BIOACTIVE COMPOUNDS FROM GOJI BERRY FOR COSMETIC PRODUCTS

CHALINEE JANTA

MASTER OF SCIENCE
IN
APPLIED CHEMISTRY

SCHOOL OF SCIENCE
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Chalinee Janta

Thesis Title	Bioactive Compounds from Goji Berry for Cosmetic Products
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ABSTRACT

The fruit of *Lycium ruthenicum* (goji berry) is widely used worldwide to be functional food and traditional medicinal herbs. This study demonstrated promising results for microwave-assisted extraction (MAE) of antioxidative from the fruit of *L. ruthenicum*. The crude extract was further separated by solid-phase extraction (SPE) led to water and ethanolic fractions. The chemical compositions of ethanolic fraction from *L. ruthenicum* have been investigated by high- performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS/MS). A total of 32 compounds were identified in the fruit of *L. ruthenicum*. Spermidine alkaloids were detected 30 compounds. The main for this group of compounds was obtained of fragment ions at *m/z* 222 and 220. Their ethanolic fraction showed the powerful antioxidant in DPPH and ABTS assays for antioxidant activitiess. Most spermidine alkaloids showed potential in antioxidant activity.

L. ruthenicum purple color (LRP) extracted with 80% ethanol has been used to formulate cosmetic products, showing the most significant antioxidant activities (DPPH, ABTS, and FRAP assay). For this study, serum skin irritation investigation did not lead to skin irritation with serum-containing *L. ruthenicum* extract (mean irritation index at 0.00).

Keywords: *Lycium ruthenicum*, Spermidine Alkaloids, Antioxidant activity, Skin Irritation

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ABBREVIATION AND SYMBOL

LC-MS	liquid chromatography-mass spectrometry
UPLC-Q-TOF-MS	ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry
UPLC-HR-MS	ultra-high performance liquid chromatography coupled with high resolution mass spectrometry
HPLC-Q-TOF-MS	high-performance liquid chromatography fingerprinting combined with quadrupole time-of-flight mass spectrometry
UPLC-Q-TOF-MSE	ultra-performance liquid chromatography coupled with Q-TOF high-resolution mass spectrometry methods along with MSE data acquisition
HAAs	hydroxylated aromatic acids
ORAC assay	oxygen radical absorbance capacity assay
SIRT1	nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
$K_2S_2O_8$	potassium persulphate
H_2SO_4	sulfuric acid
DMEM	dulbecco’s modified eagle medium
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
LPS	lipopolysaccharide
SPE	solid-phase extraction
DMSO	dimethyl sulfoxide

ABBREVIATION AND SYMBOL (continued)

g	gram
W	watt
mg	miligram
mm	millimeter
nm	nanometer
mM	milimolar
mL	milliliter
μM	micromolar
μg	microgram
μL	microliter
min	minute
h	hour
IC_{50}	half maximum inhibitory concentration
CO_2	carbon dioxide gas
°C	degree celsius
m/z	mass to charge
eV	electron voltage
psi	pound per square inch
RAW 264.7 cells	monocyte/macrophage-like cells
Abs	absorbance
RT	retention time
CID	collision energy
dhc	dihydrocaffeyl
hex	hexose
cou	*p*-coumaroyl
ppm	part per million

ABBREVIATION AND SYMBOL (continued)

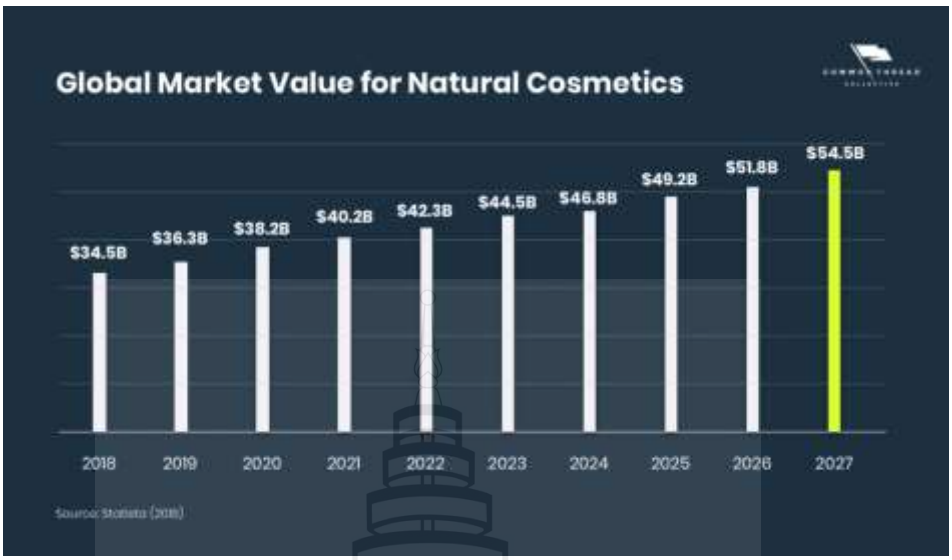
Da	dalton
TPTZ	2, 4, 6-tris(2-pyridyl)-s-triazine
$FeCl_3 \cdot 6H_2O$	iron (III) chloride hexahydrate
$FeSO_4 \cdot 7H_2O$	iron(II) sulfate heptahydrate
L. ruthenicum	*Lycium ruthenicum*
L. barbarum	*Lycium barbarum*
L. chinense	*Lycium chinense*
LRP	*Lycium ruthenicum* brown color
LRB	*Lycium ruthenicum* purple color
HCl	hydrochloric acid
ATCC	american type culture collection
DMST	department of medical sciences Thailand
MICs	minimum inhibitory concentrations
EDTA	ethylene diamine tetraacetic acid
TEA	triethanolamine
H_2O	water
ND	not detected
MAE	microwave-assisted extraction

CHAPTER 1

INTRODUCTION

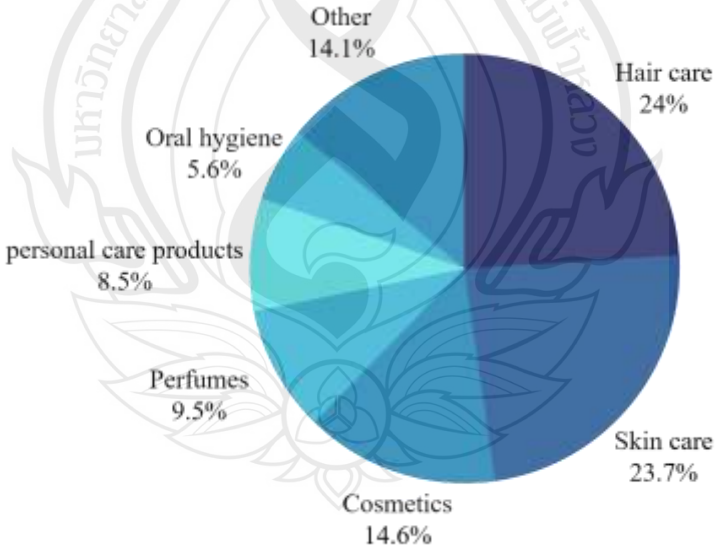
1.1 Rationale

The Asia Pacific region held the largest proportion of the beauty industry market in 2020, with 46%, followed by North America (24%), and Western Europe (18%) [1]. Geographically, the Asia Pacific and North America dominate the industry, accounting for more than 70% of the total market size. Meanwhile, the cosmetics business is growing at a rate of 4.75 % per year, with revenue estimated to reach $716 billion by 2025 [1]. The cosmetics industry is currently a fast-growing industry that is expanding both domestically and internationally. Furthermore, consumers are becoming more interested in natural health. Especially, the medicinal herbs or traditional medicine to cure various disorders. From 2018 to 2027, the global market value for natural cosmetics has steadily increased (Figure 1.1). 24.0 percent of hair care products and 23.7 percent of skincare products are among the most popular cosmetics on the market (Figure 1.2) [1].



Source [1]

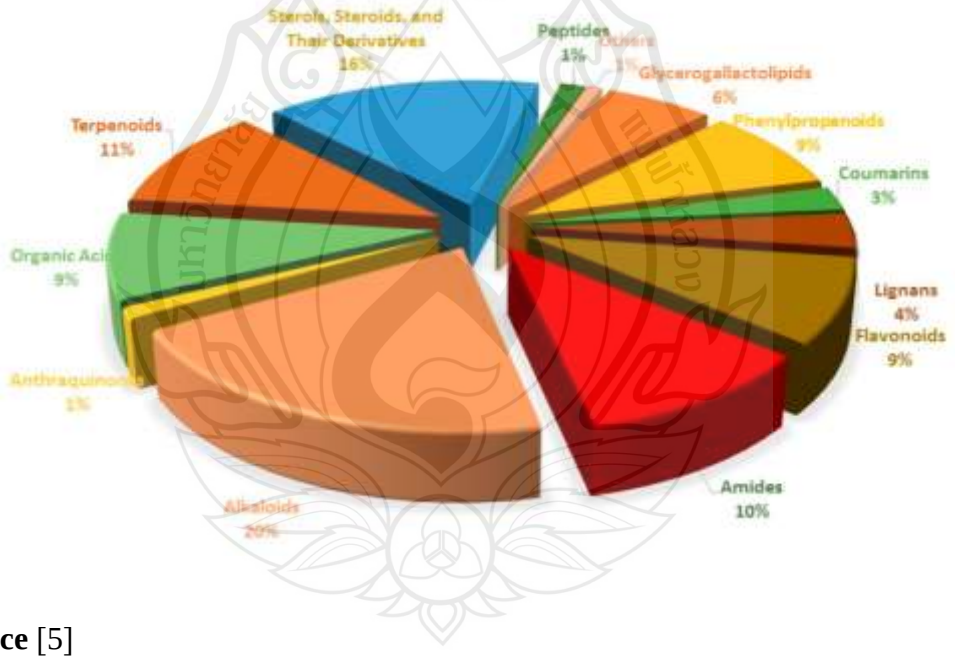
Figure 1.1 Global market value for natural cosmetics from 2018 to 2027



Source [1]

Figure 1.2 The popular cosmetics products in the market

Goji berries is one of the most popular natural extract in cosmetics. Goji berries, also known as *Gouqizi* in Chinese, are the generic names for fruit of *Lycium* genus [2]. The genus *Lycium,* belong to the Solanaceae family, is widely 80 species used as food and/or medicine worldwide included with South America, North America, Africa, and Eurasia [3]. Only three species (*L. barbarum, L. chinense*, and *L. ruthenicum*) have been utilized as medicine and nutritional supplements, particularly in China [4]. Antioxidant, anti-aging, skincare, antidiabetic, anti-fatigue, immunomodulation, neuroprotective, hepatoprotective, retinal protection, anti-microbial, and anti-tumor properties of goji berries have been documented of pharmacological activities [3, 5]. Glycerogalactolipids, amides, anthraquinones, coumarins, phenylpropanoids, flavonoids, alkaloids, organic acids, terpenoids, sterols, and steroids have been identified in the genus *Lycium* (Figure 1.3) [5].



Source [5]

Figure 1.3 Chemical constituents in genus *Lycium*

Meanwhile, *L. ruthenicum* is employed in Chinese health-care goods and has a greater function value than *L. barbarum* because it contains more antioxidants, polyphenols, and polysaccharides [6-8]. In China, *L. ruthenicum* Murr. is widely

used for medications and dietary supplements [2, 5]. This plant, known as the "black goji berry", is a thorny perennial shrub native to Northwestern China [9]. The fruit of this plant was used for traditional medicine in Tibetan medical classics *Jing Zhu Ben Cao* and *Si Bu Yi Dian* to treat hypotension, diabetes, abnormal menstruation, heart disease, and menopause [4, 9-13]. Goji berries have been demonstrated biological activities including immunomodulatory, anti-tumor, anti-aging, antioxidant, anti-fatigue, anti-cardiovascular disease, and hepatoprotective properties [12-14]. Polysaccharides, anthocyanin, alkaloids, amides, flavonoids, and organic acids have been shown in the fruit of *L. ruthenicum* [13-15]. Chemical constituents as the bioactive compounds in this plant are spermidine alkaloids, a type of alkaloid group acylated hydroxycinnamic acid amides [13,15-16]. These secondary metabolites compounds in plants are connected with the caffeoyl group and spermidine or spermine via amide bonds (Figure 1.4) [17-19]. The chemicals in this group are often identified using the liquid chromatography-mass spectrometry (LC-MS) technique. Characteristic fragments ion at *m/z* 310/308, 293/291, 222/220 and/or 165/163 are mainly found in spermidines alkaloids (Figure 1.5) [20].

