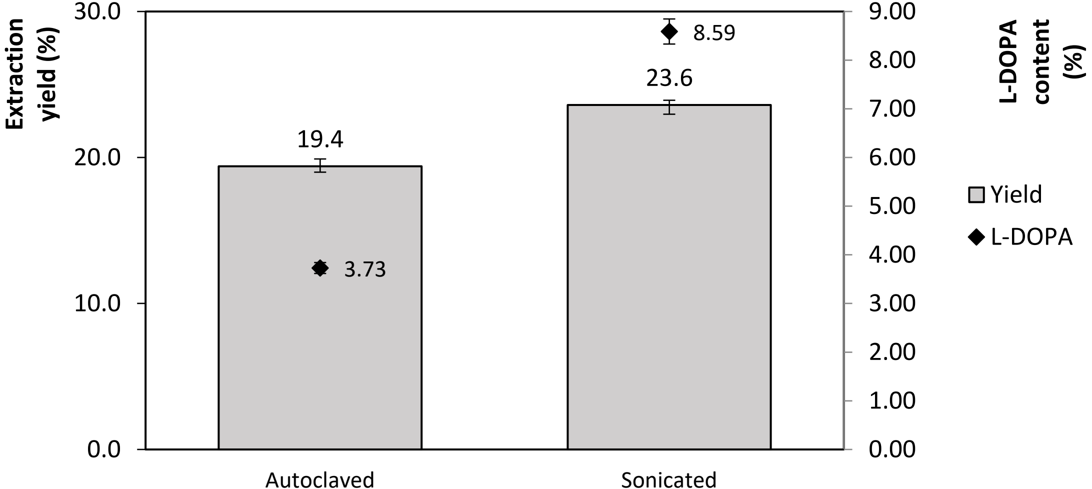


Figure 8 That compared extracted method between autoclaved and Sonicated. The report was shown as a mean value ± standard deviation (SD) (n = 3).

3.1.2 The effect of drying methods (freeze drying, Vacuum drying)

The drying process is another important factor for the use of the extract product and affects the quality and stability of the extract. It is an important issue in production to consider the cost of the production process at the industrial level. This study compares the quality of the extracts after the drying process. The results showed that both drying methods were not statistically significant. The dry method shows L-dopa content and yield were 8.06, 22.5 % (w/w). While the freeze-dry

method shows L-dopa content and yield were 7.88, 23.3 % (w/w). (Figure 9) However, vacuum drying used a time that was less than freezing drying. It takes only 10 hours, while the freeze dry method takes more than 26 hours to dry, which means more production costs, which is similar to the study report of Santivarangkna *et al.* (2007) that has gathered information on the cost of drying by various methods.[57] Therefore, the vacuum drying method was considered to be used in the study in the next section.

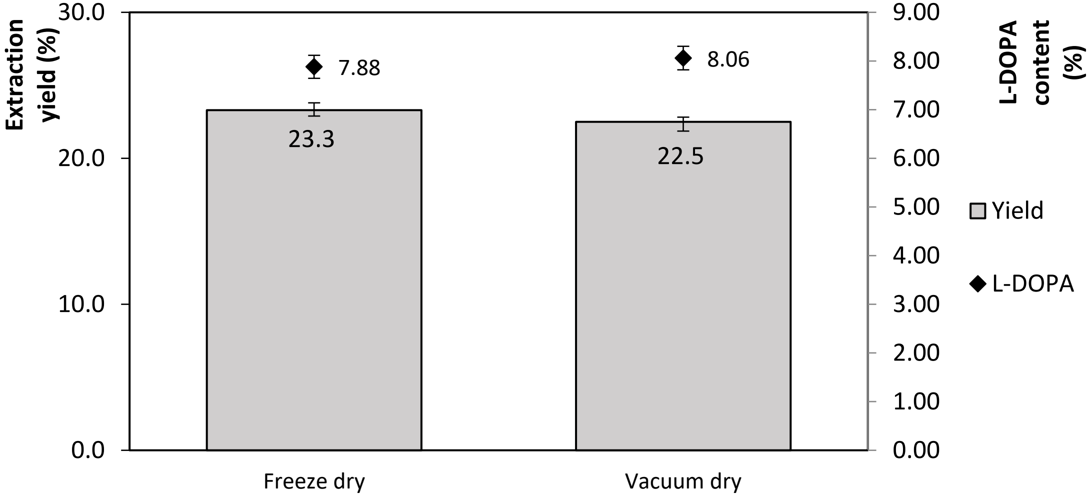


Figure 9 That compared drying method between Freeze Dry and Vacuum dry. The report was shown as a mean value ± standard deviation (SD) (n = 3).

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3.1.3 The effect of difference acid solvents

In MP seed extraction experiments for the proof of the hypothesis, the results were compared with different acid solvents. The amount of extractable L-DOPA per g of extract powder was used to compare the extraction results obtained by the process in this work. The quantification of the L-DOPA content by the HPLC analysis technique confirmed the method results and the applied in the analysis.