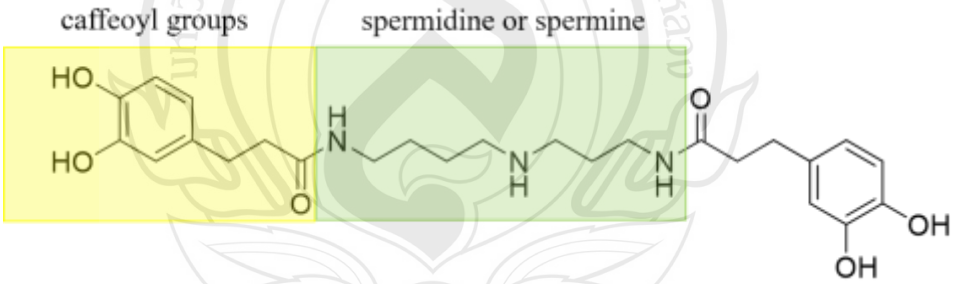


Figure 1.4 Structure of spermidine alkaloid compound

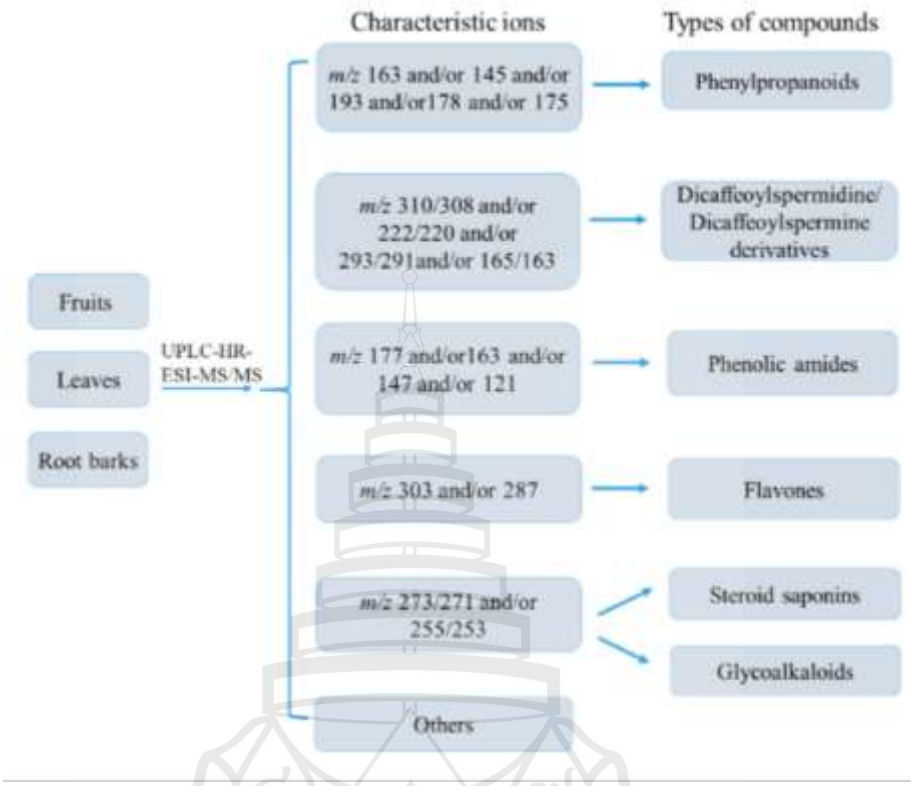


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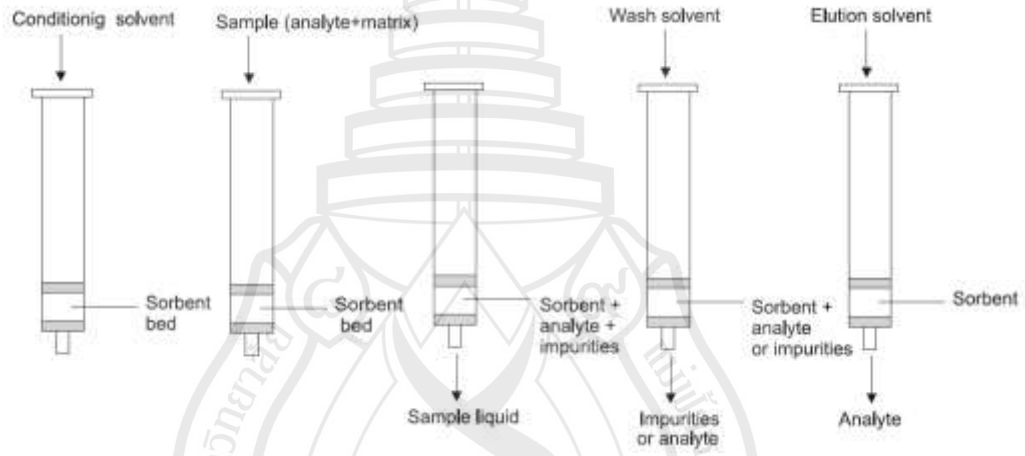
Figure 1.5 Characteristic fragments ion of spermidine alkaloid compound

1.1.1 Microwave-assisted Extraction

Microwave-assisted extraction (MAE) is an environmentally friendly technique known as "green extraction". MAE is the technique extraction of natural products by absorption of microwave energy through heating the water or alcohol as a solvent, hence accelerate the solvent dissipation into the material [21]. The advantages of MAE technique are more rapid, less solvent, more compact produces, and energy consumption compared to conventional extraction processes [22, 23]. This method can extract bioactive compounds, including phenolic, flavonoid, and antioxidant compounds, that have the rich electron-donating ability of crude extract [24]. Therefore, the MAE process has been used to extract antioxidants or bioactive compounds from natural products.

1.1.2 Solid Phase Extraction

Solid phase extraction (SPE) is the most widely used technique for preparing samples quickly and selectively [25]. Variable sorbents provide different selectivity to the targets, which is important in the vast range of SPE applications [15]. SPE processes are used to extract traces of organic compounds from environmental samples as well as to remove interfering components of complex matrices, resulting in a cleaner extract containing the analyses of interest [25]. The steps of the solid phase extraction process are shown in Figure 1.6.



Source [25]

Figure 1.6 Solid phase extraction steps

1.1.3 Antioxidants

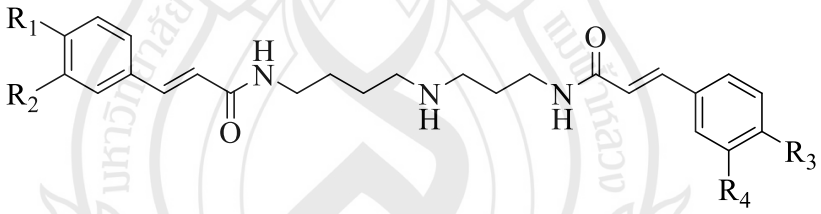
Antioxidants are molecules or compounds that can be inhibitors in oxidation processes [26]. They can protect cells from the damage caused by unstable molecules or free radicals [27-28]. An effect from free radicals can occur in the human body or from outside factors such as exposure to X-rays, ultraviolet light, air pollutants, smoking, alcohol, industrial chemicals, and cooking (burned meat, fried food) [29-30]. Free radicals can produce cardiovascular disease, inflammatory disease, diabetes mellitus, neurodegenerative diseases, cancer, cataract, asthma, autism, and rheumatoid

arthritis [30-32]. However, many foods or plants contain chemical constituents for eliminating free radical scavenging molecules that have hydroxyl group and a double bond, e.g., phenolic compound (phenolic acid, etc.) nitrogen compounds (alkaloids, amines, etc.) which enhance antioxidants [32-33].

From literature review of *L. ruthenicum,* the bioactive compounds in this family are spermidine alkaloids. Therefore, the study interested bioactive compounds by microwave-assisted extraction, solid phase extraction and improving cosmetic product from fruits *L. ruthenicum*.

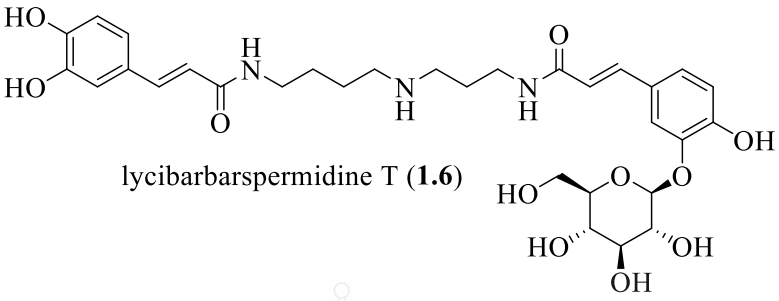
1.2 Literature Review

In 2021, Chen et al. reported nine alkaloids (**1.1**-**1.9**) (Figure 1.7) from fruit of *L. barbarum*. All compound expects **1.1** showed suppress NO production in RAW 264.7 cells stimulated by lipopolysaccharide (LPS) [34].

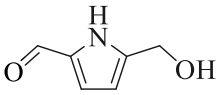


lycibarbarspermidine A (**1.1**): R_1 = β-D-glu, R_2 = OH, R_3 = OH, R_4 = OH
lycibarbarspermidine B (**1.2**): R_1 = OH, R_2 = OH, R_3 = OH, R_4 = β-D-glu
lycibarbarspermidine D (**1.3**): R_1 = OH, R_2 = OH, R_3 = β-D-glu, R_4 = OH
lyciamarspermidine C (**1.4**): R_1 = β-D-glu, R_2 = OH, R_3 = β-D-glu, R_4 = OH
scotanamine D (**1.5**): R_1 = OH, R_2 = OH, R_3 = O, R_4 = OH

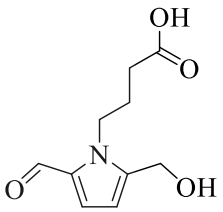
Figure 1.7 The isolated compounds from fruit of *L. Barbarum*



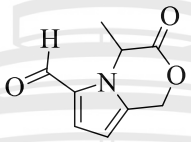
lycibarbarspermidine T (**1.6**)



5-hydroxymethyl-pyrrole-2-carbaldehyde (**1.7**)



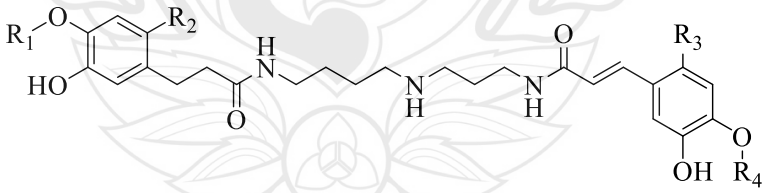
5-hydroxymethyl-pyrrole-2-carbaldehyde (**1.8**)



2-(5-hydroxymethyl-2-formylpyrrol-1-yl)propionic acid lactone (**1.9**)

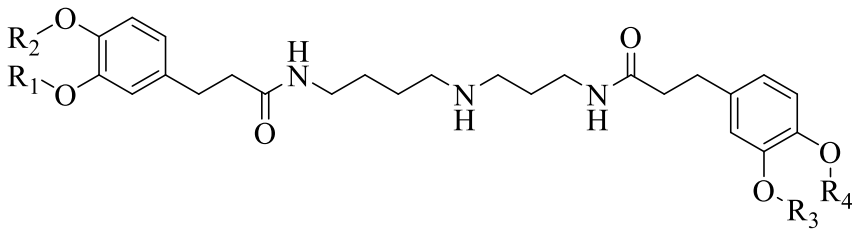
Figure 1.7 (continued)

In 2020, Qian et al. reported seven spermine or spermidine alkaloids (**1.1** and **1.10**-**1.15**) (Figure 1.8) from bitter goji (*L. barbarum*) [2].

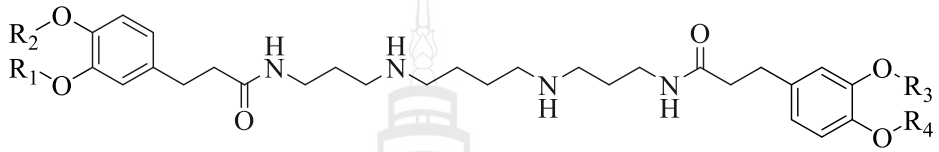


lycibarbarspermidine A (**1.1**): R_1 = H, R_2 = H, R_3 = H, R_4 = β-D-Glu
lyciamarspermidine A (**1.10**): R_1 = β-D-Glu, R_2 = H, R_3 = SO_3H, R_4 = H
lyciamarspermidine B (**1.11**): R_1 = H, R_2 = SO_3H, R_3 = H, R_4 = β-D-Glu
lyciamarspermidine C (**1.12**): R_1 = β-D-Glu, R_2 = H, R_3 = H, R_4 = β-D-Glu

Figure 1.8 The isolated compounds from bitter goji (*L. barbarum*)



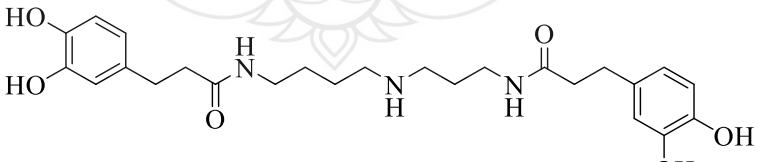
lycibarbarspermidine L (**1.13**): R_1 = H, R_2 = β-D-Glu, R_3 = H, R_4 = β-D-Glu



lyciamarspermine A (**1.14**): R_1 = H, R_2 = β-D-Glu, R_3 = H, R_4 = β-D-Glu
lyciamarspermine B (**1.15**): R_1 = β-D-Glu, R_2 = β-D-Glu, R_3 = β-D-Glu, R_4 = β-D-Glu

Figure 1.8 (continued)

In 2019, Ahad et al. reported four spermidine alkaloids (Figure 1.9), including N^1, N^3- *bis*(dihydrocaffeoyl) spermidine (**1. 16**), N^3- dihydrocaffeoyl- N^1- caffeoyl- spermidine (**1. 17**), N^1- caffeoyl- N^3- dihydrocaffeoyl- spermidine (**1. 18**), and N^1, N^3- *bis*(caffeoyl) spermidine (**1. 19**) from fruit of *L. ruthenicum*. Four structures of spermidine used as standard for identification of chemical profiling spermidines in goji berry by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). The key characteristic for this group of spermidine alkaloids was obtained of fragment ion at *m*/*z* 222 and 220. A total of 41 out of 51 spermidines were approximately identified [15].



N^1, N^3-*bis*(dihydrocaffeoyl)spermidine (**1.16**)

Figure 1.9 The isolated compounds from *L. ruthenicum*

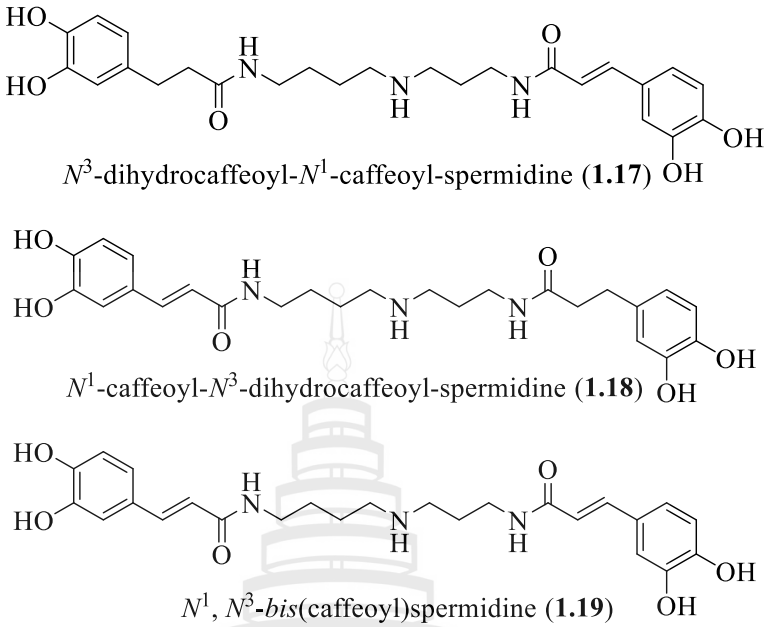


Figure 1.9 (continued)

In 2019, Xiao et al. reported seven compounds (Figure 1.10) including chlorogenic acid (**1.20**), scopolin (**1.21**), *N-p-trans*-coumaroyl tyramine (**1.22**), kukoamine B (**1.23**), rutin (**1.24**), solasonine (**1.25**), and 5,6-dihydrosolasonine (**1.26**), as standard compounds in different groups. A total of 131 compounds were identified by ultra-high performance liquid chromatography coupled with high resolution mass spectrometry (UPLC-HR-MS) in fruits, root barks, and leaves of *L. barbarum*. Spermidine alkaloids were found large amounts of compounds (74/131) and key characteristic fragment ion at *m/z* 310/308, 293/291, 222/220, or 165/163. The root barks extract exhibited potent antioxidant activity and cytotoxicity [20].

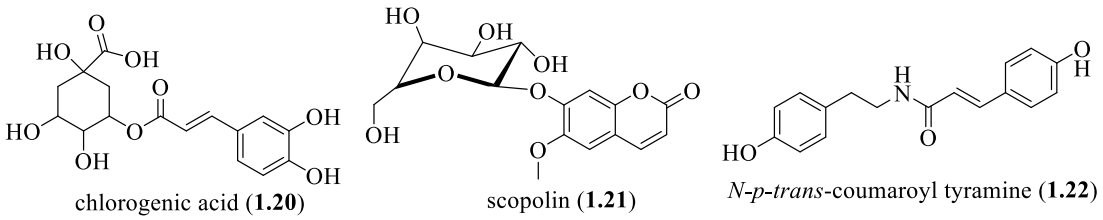


Figure 1.10 The standard compounds in analysis for the extracts of *L. barbarum*

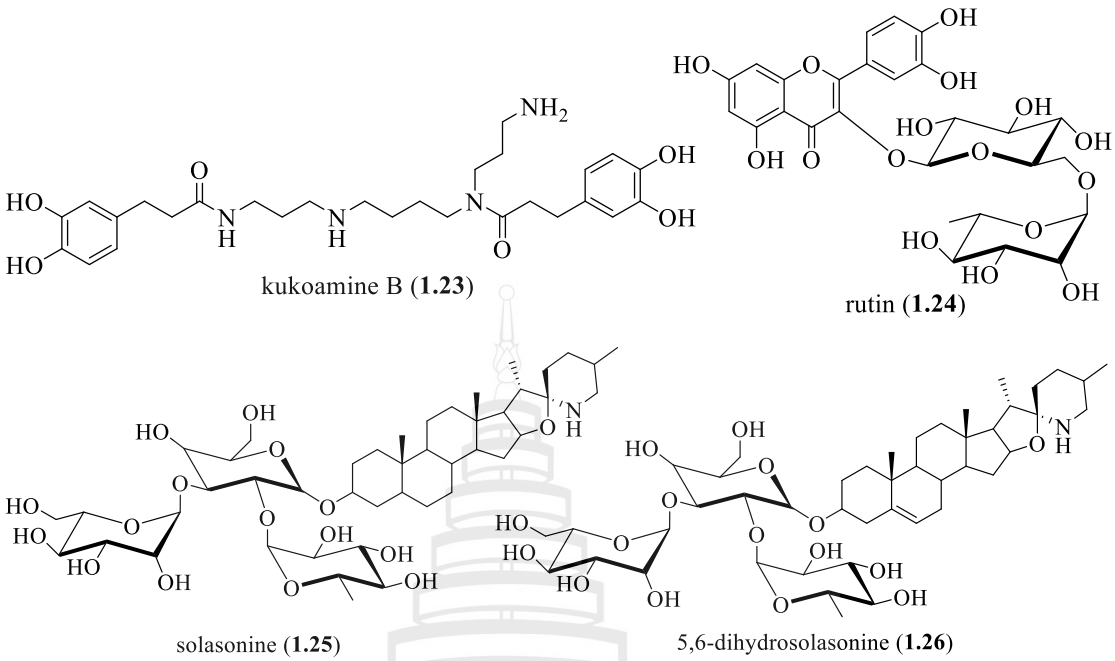


Figure 1.10 (continued)

In 2018, Qi et al. isolated a novel flavonoid glucoside (**1.27**) and eight known compounds (**1.18, 1.22, 1.28-1.33**) (Figure 1.11) from fruit of *L. ruthenicum*. Compounds **1.18** and **1.28** showed inhibitory activity against SIRT1 (a nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase) [35].

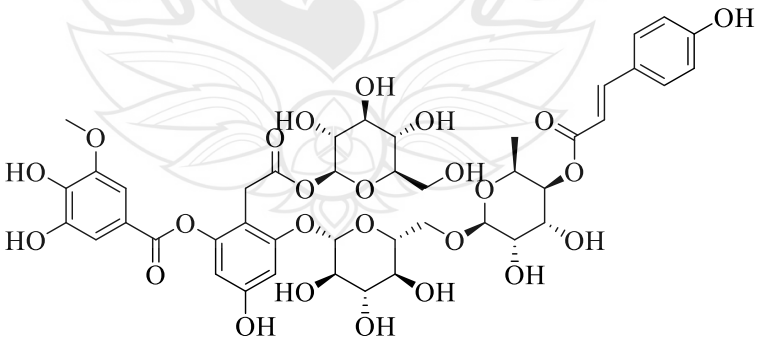
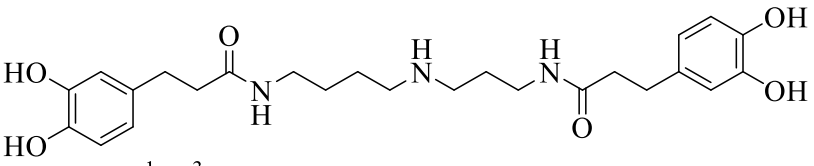
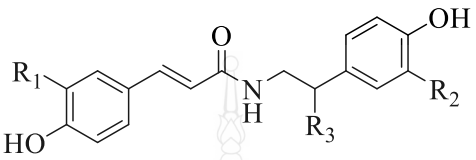


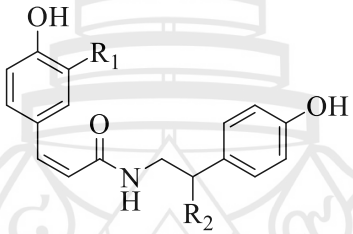
Figure 1.11 The isolated compounds from fruit of *L. ruthenicum*



N^1, N^3-*bis*(dihydrocaffeoyl)spermidine (**1.18**)



N-trans-feruloyltyramine (**1.22**): R_1 =OCH_3, R_2 = R_3 = H
N-trans-coumaroyltyramine (**1.28**): R_1 = R_2 = R_3 = H
N-trans-feruloyl 3'-*O*-methyldopamine (**1.29**): R_1 = R_2 = OCH_3, R_3 = H
N-trans-feruloyloctopamine (**1.30**): R_1 = OCH_3, R_2 = H, R_3 = OH



N-cis-coumaroyltyramine (**1.31**): R_1 = R_2 = H
N-cis-feruloyltyramine (**1.32**): R_1 =OCH_3, R_2 = H
N-cis-feruloyloctopamine (**1.33**): R_1 = OCH_3, R_2 = OH

Figure 1.11 (continued)

In 2017, Nzeuwa et al. identified 26 phenolic compounds by high-performance liquid chromatography fingerprinting combined with quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS) in fruit of *L. ruthenicum*. Standard compounds used to confirm structure (Figure 1.12) including chlorogenic acid (**1.20**), rutin (**1.24**), 1,3-dicaffeoylquinic acid (**1.34**), and kukoamine A (**1.35**) [12].

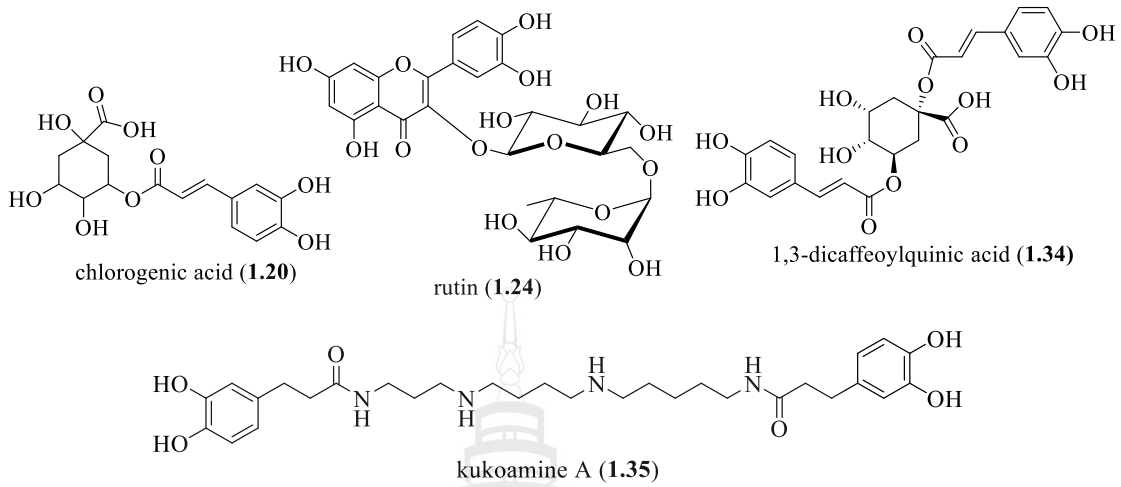
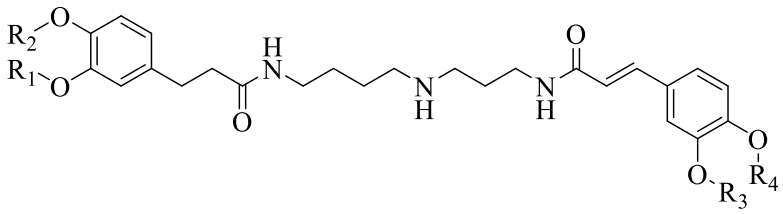


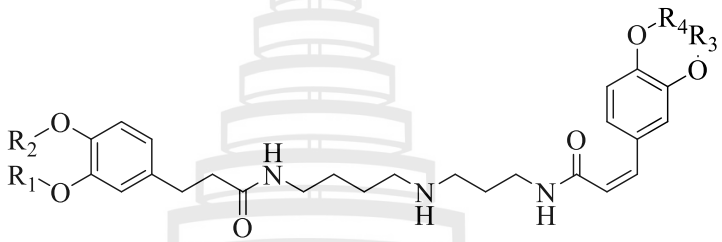
Figure 1.12 Standard compounds used to confirm structure in fruit of *L. ruthenicum*

In 2016, Wu et al. identified 26 polyphenol compounds by ultra-performance liquid chromatography coupled with Q-TOF high-resolution mass spectrometry methods along with MSE data acquisition (UPLC-Q-TOF-MSE) in extract of *L. ruthenium* fruit. One of compounds is found as hydroxylated aromatic acids (HAAs) or spermidine. The extract showed protection to Caco-2 cells against H_2O_2-inducde oxidative damage based on microscopic fluorometic imaging [9].

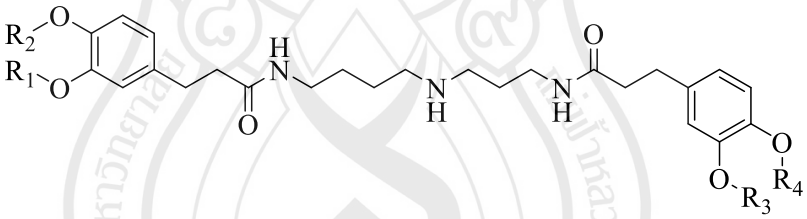
In 2016, Zhou et al. reported new nineteen dicaffeoylspermidine derivatives (Figure 1.13) combined with lycibarbarspermidines A−S (**1.1**-**1.3**, **1.13**, **1.36**-**1.50**) in fruit of *L. barbarum*. All compounds showed different level in Alzheimer's disease activity for short-term memory capacity. Compounds **1.3**, **1.37**, and **1.39** exhibited the strongest oxygen radical absorbance capacity (ORAC) assay [16, 18].



lycibarbarspermidine A (**1.1**): R_1 = H, R_2 = β-D-Glu, R_3 = H, R_4 = H
lycibarbarspermidine B (**1.2**): R_1 = H, R_2 = H, R_3 = β-D-Glu, R_4 = H
lycibarbarspermidine C (**1.36**): R_1 = β-D-Glu, R_2 = H, R_3 = H, R_4 = H
lycibarbarspermidine D (**1.3**): R_1 = H, R_2 = H, R_3 = H, R_4 = β-D-Glu
lycibarbarspermidine E (**1.37**): R_1 = H, R_2 = β-D-Glu, R_3 = β-D-Glu, R_4 = H
lycibarbarspermidine F (**1.38**): R_1 = β-D-Glu, R_2 = H, R_3 = β-D-Glu, R_4 = H

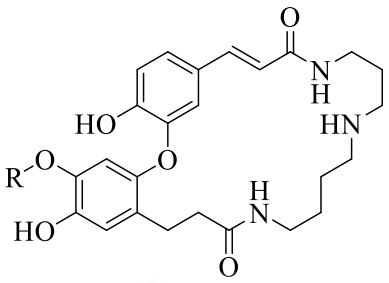


lycibarbarspermidine G (**1.39**): R_1 = β-D-Glu, R_2 = H, R_3 = β-D-Glu, R_4 = H

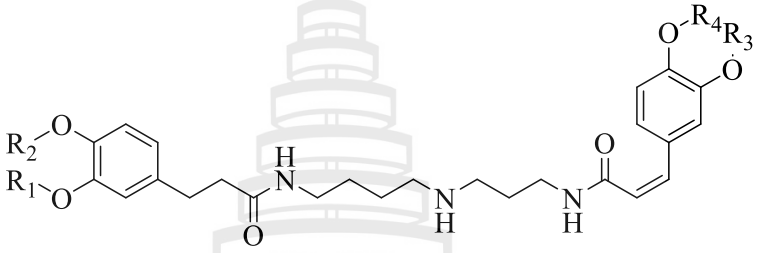


lycibarbarspermidine H (**1.40**): R_1 = H, R_2 = H, R_3 = H, R_4 = β-D-Glu
lycibarbarspermidine I (**1.41**): R_1 = H, R_2 = β-D-Glu, R_3 = H, R_4 = H
lycibarbarspermidine J (**1.42**): R_1 = H, R_2 = H, R_3 = β-D-Glu, R_4 = H
lycibarbarspermidine K (**1.43**): R_1 = β-D-Glu, R_2 = H, R_3 = β-D-Glu, R_4 = H
lycibarbarspermidine L (**1.13**): R_1 = H, R_2 = β-D-Glu, R_3 = H, R_4 = β-D-Glu
lycibarbarspermidine M (**1.44**):R_1 = β-D-Glu, R_2 = H, R_3 = H, R_4 = β-D-Glu

Figure 1.13 The isolated compounds from fruit of *L. barbarum*



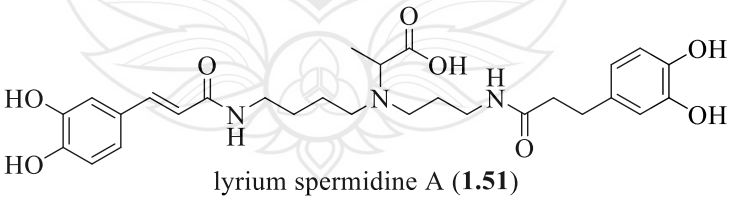
lycibarbarspermidine N (**1.45**): R = β-D-Glu,
lycibarbarspermidine O (**1.46**): R = β-D-Glu-β-D-Glu



lycibarbarspermidine P (**1.47**): R_1 = H, R_2 = H, R_3 = β-D-Glu, R_4 = H
lycibarbarspermidine Q (**1.48**): R_1 = H, R_2 = H, R_3 = H, R_4 = β-D-Glu
lycibarbarspermidine R (**1.49**): R_1 = H, R_2 = β-D-Glu, R_3 = H, R_4 = H
lycibarbarspermidine S (**1.50**): R_1 = H, R_2 = β-D-Glu, R_3 = H, R_4 = β-D-Glu

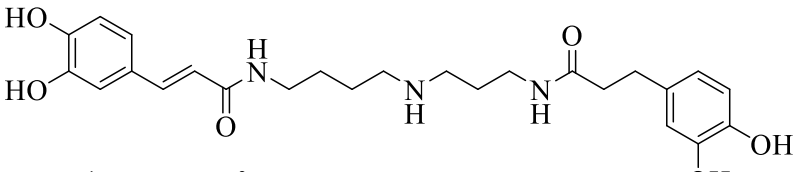
Figure 1.13 (continued)

In 2014, Zhao et al. isolated a new spermidine (**1.51**) and ten known compounds (**1.18, 1.52-1.60**) (Figure 1.14) from fruit of *L. ruthenicum* [36].