The results of PEW coextracts were confirmed to result similar extraction results. Hydrochloric acid (HA) control group. The results showed that the use of concentration at 2.0% PE shows the same effect as HA, an acid solution commonly used in manufacturing. It shows that the yields were not different in both extract value and L-DOPA content. However, increasing the high % PE concentration in extraction contributed to a higher % of yield. On the other hand, it

showed a significant decrease in the content of L-DOPA because the concentration of PE extract was too high. The extract powder mainly consisted of PE instead of L-DOPA. Compared to other methods, the use of ascorbic acid (AA) in extraction has the least content of L-DOPA 3.88%, which may be because AA is easily degraded. This is like the study of Ariahu *et al.* (2011), They reported the kinetics loss of the ascorbic acid solution in heat. They found that the ascorbic residual tendency decreased when heating in the temperature range 60-90 ° C and that the heating time resulted in a reduction trend. down as well[94]. Other studies by Pappert *et al*., (1996) have used AA to stabilise L-DOPA, but only under non-thermal extraction. They prepared levodopa with an ascorbate solution to study its stability. L-dopa was found to be the most stable in quality when stored in the freezer or refrigerator.[95] In 2007, Misra and Wagner experimentally extracted MP extracted with ethanol: water (1:1) with the addition of a small amount of Ascorbic acid, which also produced a non-thermal treatment of L-DOPA.[46] When analysing citric acid extraction, the results were more or less consistent with previous studies reporting that the introduction of CA at a concentration of 58% improved the extraction process efficiency by a ratio. MP and CA (1:7) showed that the extract L-DOPA up to 8.0%.[96] While, Benfica et al., 2020, discovered that higher solid to solution ratios and higher CA concentrations yielded similar effects of L-DOPA content effects in this thesis study, the use of CA could be another option for extraction. However, it must be taken in the form of an eutectic solvent compound. (Co-extract between Critic acid and colinium chloride), which gives a good effect of the dosage L-DOPA was 7.2% (w/w).[97] Nevertheless, that studies aim to separate solvent for purified the compound and reused solvent the extraction solution in the process again .While the development method of extraction in this thesis aims to immediately use extract as a product without separation solvent process.

The experimental results in this thesis showed that using PE at a concentration of 2.0% could produce L-DOPA at the same percentage as previously reported.[46] In this study, different percentages of PE were found to be used as L-DOPA extract. The results showed that the extraction yield was significantly increased. (Fig. 10). However, increasing the concentration of PE above 5.0% resulted in a lower L-DOPA. This may be causing the percentage of extracted L-DOPA to be diluted with the increase in the extraction yield of PE. Whereas the higher extraction value of extract when using higher concentrations at 10 and 20% PE. This may be affected by the

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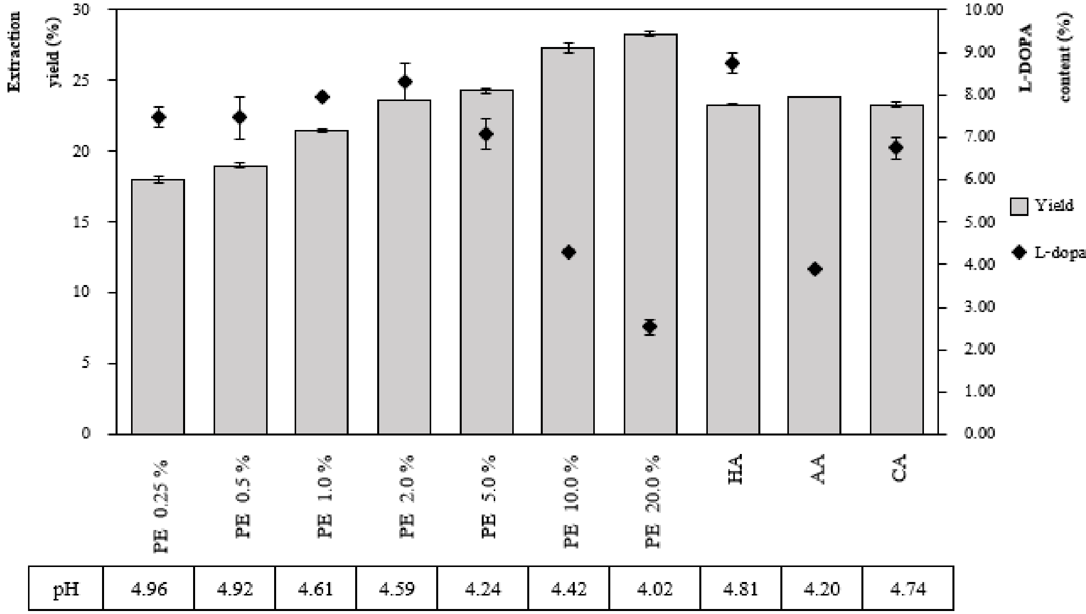
weight of the PE powder remaining after drying. Therefore, the choice of % PE is a decision based on the cost of the raw material and the amount of active ingredient is important in determining the equilibrium point of the production process.



pH	4.96	4.92	4.61	4.59	4.24	4.42	4.02	4.81	4.20	4.74

Figure 10 The yield percentage of extraction and L-DOPA content with various% concentrations of PE compared to other acids solvents (HA, AA and CA). The report was shown as a mean value ± standard deviation (SD) (n = 3).