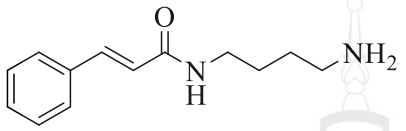


lyrium spermidine A (**1.51**)

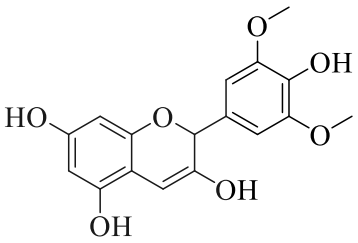
Figure 1.14 The isolated compounds from fruit of *L. ruthenicum*



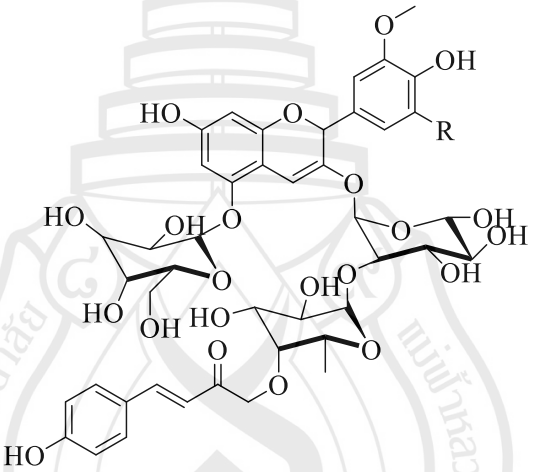
N^1-caffeoyl-N^3-dihydrocaffeoyl-spermidine (**1.18**)



N-monocinnamoylputrescine (**1.52**)

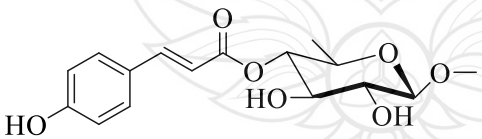


malvidin (**1.53**)

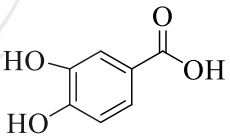


peonidin 3-*O*-[6-*O*-(4-*O*-*E*-*p*-coumaroyl-*O*-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside (**1.54**): R =H

petunidin 3-*O*-[6-*O*-(4-*O*-*E*-*p*-coumaroyl-*O*-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside (**1.55**): R = OH

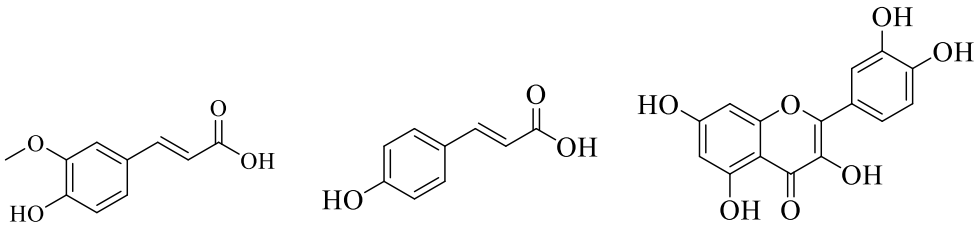


4-(*p*-cumaroyl)-methyl-*L*-rhamnoside (**1.56**)



protocatechuic acid (**1.57**)

Figure 1.14 (continued)



ferulic acid (**1.58**) *p*-coumarinic acid (**1.59**) quercetin (**1.60**)

Figure 1.14 (continued)

1.3 Research Objective

The aims of this study were to establish chemical profiling of spermidine alkaloids from *L. ruthenicum* by HPLC-ESI-QTOF-MS/MS coupled with microwave-assisted extraction (MAE) and solid-phase extraction (SPE) methods, along with their antioxidant properties and to find greatest value antioxidant activities of *L. ruthenicum* for formulate goji serum.

CHAPTER 2

CHEMICAL CONSTITUENTS PROFILE OF SPERMIDINE ALKALODIS IN FRUIT OF *L. ruthenicum*

2.1 Materials and Methods

2.1.1 Raw Materials

The dried fruit of *Lycium ruthenicum* (Brown color) was purchased from China in May 2018.

2.1.2 Reagents and Chemicals

HPLC-grade acetonitrile, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid, (+)-glucose, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium persulphate ($K_2S_2O_8$) was purchased from Ajax Finechem (Australia). Dimethyl sulfoxide (DMSO) and phenol were purchased from Fisher Scientific (Pennsylvania, USA). Concentrated sulfuric acid (H_2SO_4) was purchased from RCI Labscan (Bangkok, Thailand). Water for LC-MS mobile phase was reverse osmosis Milli-Q water (Millipore, USA). AR-grade ethanol and methanol were purchased from Merck (Darmstadt, Germany).

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were purchased from Hyclone (USA). Penicillin-Streptomycin was purchased from Gibco (USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Invitrogen (USA). Griess reagent, Lipopolysaccharide (LPS), and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.3 Sample Preparation

The dried fruit of *L. ruthenicum* (5.0 g) were ground and further extracted under a microwave-assisted extractor (ETHOS X, Italy) with 50% methanol/water (1:10, w:v). In brief, the program was performed at 1000 W for 5 min, extraction temperature set as 85 °C to give crude extract (1.64 g). The solvent was then removed under reduce pressure, and residue afforded a crude extract after lyophilization. The crude extract was further separated by solid-phase extraction (SPE, 10 g C_{18} cartridge, Water™, Milford, MA, USA) in a portion of 5 g dissolved in 2 mL of distilled water. Two fractions were collected in each separation eluented with 200 mL of distilled water and ethanol to obtain F1 (1.4 g) and F2 (190 mg), respectively. The solvents of each fraction were removed under reduce pressure distillation, and the residues were dissolved in distilled water and freeze-dried. HPLC-ESI-QTOF-MS/MS analysis (1 mg of the sample) was dissolved with methanol and filtered with a 0.22 mm filter membrane.

2.1.4 Chromatographic Condition

Agilent 1290 Infinity LC instrument (Agilent, USA) connected to an Agilent 6540 series Q-TOF-MS (Agilent, USA) equipped with an ESI source, a diode-array detector (DAD), an automatic sample injector, a degasser, and a column Agilent ZORBAX Eclipse XDB column (100 × 2.1 mm i.d.; particle size 1.7 μm; Palo Alto, CA, USA). HPLC-ESI-QTOF-MS analysis was maintained at 35 °C was finally chosen for separation of these extracts. At a flow rate of 200 μL/min, the mobile phase was water (0.1% formic acid, A) mixed with acetonitrile (B). The elution gradient mode was following: 0-5 min, 5% to 17% B; 5-6 min, 17% B, 6-30 min, 17% to 100% B, 30-35 min, 100% B; 35-40 min, 100% to 5% B; 40-45 min, 5% B. The injection volume was 1.0 μL and samples were set at 4 °C.

Both negative and positive ion modes were utilized to confirm fragment ions in MS/MS data by energy collision dissociation within the *m/z* ratio range of 50–1000 at a resolution of 4,000 to establish chemical profile (HCD: 40, 20, and 10 eV). Gas temperature 350 °C, drying gas flow rate 12 L min^{-1}, nebulizer gas pursuer 45 psi, sheath gas temperature 250 °C, and sheath gas flow rate 12 Arb were the other characteristics. Agilent mass Hunter workstation software B.08.00 was used analysis components for LC-MS/MS control and data handling.

2.1.5 DPPH Radical Scavenging Assay

The plant extract was tested for free radical scavenging activities using a modified methodology [36]. The hydrogen atom donating ability of the extract to react with a solution of 2,2-diphenyl-1-picrylhydrazyl was used to determine the DPPH radical scavenging activity (DPPH). In the presence of antioxidants, DPPH showed a violet purple color in an ethanol solution. A solution of 60 μM DPPH radical solution in ethanol (100 μL) was mixed with 100 μL of extract in ethanol at different concentrations. The reaction was incubated for 30 min at room temperature in the dark, and the absorbance was measured at 517 nm (TECAN, infinite 200 PRO). Ascorbic acid was used as a positive control. The percentage of inhibitory concentration (IC_{50}) of crude extract was calculated by plotting inhibition percentages against the concentrations of extracts. Percentage DPPH radical scavenging activity was calculated as follow:

$$\% \text{ DPPH radical scavenging activity} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of DPPH radical solution, and A_1 is the absorbance of DPPH radical solution for the extractives/standard. The experiment was done in triplicate.

2.1.6 ABTS Radical Scavenging Assay

The ABTS radical scavenging assay of samples was evaluated by modification [37]. The radical cations were prepared by mix 7 mM aqueous ABTS with 2.45 mM potassium persulphate (1:1) in the dark at room temperature for 16-18 h before use. Dilute $ABTS^{\circ+}$ solution was mixed with distilled water to an absorbance of 0.700±0.020 at 734 nm. The reaction of $ABTS^{\circ+}$ solution (160 μL) was added to 40 μL of sample in 50% ethanol at different concentrations, and the absorbance was recorded after 5 min at 734 nm (TECAN, infinite 200 PRO). Ascorbic acid was used as a standard substance. The percentage inhibition and IC_{50} were calculated using the formula:

$$\% \text{ ABTS radical scavenging assay} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of $ABTS^{\bullet+}$ solution, and A_1 is the absorbance of $ABTS^{\bullet+}$ solution for tested samples/standard. All the measurements were performed in triplicates.

2.1.7 Total Polysaccharide Content

Previous studies [38] were used to determine the total polysaccharide content. In distilled water, a sample and glucose dissolve solution was made. 200 μL of 100 μg/mL material was mixed with 200 μL of 5% phenol solution and 1 mL of concentrated H_2SO_4 for the reaction. The absorbance was measured for 10 minutes at 488 nm (TECAN, endless 200 PRO) and glucose standards (100-10 μg/mL) was used to calculated the calibration curve.

2.1.8 Nitric Oxide Production Inhibitory Assay

The nitric oxide production inhibitory assay was previously described using Griess reagent [39-40]. RAW 264.7 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin at 37 °C under 5% CO_2. Cells (4×10^3 cell/mL) were cultured in 96 well-plate for 24 h. After the incubation period, cells were stimulated with 1 μg/mL of lipopolysaccharide (LPS) in the presence or absence of the samples at 37 °C under 5% CO_2 for 24 hr. The supernatant (50 μL) was transferred to a new plate and added 50 μL of Griess reagent. Indomethacin was used as a positive control. The samples were detected at 570 nm using a microplate reader (TECAN, infinite 200 PRO). The percentage of nitric oxide inhibition activity was calculated from the formula:

$$\% \text{ NO} = [(A_{LPS}/A_{sample})/ (A_{LPS}/A_{control})] \times 100$$

A_{LPS} Abs of LPS-treated cells

A_{sample} Abs of sample-treated cells

$A_{control}$ Abs of untreated cells

2.1.9 Cell Viability Assay

The cell viability was used to determine by MTT assay, as previously described [25]. RAW 264.7 cells were seeded at 4×10^3 cell/mL in DMEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin at 37°C under 5% CO_2. Cells were treated with samples at 37°C under 5% CO_2. The cells were added 0.5 mg/mL of MTT and detected at 570 nm using a microplate reader. Indomethacin was used as a positive control. The percentage of cell viability was calculated from the formula:

$$\% \text{ cell viability} = (A_{sample}/A_{control}) \times 100$$

A_{sample} Abs of sample-treated cells

$A_{control}$ Abs of untreated cells

2.1.10 Statistical Analysis

Significant differences were analyzed using Graph-Pad Prism (GraphPad Software Inc.) by one-way ANOVA. The level of statistical significance was $p < 0.05$. Agilent mass Hunter workstation software version B.08.00 (Agilent Technologies, Palo Alto, CA, USA) was used analysis components. Parameters for the program were using compound discovery workflow, molecular feature function, *m/z* range 50–1000, retention time (RT) range 0.1–2 min, with a minimum absolute abundance of 1000 counts. For identification of the compounds was performed by comparing specific fragments from databases including Mass Hunter PCDL Manager (Version B.08.00), Pubmed, Chemspider, MassBank, and literature data [12, 15, 16].

2.2 Result and Discussion

2.2.1 Multi-component Analysis of Extracts by HPLC-ESI-QTOF-MS/MS

The extraction of *L. ruthenicum* fruit was analyzed chemical constituents profile by comparison mass fragment in negative and positive ions. The MS conditions were optimized sensitivity for analysis components that key parameter is collision energies. CID represented an attractive approach for selectively yielding fragment ions. Different

collision energies 10, 20, and 40 eV was tested for extraction. Abundant fragmentation ions were generated in the MS/MS spectra when the collision energy was raised to 40 eV. The accuracy of the assigned chemical formula was determined using a mass difference tolerance of ± 5 ppm, which was calculated by the difference between the calculated mass and experimental mass [41]. The results in the MS/MS spectra showed that dicaffeoylspermidine derivatives (spermidine alkaloids) were the most detected compounds in positive ion mode. According to the fragment ions and fragmentation analysis, some of the peaks in ethanol fraction (Figure 2.1) were identified as spermidine alkaloids. The detailed information of the 32 compounds found in fruit of *L. ruthenicum* is performed in Table 2.1.

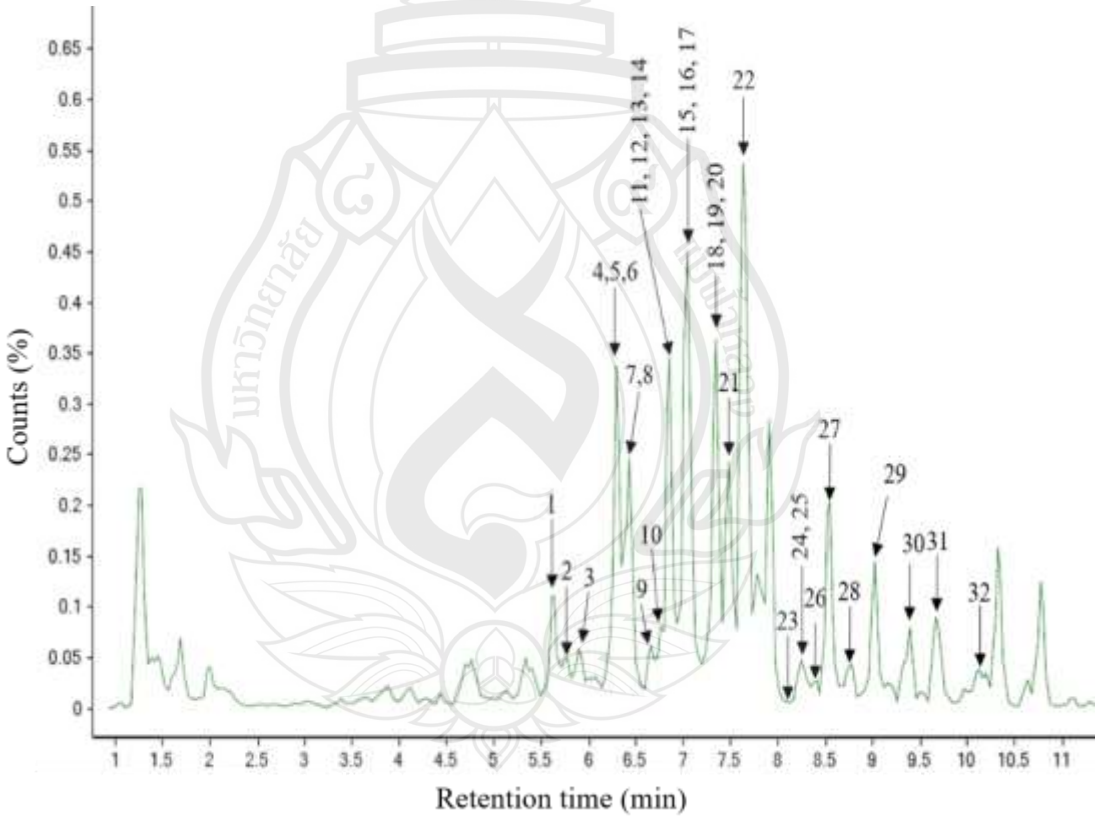


Figure 2.1 Chromatogram of ethanolic fraction from *L. ruthenicum* (positive ion)

Table 2.1 Compound identification from ethanolic fraction of *L. ruthenicum* fruits by HPLC-QTOF-MS/MS (positive ion)

No	RT[1]	Formula	$[M + H]^+$	Mass error (ppm)	MS/MS fragment ion[2]	Identification[3]
1	5.724	$C_{37}H_{53}O_{16}N_3$	796.3464	1.10	**634.2908**; 472.2582; 310.2221; 220.0947; 163.0371	N^1-caffeoyl-N^3-dhc-spermidine-di-hex
2	5.769	$C_{37}H_{55}O_{16}N_3$	798.3640	0.31	**636.3101**; 474.2568; 384.1652; 236.1263; 222.1131; 100.7360	N^1-N^3-bis-dhc-spermidine-di-hex
3	5.910	$C_{37}H_{53}O_{16}N_3$	796.3507	-0.37	**634.2981**; 472.2686; 384.1626; 310.2120; 293.1858; 234.1111; 222.1093; 163.0380	N^1-caffeoyl-N^3-dhc-spermidine-di-hex
4	6.223	$C_{25}H_{33}O_7N_3$	488.2395	0.35	308.1972; 234.1118; 220.0969; 185.5188; 172.1423; **163.0392**; 72.0811	dihydrocaffeoyl spermidine derivative
5	6.244	$C_{31}H_{43}O_{11}N_3$	634.2962	-0.17	472.2426; 310.2102; 236.1267; **220.0966**; 163.0388	N^1-caffeoyl-N^3-dhc-spermidine-hex
6	6.325	$C_{31}H_{45}O_{11}N_3$	636.3120	0.77	474.2595; 310.2124; 293.1872; **222.1126**; 165.0550; 123.0430; 100.0752; 72.0808	N^1-N^3-dhc-spermidine-hex
7	6.405	$C_{16}H_{18}O_9$	355.1024	-0.03	**163.0392**; 145.0286; 135.0444; 117.0343	chlorogenic acid
8	6.430	$C_{31}H_{43}O_{11}N_3$	634.2975	-1.49	474.2620; 310.2123; 293.1919; 236.1254; **222.1111**; 165.0543; 72.0807	N^1-caffeoyl-N^3-dhc-spermidine-hex
9	6.587	$C_{31}H_{39}O_9N_3$	598.2749	0.91	472.2412; 454.2351; 336.1897; 310.2142; 234.1110; **222.1126**; 163.0383	dihydrocaffeoyl spermidine derivative
10	6.650	$C_{31}H_{43}O_{11}N_3$	634.2972	-0.03	472.2510; 310.2117; 293.1880; 236.1271; **220.0966**; 163.0392; 123.0469	N^1-caffeoyl-N^3-dhc-spermidine-hex
11	6.826	$C_{31}H_{41}O_{11}N_3$	632.2808	0.98	470.2297; 308.1952; 293.1784; 234.1130; **220.0936**; 163.0399	dhc-caffeoyl-cyclic-spermidine-hex

Table 2.1 (continued)

No	RT[1]	Formula	$[M + H]^+$	Mass error (ppm)	MS/MS fragment ion[2]	Identification[3]
12	6.923	$C_{31}H_{39}O_9N_3$	598.2741	-0.14	472.2412; 455.2176; 310.2093; 23.1122; **222.1118**; 220.0962; 163.0398	dihydrocaffeoyl spermidine derivative
13	6.930	$C_{31}H_{43}O_{11}N_3$	634.2967	0.07	472.2418; 384.1658; 310.2104; 293.1820; 234.11120; 222.1122; **163.0385**; 145.0281; 72.0807	N^1-caffeoyl-N^3-dhc-spermidine-hex
14	6.970	$C_{24}H_{25}O_7N_{11}$	580.2000	-1.54	455.1494; 293.0994; 275.0982; 231.1096; 147.0473; 129.0543; **126.0549**	dihydrocaffeoyl spermidine derivative
15	7.033	$C_{25}H_{35}O_6N_3$	474.2564	-0.51	310.2116; 293.1895; 236.1272; **222.1124**; 165.0546; 123.0444; 100.0754; 72.0811	N^1,N^3-*bis*(dhc)spermidine
16	7.202	$C_{25}H_{35}O_6N_3$	474.2593	1.25	310.2176; 236.1250; **222.1121**; 165.0543;123.0445; 100.0748	N^1,N^3-*bis*(dhc)spermidine
17	7.249	$C_{15}H_{22}O_3N_4$	307.1768	-0.95	177.0546; 149.0591; **145.0282**; 117.0332	feruloylagmatine
18	7.355	$C_{31}H_{43}O_{11}N_3$	634.2963	1.27	472.2441; 310.2118; 293.1853; 291.1679; 236.1271; 234.1094; **222.1105**; 220.1105; 163.0249; 123.0413	N^1-caffeoyl-N^3-dhc-spermidine-hex
19	7.434	$C_{26}H_{33}O_3N_{13}$	576.2906	-0.94	522.2633; 414.2371; 397.2071; 331.0507; 265.1436; 236.1205; **162.0912**; 147.0459	dihydrocaffeoyl spermidine derivative
20	7.465	$C_{25}H_{33}O_6N_3$	472.2442	-0.02	310.2131; 293.1835; 253.1521; 236.1269; 220.0961; **163.0386**; 145.0287; 72.0812	N^1-dhc-N^3-caffeoyl-spermidine
21	7.525	$C_{26}H_{35}O_6N_3$	486.2598	0.30	237.1310; 222.1114; 193.0527; 165.0540; 147.0452; 124.0460; **123.0432**	dihydrocaffeoyl spermidine derivative

Table 2.1 (continued)

No	RT[1]	Formula	$[M + H]^+$	Mass error (ppm)	MS/MS fragment ion[2]	Identification[3]
22	7.639	$C_{25}H_{33}O_6N_3$	472.2443	-0.02	293.1861; 234.1122; **222.1124**; 165.0542; 163.0390; 123.0440; 100.0750; 72.0821	N^1-caffeoyl-N^3-dhc-spermidine
23	8.193	$C_{24}H_{31}ON_3$	518.2848	-0.49	293.1792; **222.1116**; 173.1508; 163.0381; 84.0794; 72.0821	dihydrocaffeoyl spermidine derivative
24	8.242	$C_{25}H_{31}O_6N_3$	470.2289	-0.70	308.1954; 291.1713; 234.1112; 220.0963; **163.0392**; 145.0280; 72.0813	N^1,N^3-bis(caffeoyl)spermidine
25	8.291	$C_{26}H_{37}O_6N_3$	488.2733	0.29	293.1872; **236.1277**; 222.1133; 179.0701; 165.0548; 137.0593; 123.0436; 100.0753	dihydrocaffeoyl spermidine derivative
26	8.397	$C_{26}H_{33}O_6N_3$	484.2434	0.09	348.1928; 322.2118; 277.1550; 234.1127; **222.1116**; 163.0386; 84.0805	dihydrocaffeoyl spermidine derivative
27	8.566	$C_{25}H_{33}O_5N_3$	456.2478	1.05	293.1828; 222.1093; 204.1012; **147.0441**; 119.0506	N^1-dhc-N^{10}-cou-spermidine
28	8.777	$C_{25}H_{33}O_5N_3$	456.2486	-0.35	253.0648; **222.1151**; 165.0671; 147.0449; 123.0495; 100.0741; 84.0804	N^1-dhc-N^{10}-cou-spermidine
29	8.992	$C_{26}H_{35}O_6N_3$	486.2593	0.60	306.0398; 293.1834; 278.0463; 236.1246; 222.1116; **177.0545**; 165.0541; 145.0291; 123.0443	dihydrocaffeoyl spermidine derivative
30	9.170	$C_{26}H_{35}O_6N_3$	486.2592	1.62	293.1832; 278.0463; 236.1248; 222.1115; **177.0546**; 165.0540; 145.0288	dihydrocaffeoyl spermidine derivative

Table 2.1 (continued)

No	RT[1]	Formula	$[M + H]^+$	Mass error (ppm)	MS/MS fragment ion[2]	Identification[3]
31	9.580	$C_{26}H_{35}O_6N_3$	486.2590	1.85	469.2319; 336.1822; 293.1919; **222.1133**; 177.0528; 123.0433	dihydrocaffeoyl spermidine derivative
32	10.164	$C_{34}H_{46}O_8N_4$	639.3387	0.42	512.2678; 439.2713; 383.1984; 293.1861; 291.1703; 236.1265; **222.1115**; 219.1484; 191.0543; 165.0534; 150.0906; 123.0436	dihydrocaffeoyl spermidine derivative

Note [1] RT represent retention time (min), [2] Mass error (ppm) show chemical formula was determined using a mass difference tolerance of ± 5 ppm, [3] MS/MS fragment show positive ion of fragment of which the number in **bold** represent the most abundant product ion., Abbreviations: dhc = dihydrocaffeyl; hex = hexose; cou = *p*-coumaroyl

Compound **2** in RT 5.724 min contained a $[M+H]^+$ at *m/z* 798.3640 ($C_{37}H_{55}O_{16}N_3$, cal. 798.3655, mass error 0.31 ppm) and obtained of fragment ions at *m/z* 636.3101, 474.2568, 384.1652, 236.1263, 222.1130, and 100.7360 (Figure 2.2) [15, 20]. The ion was detected at *m/z* 636 by the loss hexose unit. After the loss of two glucosyl units, the one ion *m/z* 474 is conducted. The fragment ions *m/z* 310 and 163 were produced by the cleavage of the caffeoyl unit. The dihydrocaffeoyl unit was cleaved, yielding the fragment ions *m/z* 310 and 165. The ammonia unit was lost due to the ion at *m/z* 293. The cleavage of a rearrangement ion at *m/z* 72 produced the ion *m/z* 222. The one ion *m/z* 123 was caused after losing of molecule ion at *m/z* 100 from fragment ion *m/z* 222. Therefore, compound **2** was characterized as N^1-N^3-bis-dihydrocaffeyl-spermidine-di-hexose or lyciumbarbarspermidine M isomer (**2.1**) (Figure 2.3).

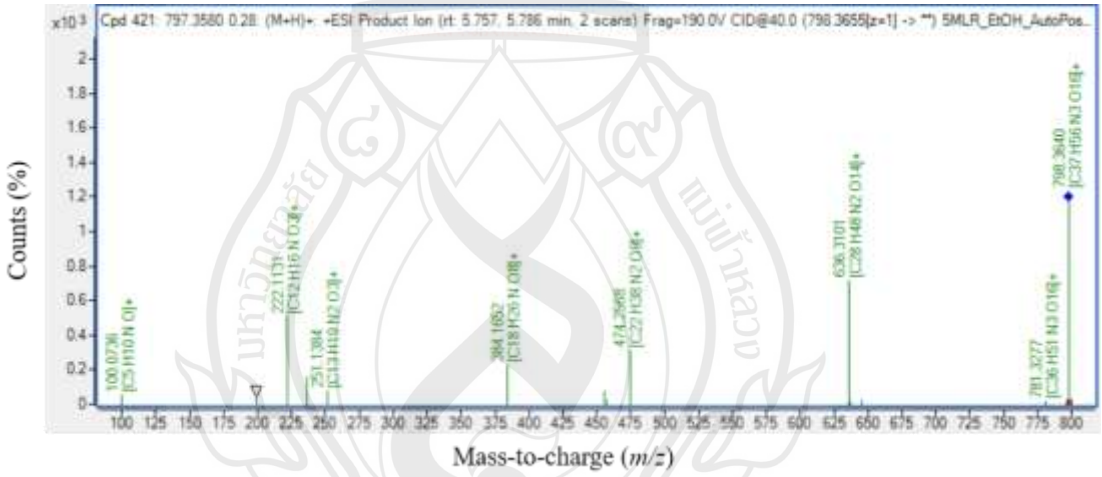
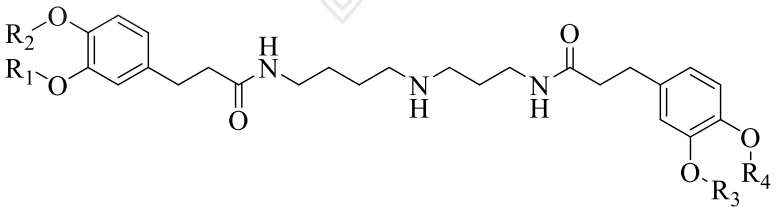


Figure 2.2 Mass spectrum fragment ions of compound **2**



lycibarbarspermidine M (**2.1**):R_1 = β-D-Glu, R_2 = H, R_3 = H, R_4 = β-D-Glu

Figure 2.3 Structure of lyciumbarbarspermidine M isomer

Compound **3** in RT 5.910 min had molecular ion $[M+H]^+$ at *m/z* 796.3507 ($C_{37}H_{53}O_{16}N_3$, cal. 796.3499, mass error -0.37 ppm) and fragment ions at *m/z* 634.2981, 472.2686, 384.1626, 310.2120, 293.1858, 234.1111, 222.1093, and 163.0380 (Figure 2.4) [15, 20]. The ion at *m/z* 634 $[M+H-C_6H_{10}O_5]^+$ and *m/z* 472 $[M+H-C_6H_{10}O_5-C_6H_{10}O_5]^+$ were performed by the loss of one and two hexose units, respectively. The fragment ion *m/z* 310 was formed by the cleavage of the caffeoyl unit (163 Da). The ion at *m/z* 293 was detected with loss ammonia unit from the ion at *m/z* 310. The ion at m/z 222 was presented by leaving a rearrangement ion at m/z 72 from molecule ion m/z 293. The ions at m/z 220 and 253 were produced from another pathway after the ion m/z 253 appeared m/z 234 by the loss of ammonia unit. This compound showed same fragment ion with compound **1**. Thus, compounds **1** and **3** were identified as N^{1}-caffeoyl-N^{3}-dihydrocaffeyl-spermidine-di-hexose or lyciumbarbarspermidine F isomer (**2.2**) (Figure 2.5).