The evaluation of the quality of raw materials, ingredients and finished products in herbal medicines, and the results of the chemical profile tests are important and identify the authenticity.[98] HPTLC is an alternative for analysis. It is a simple testing technique and is widely applied in the field of herbal quality assurance. which can be applied to inspection in conjunction with the HPLC technique to confirm the quality of the product. The results of the HPTLC chromatogram test were used to compare the chemical properties of PE-MP extract, MP seed, PE and other acids (HA, AA, CA) using L-DOPA as markers. (Figure 11) shows chemical profile of standard L-DOPA Rf =0.45 (Track 1), raw material (Track 2), PE water (Track 3), MP co-extracted with the concentration of PE water difference (PE-MP) as follows 0.25, 0.5, 1.0, 2.0, 5.0, 10.0 and 20%, respectively (Track 4-10), MP seed extracted with HA(Track 11), MP seed extracted with AA (Track 12), and MP seed extracted with CA (Track 13),The chemical profile of PE-MP was similar to that of a single substance of MP and PE extract, and L-DOPA was also

detected. Although the PE-MP sample was a combination of two herbs, it was shown that it could be distinguished by this technique compared to a standard sample.

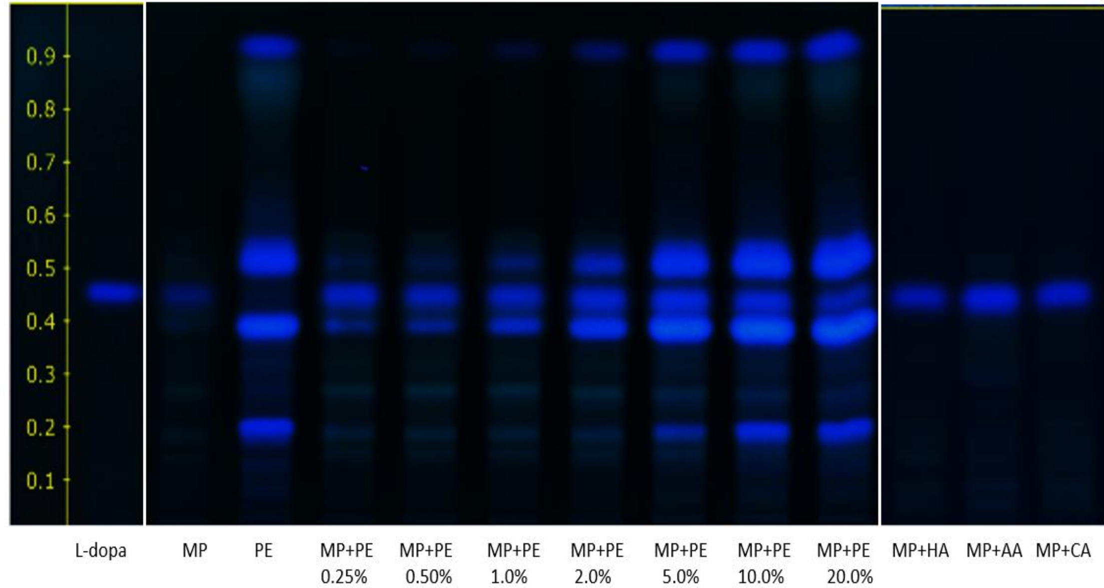


Figure 11 Chemical profile fingerprinting result of the HPTLC. MP co-extracts with PE water at various concentrations when compared with other acid solvents. Track (1) is L-DOPA; track (2) is the Authentic MP seed; track (3) is the PE single component extract; track 4 -10 is the sample extract from MP seeds with PE different concentrations as follows 0.25, 0.5, 1.0, 2.0, 5.0, 10.0 and 20 % respectively; track 11 is MP seeds extracted with HCA; track 12 is MP seeds extracted with ASA; track 13 is MP seeds extracted with CTA

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3.1.4 Physicochemical stability test

3.1.4.1 Colour

The physical value of the sample extract indicates colour exchange. The initial (0 months) showed the brightness colour all sample values of lightness (L*) range 91.599-96.442. When all samples are stored under accelerate condition after 6 months. The samples showed that the colour of the extract was changed to decrease the brightness of all samples. However, the MP-PE samples had the highest residual (L*) = 70.365 followed by hydrochloric acid, vitamin C, and citric acid. Their brightness values were 60.091, 57.176, and 45.811, respectively. Then storage after 12 months, all extracts were changed their colour to an increase dark brown shade. (Table 6).