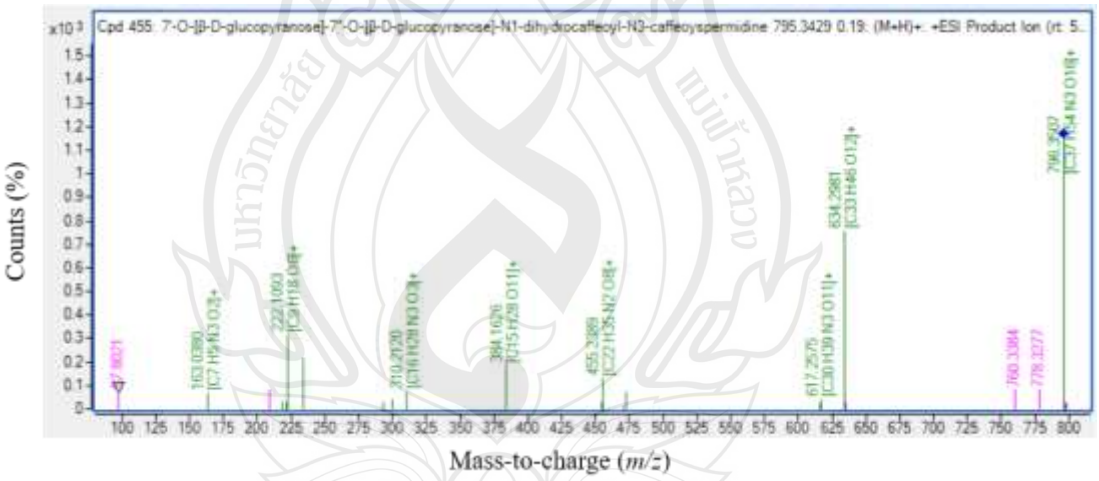
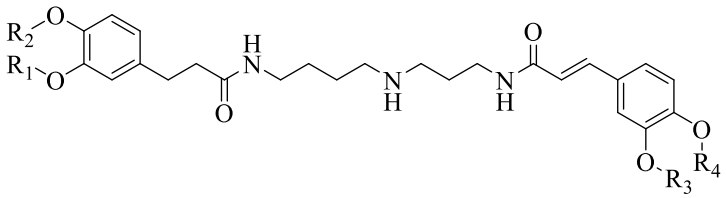


Figure 2.4 Mass spectrum fragment ion of compound **3**



lycibarbarspermidine F (**2.2**): R_1 = β-D-Glu, R_2 = H, R_3 = β-D-Glu, R_4 = H

Figure 2.5 Structure of lyciumbarbarspermidine F isomer

Compound **5** in RT 6.244 min had a $[M+H]^+$ at *m/z* 634.2962 ($C_{37}H_{43}O_{11}N_3$, cal. 634.2970, mass error -0.17 ppm) and produced fragment ions at *m/z* 472.2426, 310.2102, 236.1267, 220.0966, and 163.0388 (Figure 2.6) [15, 20]. The one ion *m/z* 472 $[M+H-C_6H_{10}O_5-C_6H_{10}O_5]^+$ is performed after the loss of two glucosyls. The fragment ion *m/z* 310 and 163 resulted from the leave of the caffeoyl unit. The ion at *m/z* 293 was generated of ammonia unit from the ion at *m/z* 310. The ion at *m/z* 222 was produced by leaving a cyclization ion at *m/z* 72 from molecule ion *m/z* 293. The ions at m/z 220 and 253 were observed from another pathway after the ion 253 appeared a 236 by the cleaving of the ammonia unit. Compounds **5**, **8**, **10**, **13**, and **18** had the same $[M+H]^+$ at *m/z* 634. Therefore, these compounds were confirmed to be N^1-caffeoyl-N^3-dihydrocaffeyl-spermidine-hexose or lyciumbarbarspermidine B isomer (**2.3**) (Figure 2.7).

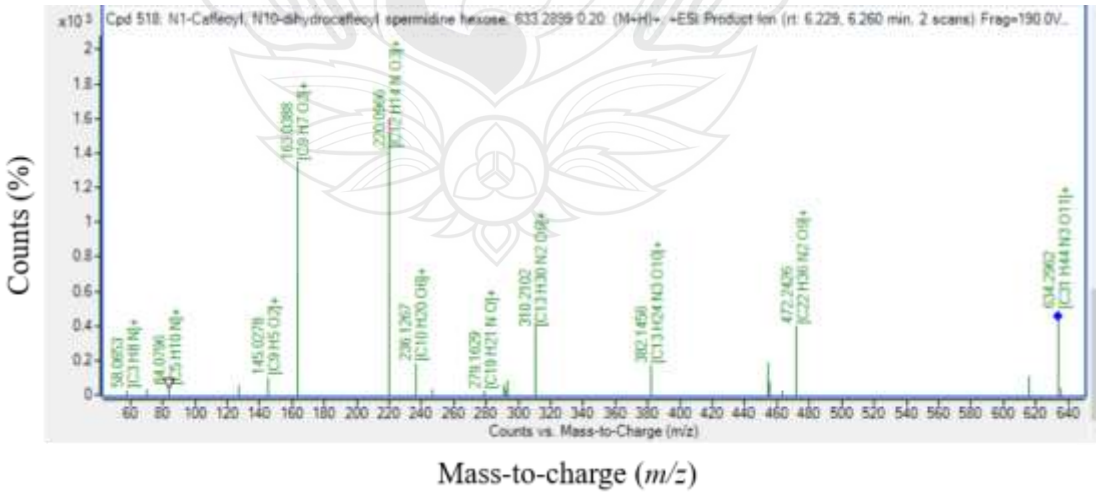
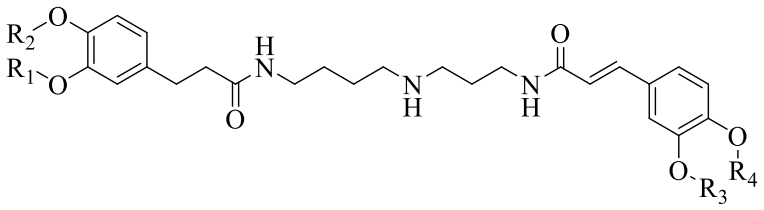


Figure 2.6 Mass spectrum fragment ion of compound **5**



lycibarbarspermidine B (**2.3**): R_1 = H, R_2 = H, R_3 = β-D-Glu, R_4 = H

Figure 2.7 Structure of lyciumbarbarspermidine B

Compound **6** in TR 6.325 min had a $[M+H]^+$ at *m/z* 636.3120 ($C_{37}H_{45}O_{11}N_3$, cal. 636.3127, mass error 0.77 ppm) and yielded molecule ions at *m/z* 474.2595, 310.2124, 293.1872, 222.1126, 165.0550, 123.0430, 100.0752, and 72.0808 (Figure 2.8) [15, 20]. The ion at *m/z* 474 $[M+H-C_6H_{10}O_5]^+$ were detected by the hexose unit from the ion at *m/z* 636. The ion *m/z* 310 appeared after the loss of ion at *m/z* 165. The ion at *m/z* 293 was indicated when losing the ammonia unit from molecule ion at *m/z* 310. The ion at *m/z* 222 was presented by the left of a rearrangement ion at *m/z* 72. The fragment ion at *m/z* 123 was generated by losing the substitution rearrangement production ion at *m/z* 100 from the ion at *m/ z* 222. Thereby, compound **6** was assigned as N^1- N^3-dihydrocaffeyl-spermidine-hexose or lycibarbarspermidine J (**2.4**) (Figure 2.9).

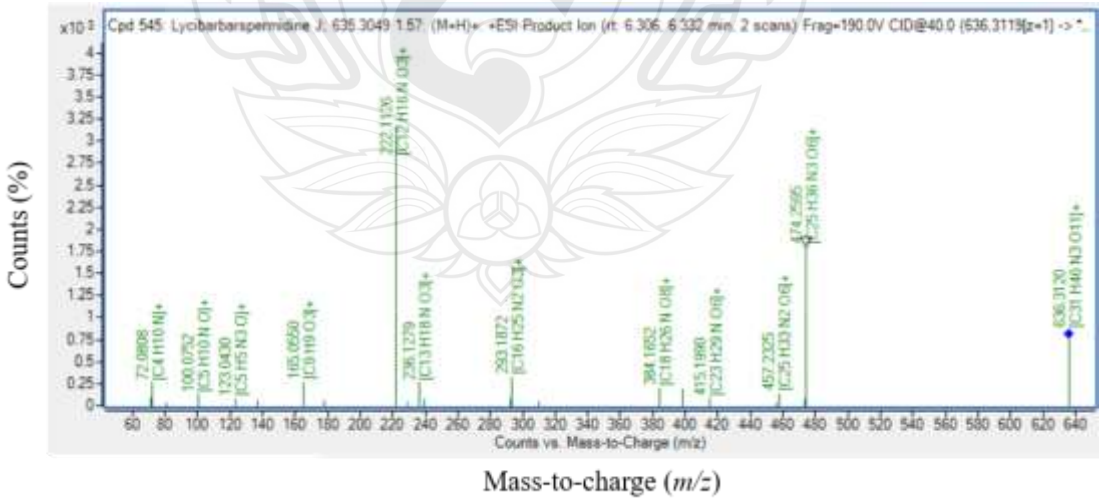
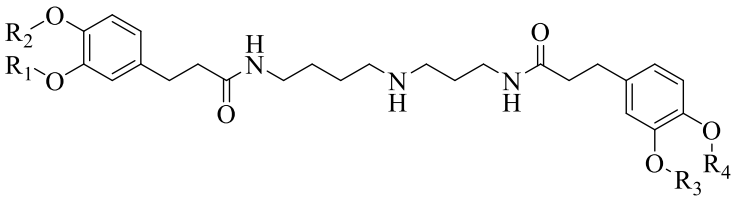


Figure 2.8 Mass spectrum fragment ion of compound **6**



lycibarbarspermidine J (**2.4**): R_1 = H, R_2 = H, R_3 = β-D-Glu, R_4 = H

Figure 2.9 Structure of lyciumbarbarspermidine J

Compound **11** in RT 6.826 min had molecular ion $[M+H]^+$ at *m/z* 632.2808 ($C_{37}H_{45}O_{16}N_3$, cal. 632.2893, mass error -0.37 ppm) and seven fragment ions at *m/z* 470.2297, 308.1952, 293.1784, 234.1130, 220.0936, and 163.0399 (Figure 2.10) [15, 20]. The ion at *m/z* 470 was formed by the loss of the sugar molecule (162 Da). The fragment ion *m/z* 308 was indicated when loss of caffeoyl unit (163 Da). The ion at *m/z* 293 was detected after the loss ammonia unit from the ion at *m/z* 308. The ion at *m/z* 220 was appeared by the loss of a rearrangement ion at *m/z* 72 from molecule ion *m/z* 291. The ions at m/z 220 and 253 were produced from another pathway after the ion 253 appeared a 234 by the loss of ammonia unit. As a result, compound **11** was determined to be dicaffeoyl- caffeoyl- cyclic- spermidine- hexose or lyciumbarbarspermidine N (**2.5**) (Figure 2.11).

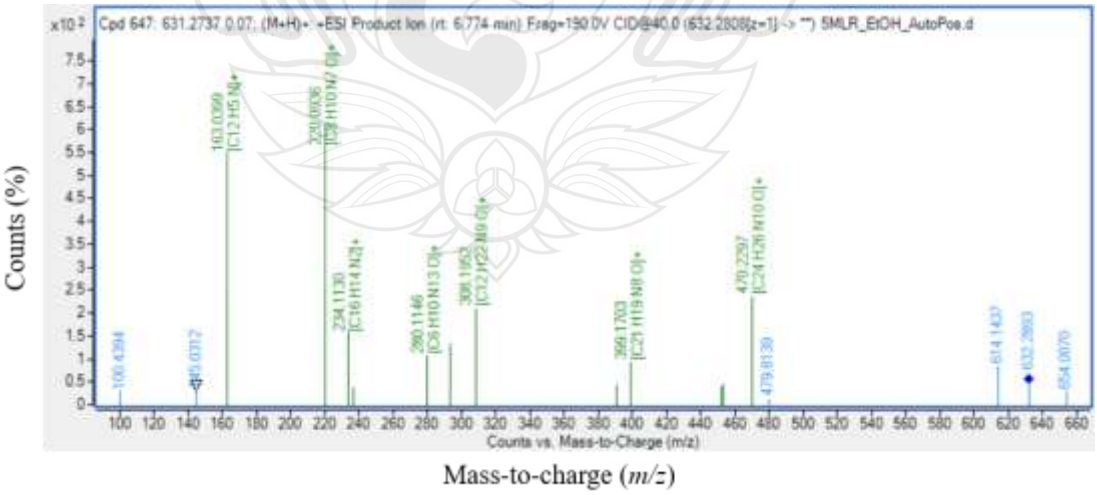
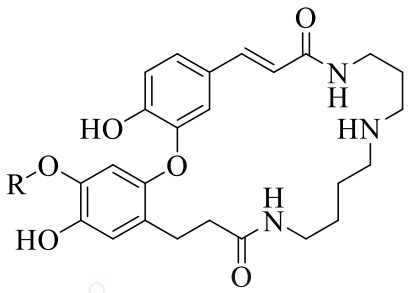


Figure 2.10 Mass spectrum fragment ion of compound **11**



lycibarbarspermidine N (**2.5**): R = β-D-Glu

Figure 2.11 Structure of lyciumbarbarspermidine N

Compound **15** in RT 7.033 min had molecular ion $[M+H]^+$ at *m/z* 474.2564 ($C_{25}H_{35}O_6N_3$, cal. 474.2599, mass error -0.51 ppm) and obtained of fragment ion at *m/z* 310.2116, 293.1895, 236.1272, 222.1124, 165.0546, 123.0444, 100.0754, and 72.0811 (Figure 2.12) [15, 20]. The cleavage of the dihydrocaffeoyl unit formed the fragment ion *m/z* 310 and 165. The loss of the ammonia unit generated the ion at *m/z* 293. The ion at *m/z* 222 was detected when cleavage of a rearrangement ion at *m/z* 72 from molecule ion *m/z* 293. The one ion at *m/z* 123 was produced after losing the substitution cyclization molecule ion at *m/z* 100 from the ion at *m/z* 222. The ions at *m/z* 220 and 253 occurred from another pathway after the ion 253 presented a 234 by losing the ammonia unit. Compound **16** had similar $[M+H]^+$ and fragment ions with compound **15**. In this way, compounds **15** and **16** were recognized as N^1,N^3-*bis*(dicaffeoyl)spermidine (**2.6**) (Figure 2.13).

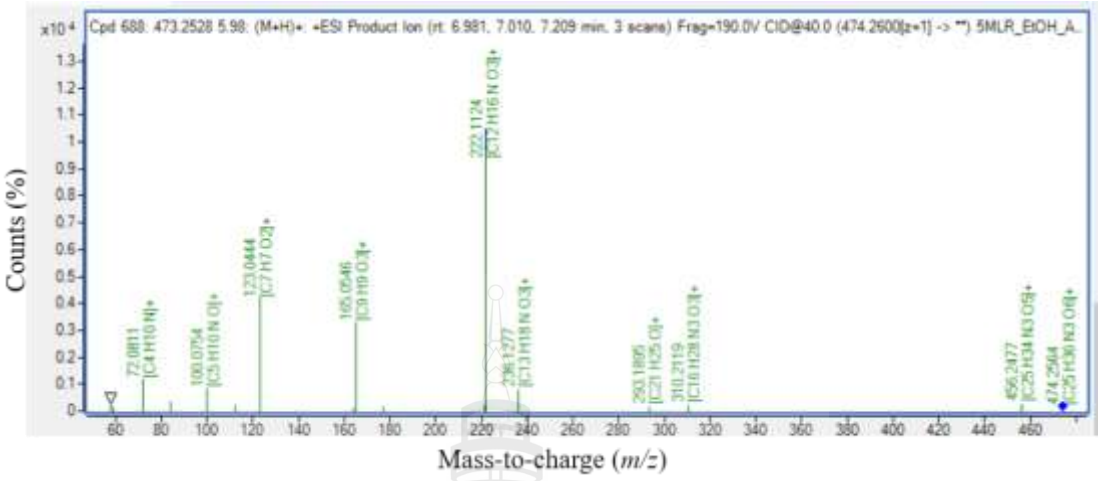
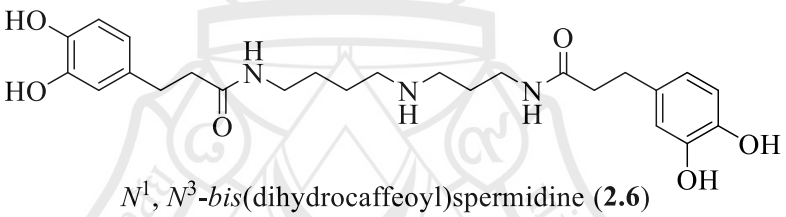


Figure 2.12 Mass spectrum fragment ion of compound **15**



N^1, N^3-*bis*(dihydrocaffeoyl)spermidine (**2.6**)

Figure 2.13 Structure of N^1,N^3-*bis(*dicaffeoyl)spermidine

Compound **20** in RT 7.456 min had a $[M+H]^+$ at *m/z* 472.2442 ($C_{25}H_{33}O_6N_3$, cal. 472.2441, mass error -0.02 ppm) and fragment ions at *m/z* 310.2131, 293.1835, 253.1521, 236.1269, 220.0961, 163.0386, 145.0287, and 72.0812 (Figure 2.14) [15, 20]. The ion at *m/z* 310 and 163 were yielded after the loss of the caffeoyl unit from the protonated parent ion. The ion at *m/z* 293 was presented with a loss of ammonia unit from the ion at *m/z* 310. The cleavage of a rearrangement ion caused the ion at *m/z* 222 and 72 from molecule ion *m/z* 293. The ions at *m/z* 220 and 253 were produced from another pathway after the ion 253 appeared a 236 by the loss of ammonia unit. Compound **20** was exhibited a similar fragmentation ion with compound **22**. Therefore, compounds **20** and **22** were proposed to be N^3-dihydrocaffeoyl-N^1-caffeoyl-spermidine (**2.6**) or N^1-dicaffeoyl-N^3-caffeoyl-spermidine (**2.7**) (Figure 2.15).

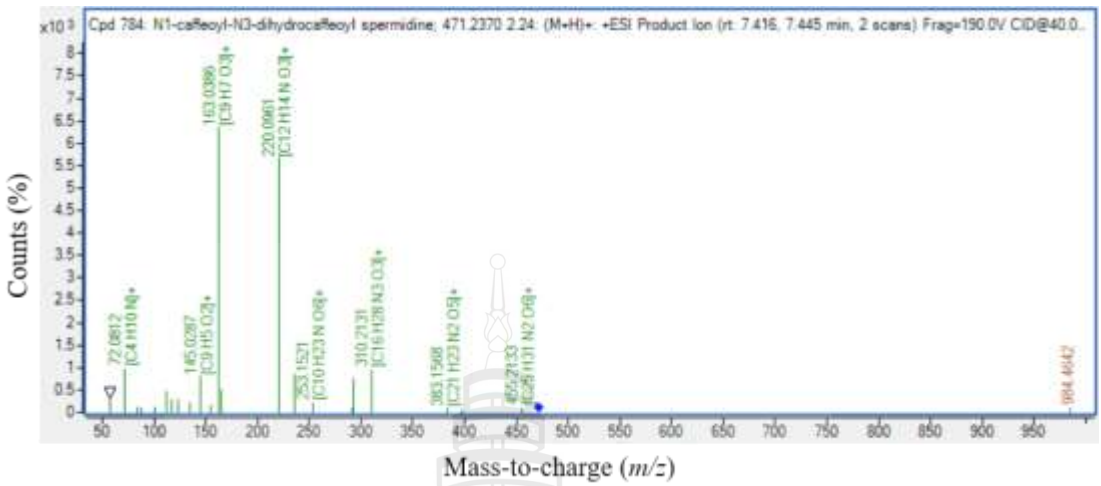
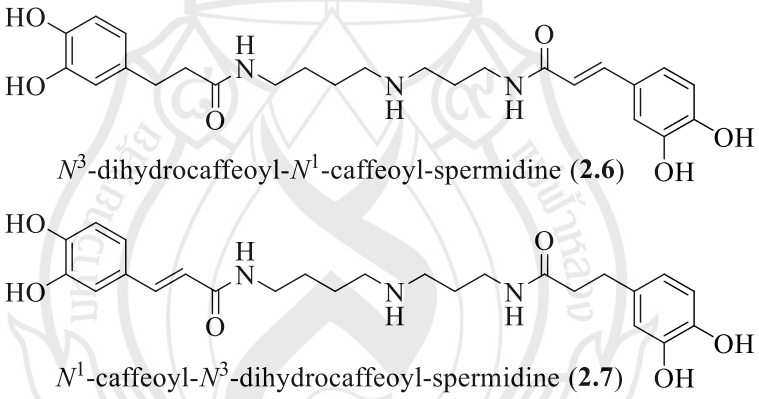
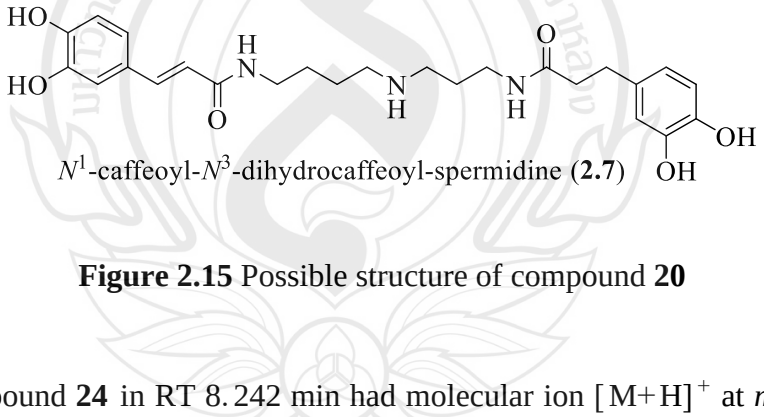


Figure 2.14 Mass spectrum fragment ion of compound **20**



N^3-dihydrocaffeoyl-N^1-caffeoyl-spermidine (**2.6**)



N^1-caffeoyl-N^3-dihydrocaffeoyl-spermidine (**2.7**)

Figure 2.15 Possible structure of compound **20**

Compound **24** in RT 8.242 min had molecular ion $[M+H]^+$ at *m/z* 470.2289 ($C_{25}H_{31}O_6N_3$, cal. 470.2283, mass error -0.70 ppm) and produced fragment ions at *m/z* 308.1954, 291.1713, 234.1112, 220.0963, 163.0392, 145.0280, and 72.0813 (Figure 2.16) [15, 20]. The fragment ion *m/z* 308 was formed by the cleavage of the caffeoyl unit (163 Da). The loss ammonia unit observed the ion at m/z 291 from the ion at *m/z* 308. The ion at *m/z* 220 was produced by the loss of a rearrangement ion at *m/z* 72 from molecule ion *m/z* 291. Thus, Compound **24** was suggested as N^1,N^3-*bis*(caffeoyl)spermidine (**2.8**) (Figure 2.17).

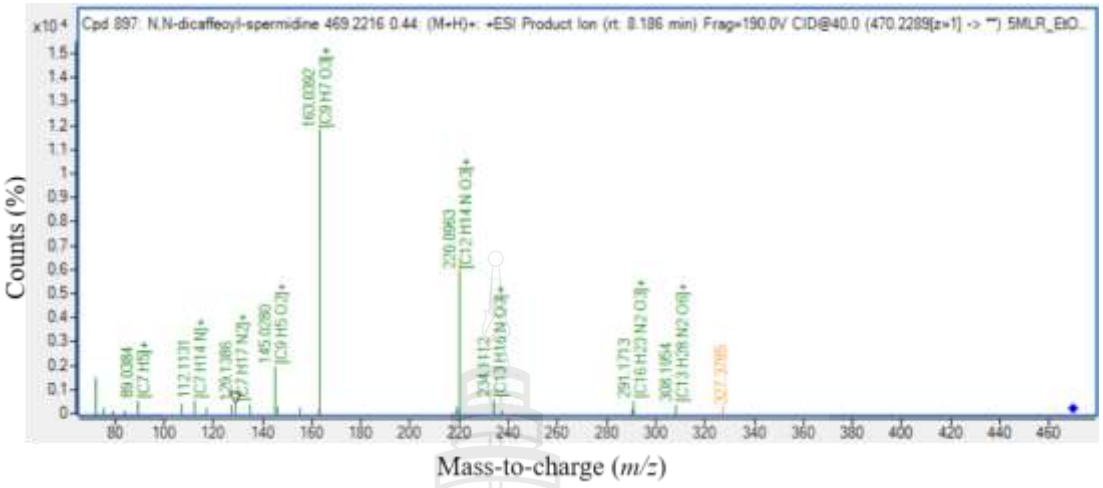
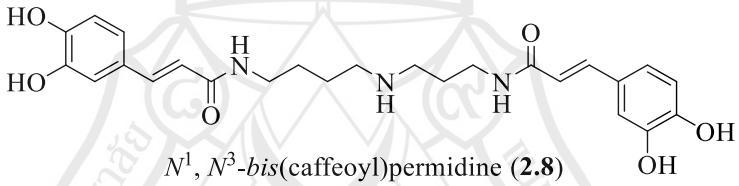


Figure 2.16 Mass spectrum fragment ion of compound **24**



N^1, N^3-*bis*(caffeoyl)permidine (**2.8**)

Figure 2.17 Structure of N^1,N^3-*bis*(caffeoyl)spermidine

Compound **28** in RT 9.170 min had a $[M+H]^+$ at *m/z* 456.2486 ($C_{25}H_{33}O_6N_3$, cal. 456.2493, mass error -0.35 ppm) and yielded molecule ions at *m/z* 253.0648, 222.1151, 165.0671, 147.0449, 123.0495, 100.0741, and 84.0804 (Figure 2.18) [15, 20]. The ion at *m/z* 293 appeared after cleavage of the caffeoyl unit (163 Da) from the protonated parent ion. The fragment ion at *m/z* 222 and 123 were indicated by the leaving of rearrangement ion at *m/z* 72 and 100, respectively. From another pathway, the one ion at *m/z* 147 was formed by the loss of a coumaroyl group from the protonated parent ion. In the same manner, compound **27** was showed $[M+H]^+$ similar to compound **28**. Hence, Compounds **27** and **28** were presented to be N^1-dicaffeoyl-N^{10}-coumaroyl-spermidine (**2.9**) (Figure 2.19).

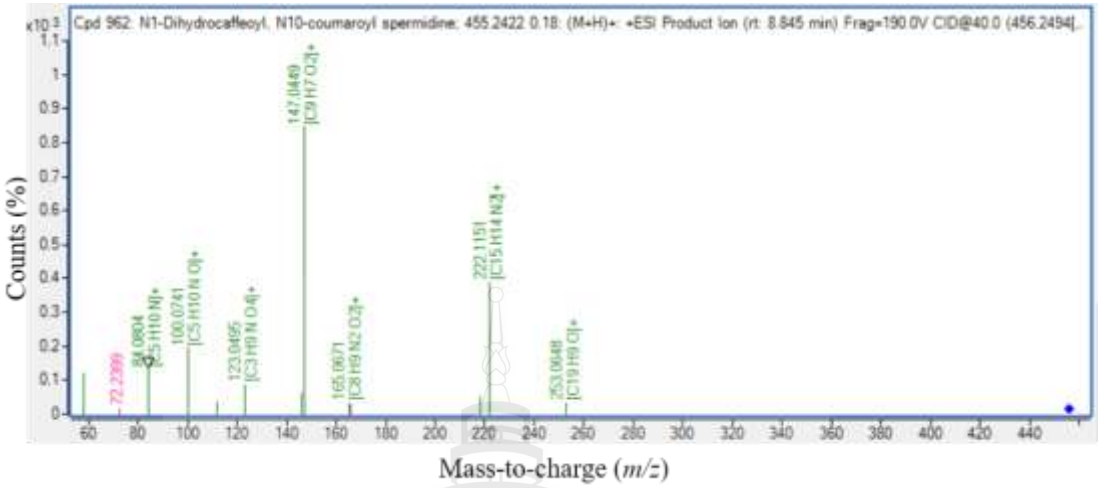
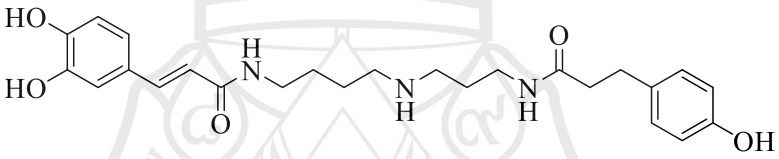


Figure 2.18 Mass spectrum fragment ion of compound **28**



N^1-dicaffeoyl-N^{10}-coumaroyl-spermidine (**2.9**)

Figure 2.19 Structure of N^1-dicaffeoyl-N^{10}-coumaroyl-spermidine

In this plant, *L. ruthenicum* can found phenolic amide compounds, which detected fragment ions at *m/z* 177 (feruloyl group), 163 (caffeoyl group), and 147 (coumaroyl group) [20, 42]. Compound **7** in RT 6.405 min had a $[M+H]^+$ at *m/z* 355.1024 ($C_{16}H_{18}O_9$, cal. 355.1039, mass error -0.03 ppm) and four fragment ions at *m/z* 163.0392, 145.0286, 135.0444 and 117.0343 (Figure 2.20). The ion at *m/z* 163 was formed by loss of caffeoyl group from molecule ion. The one ion *m/z* 135 was presented with the leaving of CO (28 Da). The ion at *m/z* 145 and 117 were produced by the loss of a water molecule from the ion at *m/z* 163 and 135, respectively. The possible fragmentation of chlorogenic acid was proposed in Figure 2.21 [42] This compound was identified as chlorogenic acid (**2.10**) (Figure 2.22).