Table 6 The Appearance color of the MP extracts seed powder with various acidic solvents. Accelerated state control was stored at initial values after 6 and 12 months.

Sample	Initial				6 Months				12 Months			
	CIE-L*a*b				CIE-L*a*b				CIE-L*a*b			
	(L)	(a)	(b)	Color	(L)	(a)	(b)	Color	(L)	(a)	(b)	Color
MP+HA	94.7735	-3.1216	28.4629		60.0908	17.8372	57.7983		33.2482	8.7708	31.9524	
MP+PE	96.4417	-7.2496	33.2448		70.3645	5.3563	32.0316		55.7375	9.5249	37.9486	
MP+AA	91.5999	1.4565	14.1484		57.1764	13.1307	48.6883		6.4405	14.2627	9.5094	
MP+CA	91.7986	-0.4415	26.3880		45.8105	4.8658	38.8984		26.1554	13.5489	30.6758	

However, the highest residue brightness remained in the samples extracted with PE extract. That showed a percentage change in brightness after 6 months of storage, approximately 50.71 % of the samples extracted with PE were darker than the acid-extracted samples. The hydrochloric acid was 64.92% darker in the samples with citric acid extraction, 71.51% darker, and the samples with 92.97% darkened by ascorbic acid extraction. The results of this test showed that the MP seed extract with PE capable retained the colour in the mostly It's compared to other samples,which may contain some compounds that have anti-oxidation properties a study reported by Luo *et al*, 2009 indicated that 6 organic acids with antioxidant activity were extracted from Indian gooseberry juice: cinnamic acid, quercetin, 5-hydroxymethylfurfural, gallic acid, β-daucosterol. and ellagic acid.[99, 100] (Figure13) The extract of the color is caused by humidity of the environment.[101] The most intense color variation of ascorbic acid extracts was due to the instability of ascorbic acid in its responsive and degraded solution form. That heated and extraction time[102]

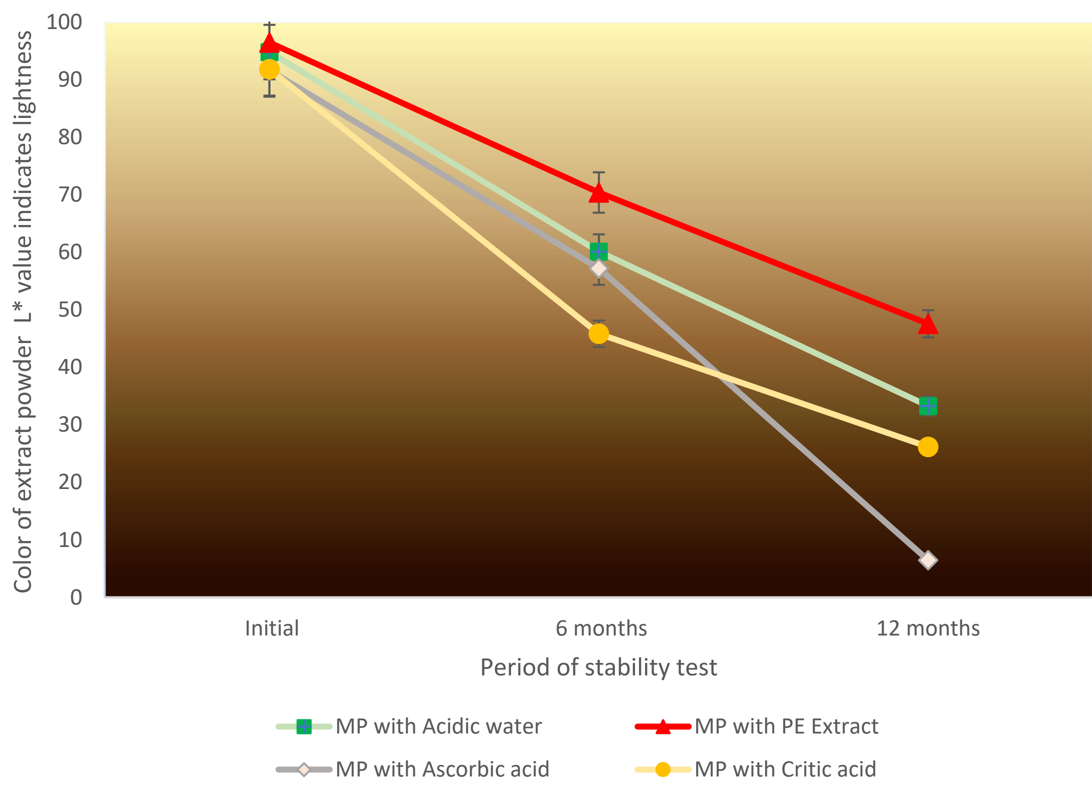

Figure 12 The decreased trend of lightness (L*) was compared to samples of different solvent extracts at the start of the test. After storage accelerated conditions for 3 months and 6 months.

Moisture oxidation $+2H^{+} + 2e^{-}$

L-dopa (white)   L-dopaquinone (yellow to brown)   Cyclodopa   Dopachrome

Figure 13 Reaction L-dopa structure when oxidized by environmental conditions changes to L-dopaquinone.

3.1.4.2 pH of sample extract solutions

pH is a quality indicator of the stability of the extract. Because of the specific properties of L-DOPA, it is stable under acidic conditions. Normally, the L-DOPA extract must have an optimum pH in the range of 2.0 to 4.0. [103] The pH of the solvent was controlled at pH 3 before

being used as a solution for MP seeds, as previous studies reported that good MP seed extraction conditions required a pH of 3. which improves extraction efficiency even further. [104] Importantly, L-DOPA has a zwitterionic ability (ion exchange property), L-DOPA can chemically change hydrogen atoms in the structure as pH changes, making it soluble in water. and alcohol is valuable low with a pH range between 2.3 (pKa1) and 8.11 (pKa2). [105, 106] It also has good solubility in the acidic range, where the acidic pH also promotes oxidation as well.[107] Therefore, acidity is important for the stability of the L-DOPA extract. According to the test results, the pH of each solution changed with only a slight increase after extraction between 4.20 and 4.81. After collection and dissolution for testing, the pH of each sample showed little change, even at 6 and 12 months, without significant differences. (Table7). In this experiment, PE was used because of its acidic nature. Although PE could be prepared at a concentration of 0.25 to 20.0%, the pH of the solution ranged from 4.02 to 4.96, as shown in Figure 10. It is possible that further adjustment of pH with other acids to reduce the pH below 2.3 may improve the solubility of L-DOPA.

Table 7 The result of the pH value in MP extracted with different acid solvents. It was not significant for the Duncan multiple-range test.

Sample	Initial	6 Months	12 Months
MP+HA	4.81 ± 0.02	4.81 ± 0.01	4.79 ± 0.02
MP+PE	4.59 ± 0.01	4.59 ± 0.01	4.58 ± 0.02
MP+AA	4.20 ± 0.02	4.19 ± 0.02	4.21 ± 0.01
MP+CA	4.74 ± 0.02	4.74 ± 0.02	4.72 ± 0.01

3.1.4.3 Stability of L-DOPA content in sample extracts

The amount of L-DOPA can be reduced in the storage environment under accelerated conditions. It is a conditional control to rapidly track changes. All samples are tested at specified intervals using the HPLC technique. (Figure 14) The amount of L-DOPA year to be converted over the longer period. At the initial (0 months), L-DOPA in the sample extract with HA is the highest percentage at 8.76% (w/w) followed by PE at 7.72% (w / w) CA at 6.74% (w/w), and AA at 3.88% (w/w). While the lowest analytical L-DOPA content obtained from extracts may be due

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to its tendency to decompose faster when AA is prepared in solution.[108] Therefore, this results in reduced efficiency of the AA property. Therefore, it could not prevent the degradation of L-DOPA, resulting in a significant loss of extraction yield compared to other solvent extracts.

The quantitative stability of L-DOPA was demonstrated after storage for 6 and 12 months in an accelerated temperature-controlled condition of 40±2°C and 75±5 %RH and for the duration specified in the experimental plan. It covers a minimum retention period of 6 months according to the ICH guidelines. [109] All samples were collected under these controlled conditions. There was a significant decrease in the L-DOPA test results in all samples after 6 and 12 months, as the result shows in Figure 15. The test results showed a rapid decrease in the L-DOPA content during the first 6 months of the study, and after 12 months of continuous storage, there was only a slight decrease in the L-DOPA rate. PEW was the highest 73.55%, followed by CA, HA, and AA, respectively.

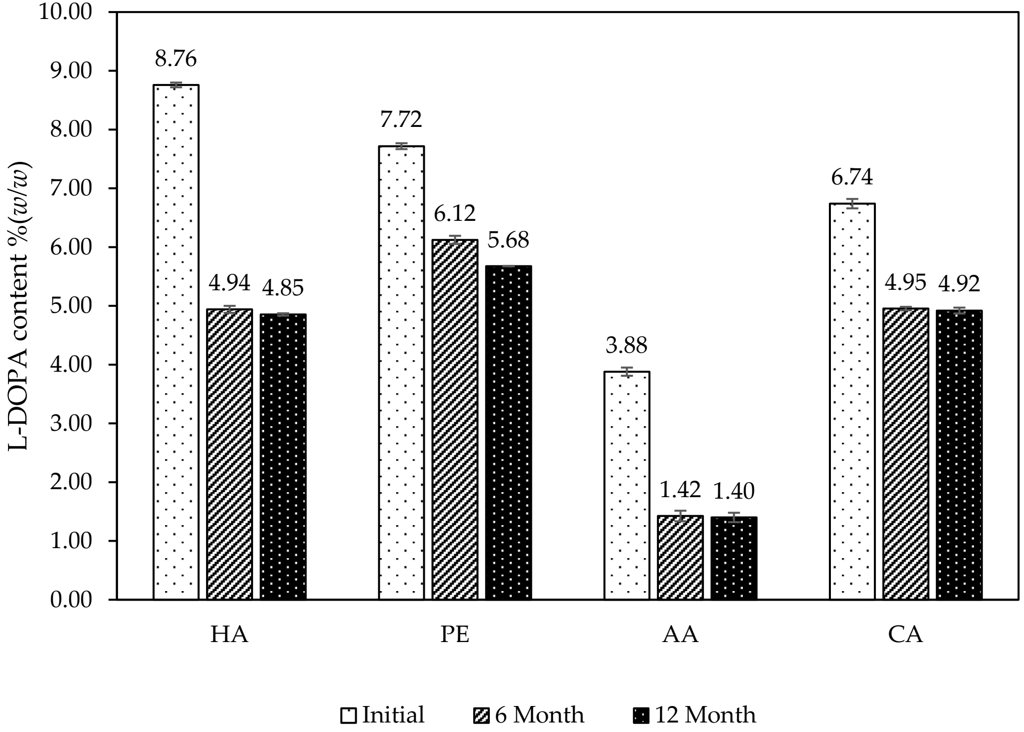

Figure 14 The result of L-DOPA content in the acid solvent of sample difference shows the stability data for the initial (0 months), 6 months, 12 months. The report was shown as a mean value ± standard deviation (SD) (n = 3).

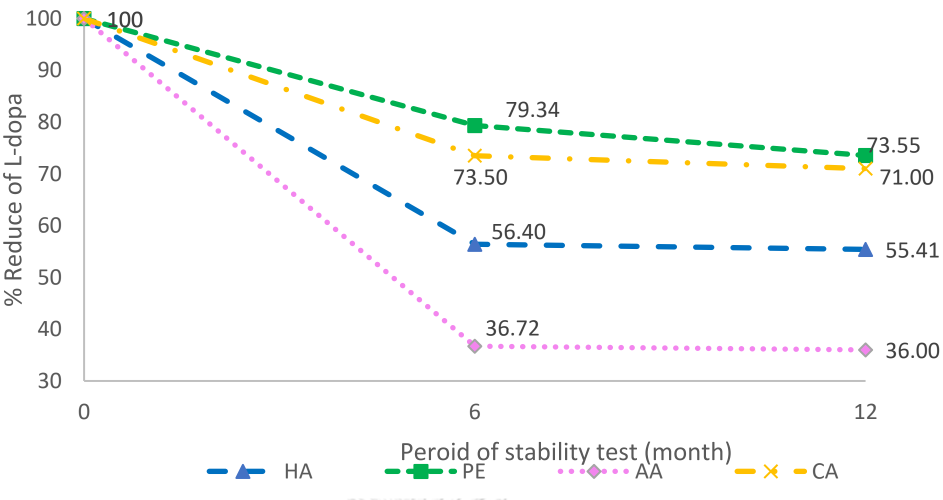

Figure 15 The percentage remained of L-DOPA remained in sample extracts compared to the initial stage (0 months). The report was shown as a mean value ± standard deviation (SD) (n = 3).

3.1.4.3 Stability of the chemical profile of Levodopa

Characterisation of the extract for chemical profile fingerprints of MP extract samples using the HPTLC technique. This experiment used standard L-DOPA (Track1); MP seed (Track2) and PE water (Track3) were used to compare the extract in this assay. Trace the chemical characteristics extracted from the PE. whereas L-DOPA is produced by extraction with the same strip of MP. Similarly, a chemical profile with PE water and MP+PE extract also appears, but other examples do not seem to be consistent with this feature. It showed a chemical expression similar to that of the original sample. Although this method was able to isolate constituents from extracts containing this combination of herbal by using standardised compounds as comparison controlled substances.[110] While the results of the study test when storing the chemical fingerprint after 6 months compared to 12 months, the extract remains the same. There was a change in expression in RF= 0.5 of all samples. and found in RF = 0.55, there are expression is yellow band. (Figure 18) These results show that. The quality of chemicals found in herbs and chemicals of natural extracts.[111] The structural changes in the chemical expressions may be caused by certain reactions or caused by the degradation of that chemical for this test, to confirm the chemical composition found in the MP seed extract compared with other samples without such compound. Analysis of the compounds in the extract samples showed that

L-dopa dissociated independently of other compounds found in PE, indicating that it did not cause a synergistic interaction, preserving the essential therapeutic value. patient.

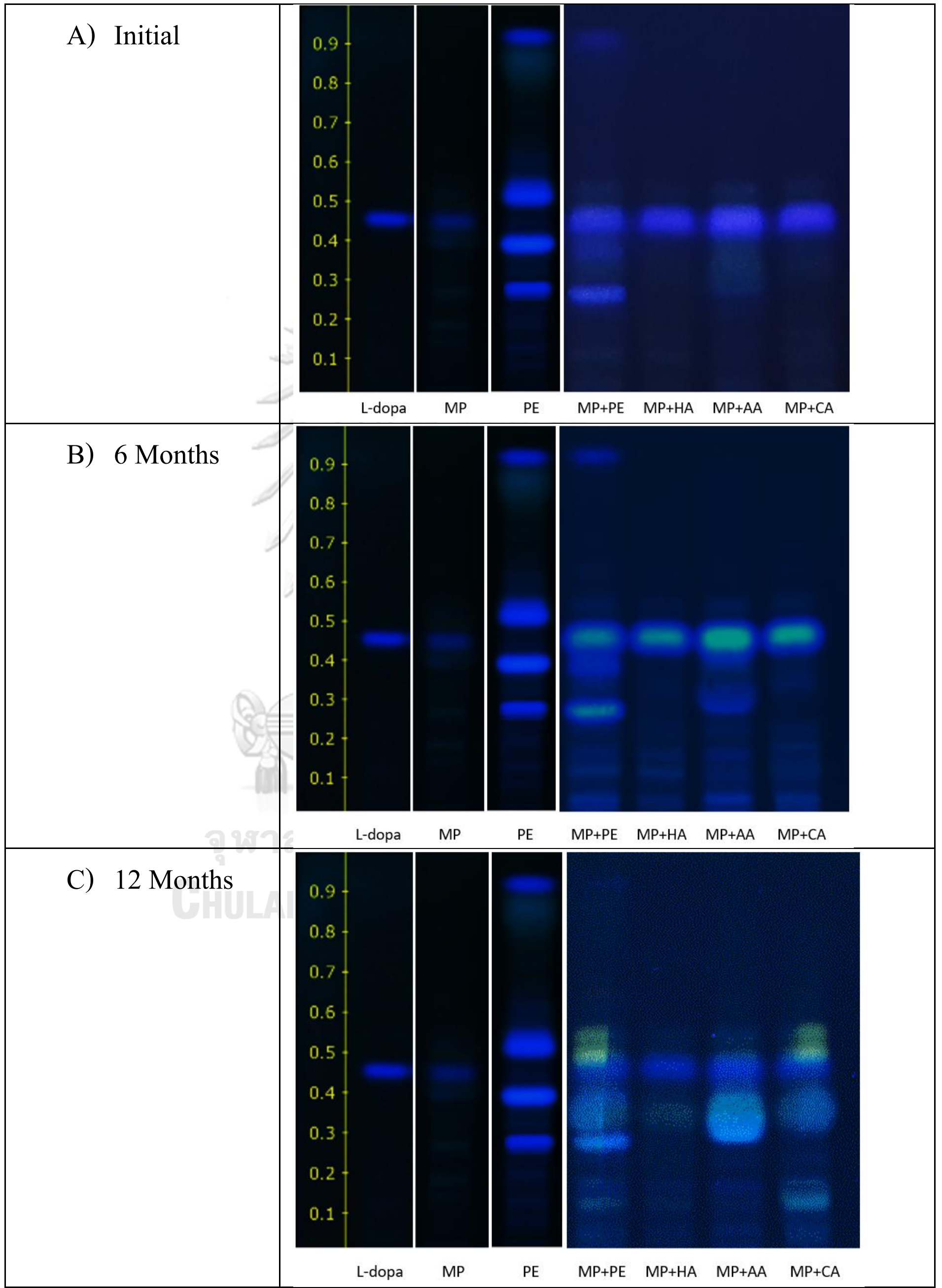

Figure 16 Chemical profile fingerprinting of sample extract by detected HPTLC. This shows a comparison of stability of three periods: initial time (0 months), 6 months, and 12 months.

Another analysis of L-DOPA in sample MP extract with PE is the HPLC chromatogram report. This is a technique used to follow the pharmacopeia. The development of an active ingredient analysis system is important for re-check control quality of pharmaceuticals. The HPLC method used in this quantification experiment was also able to detect and show the comparative results of L-DOPA in PE-MP extract. (Figure 17). This means that there is no overlap of the peaks. The L-DOPA separation retention time (RT) was 6 min. (Figure 17A). L-DOPA isolated from other compounds in PE-MP (Figure 17B, peak f) The chromatograms shown are for all the peaks of the PE compound. (Figure 17C). All peaks that were detected in the PE-MP extract could also be observed in single-compound PE. This can be clearly identified as a compound derived from PE. It can be concluded that L-DOPA and other compounds in PE do not interact with each other. This test is an indication of the quality of natural herbs and the quality of natural extracts. This experiment can be applied for quality control in the commercial herbal production process. HPLC techniques are used to detect herbal extracts. For example, a previously studied separate extract of *Andrographis paniculata (Burm f.) Ness* combination with *Phyllantus niruri L*. The andrographolide standard was used as a chemical detected for analysis by the HPLC method.[(112)] In another reported mix of standard makers (ascorbic acid gallic acid and ellagic acid) are separate components in herbal components.[(113)] HPLC analysis is a very accurate method and can also be applied to quality control.

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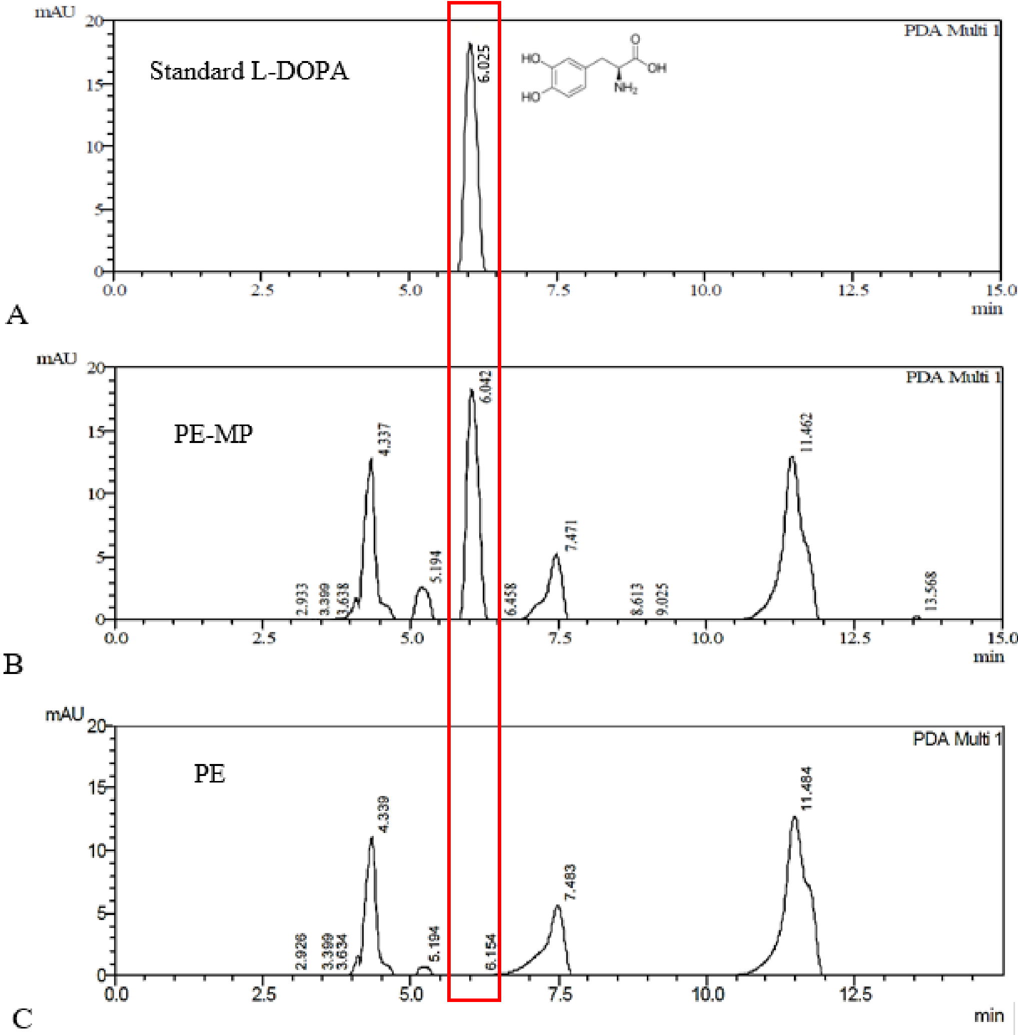

Figure 17 L-DOPA profile detected by the HPLC chromatogram. (A) L-DOPA used for standard; (B) extract MP with PE; (C) single component PE. The result peak L-DOPA is (f) is a squared line at the retention time (RT) at 6 min.

3.2 Conclusions

The results of the study can be summarised as follows:

1 Mucuna Pruriens Seed Preparation Process Before Extraction Choose to use grinding with seed shells to reduce steps in the production process.

2. Mucuna seed species were selected from Indian species because the amount of Levodopa was not significantly different from that of Thai and the process was easy to harvest and low cost.

3. The extraction process selects the sonicated method used because it has better efficiency and higher L-DOPA than the autoclaved method.

4. The drying process uses the dry vacuum method because it reduces production costs.

5. Choose an acid solvent, *Phyllanthus emblica* encourages high percent productivity and important positive appearance properties. There was little change in the colour of the powder, while the pH was unchanged, and PEW did not cause a reaction with Levodopa. Additionally, it results in a higher percentage of Levodopa compared to other acids.

Therefore, this thesis study confirms that the best extraction method was PEW using sonicate extraction and the vacuum dry method, resulting in the quality and quality of the extraction of L-DOPA from MP seeds. Good stability It is a safe way to reduce the use of chemical solvents, and PEW is also useful in treatment of patients with Parkinson's disease. Indirect benefits also promote and increase the value of agricultural crops.

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3.3 Recommendation

1. The safety and use of the extract should be studied in vitro and in vivo. for information before treating patients.

2. should study and develop methods of extraction and drying for others in the industry scale.

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