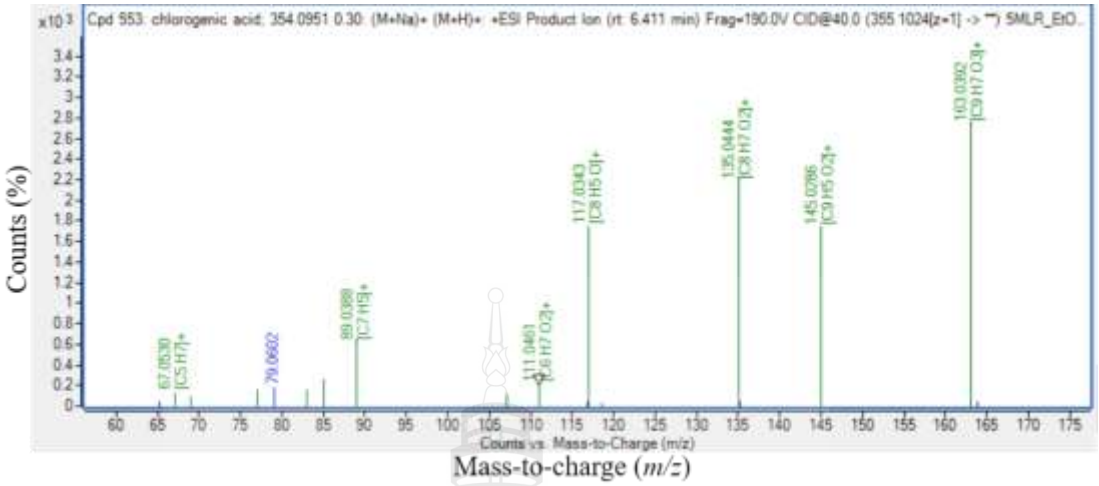
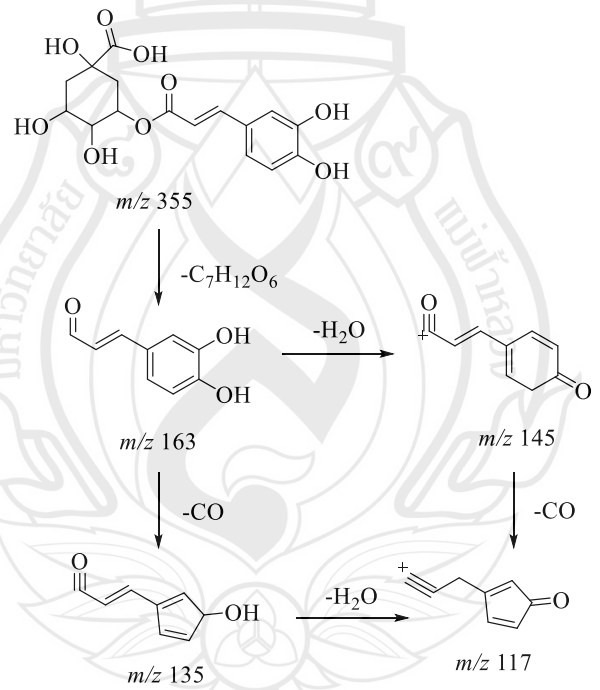
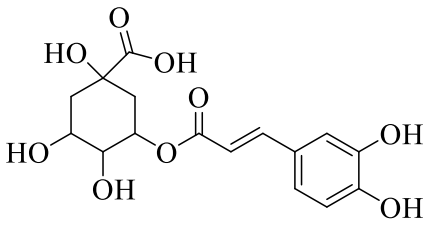


Figure 2.20 Mass spectrum fragment ion of compound **7**



Source [20]

Figure 2.21 Proposed fragmentation pathway of chlorogenic acid



chlorogenic acid (**2.10**)

Figure 2.22 Structure of chlorogenic acid

Compound **17** in RT 7.249 min had a $[M+H]^+$ at *m/z* 307.1768 ($C_{15}H_{22}O_3N_4$, cal. 307.1772, mass error -0.95 ppm) and generated fragment ions at *m/z* 177.0546, 149.0591, 145.0282, and 117.0332 (Figure 2.23). The one ion at *m/z* 177 was observed after the loss of the agmatine (130 Da) from the ion at *m/z* 307. The ion at *m/z* 145 and 117 were detected by leaving molecule CH_4O and CO from molecule ion at *m/z* 177 and 145, respectively. The possible fragmentation of feruloylagmatine was proposed in Figure 2.21 [43]. Therefore, compound **17** was confirmed to be feruloylagmatine (**2.11**) (Figure 2.23).

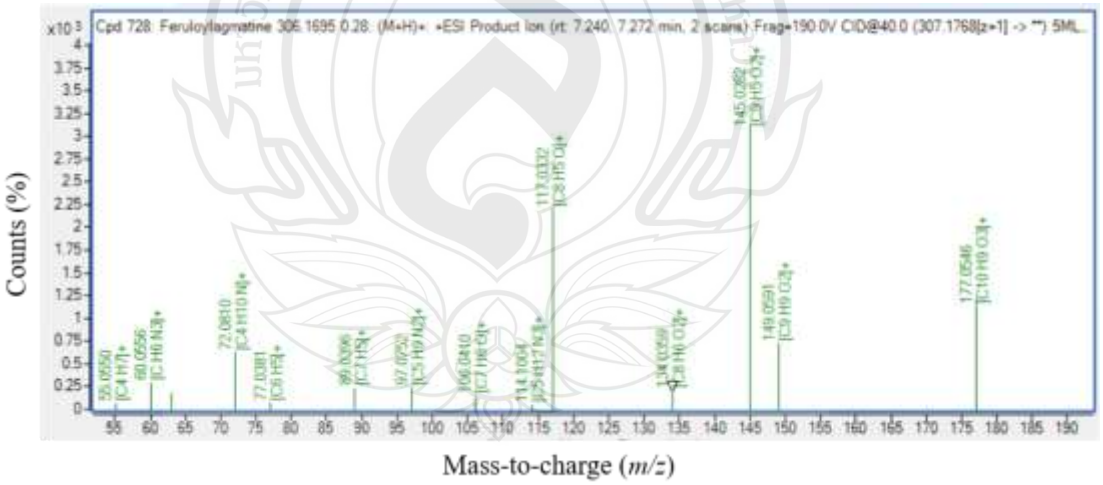
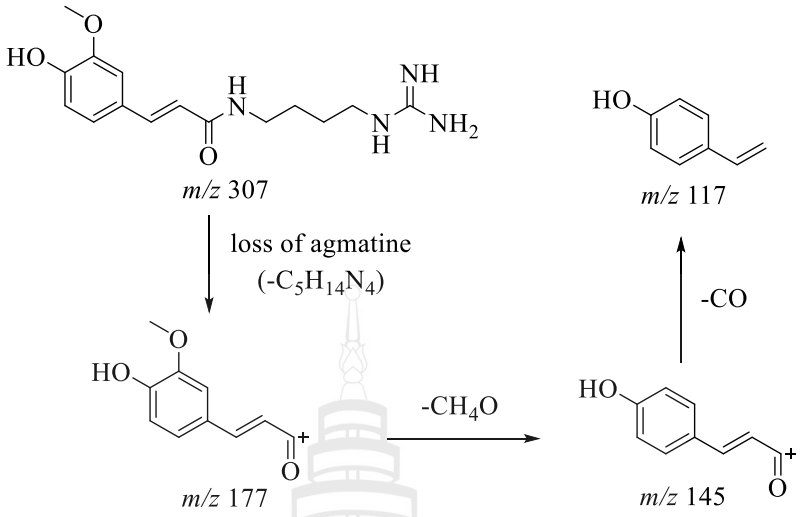


Figure 2.23 Mass spectrum fragment ion of compound **17**



Source [27]

Figure 2.24 Proposed fragmentation pathway of feruloylagmatine

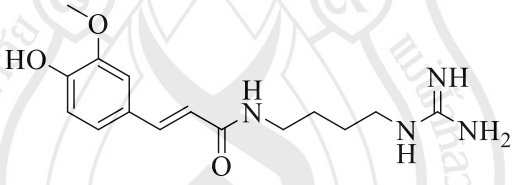
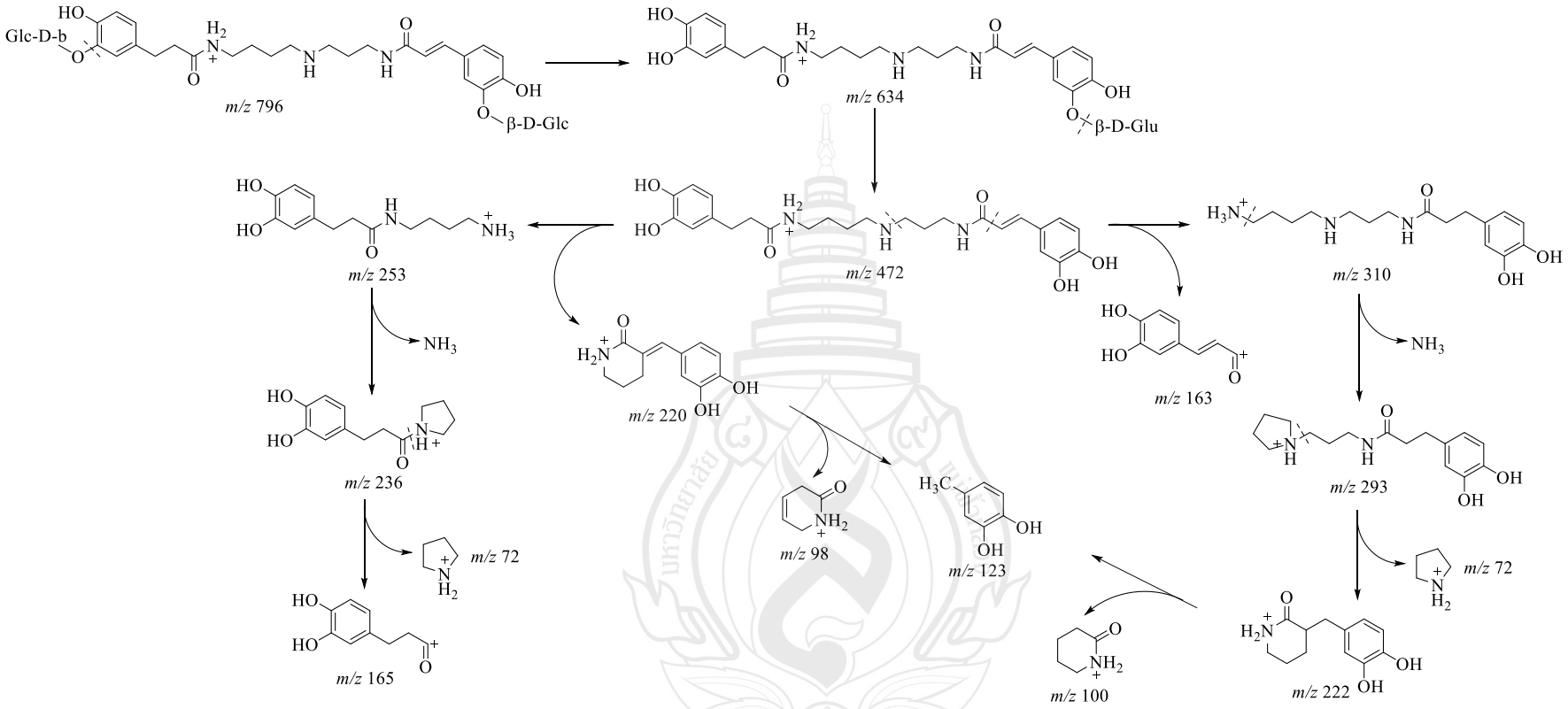


Figure 2.25 Structure of feruloylagmatine

In addition, compound **4**, **9**, **12**, **14**, **19**, **21**, **25**, **26**, **29**, **30**, **32**, and **33** were presented to be dicaffeoylspermidine derivative. The dicaffoylspermidine can be performed by abundant fragment ion peak at *m/z* 310/308, 293/291, 222/220, or 165/163 [20]. The key characteristic for this group of compounds was obtained of fragment ions at *m/z* 222 and 220 [15].

The possible fragmentation of dicaffeoylspermidine derivative was proposed in Figure 2.26. Spermidine alkaloids were appeared by fragment ions at *m/z* 310/308 was formed by the cleavage of one caffeoyl unit, 293/291 was detected with loss ammonia unit, 222/220 represented the leaving of a rearrangement ion at *m/z* 72, and 165/163

was formed by further neutral loss of the spermidine unit. Summary, 30 out of 32 permidine alkaloids in fruit of *L. ruthenicum* were identified using MS/MS analyse.



Source [15, 20]

Figure 2.26 The proposed fragmentation pathway of dicaffeoylspermidine derivatives

2.2.2 Antioxidative Activity Assays

The antioxidant assay showed that ethanolic fraction obtained the greatest value of IC_{50} at 2.04 ± 0.02 µg/mL and 4.70 ± 0.28 µg/mL in DPPH and ABTS assay, respectively. Water fraction performed IC_{50} > 100 µg/mL and 78.37 ± 1.12 µg/mL while ascorbic acid showed 1.53 ± 0.02 µg/mL and 2.92 ± 0.02 µg/mL for DPPH and ABTS assay, respectively (Table 2.2). In addition, the result significant differences in water fraction, which showed low antioxidant properties. This study could be confirmed that, the extract which contains a large amount of spermidine alkaloid showed the strongest antioxidant activity [20].

Table 2.2 Antioxidant activities of crude extracts from *L. ruthenicum* fruit

Extracts	DPPH (IC_{50}, µg/mL)[1]	ABTS (IC_{50}, µg/mL)[2]
Crude extract	27.26 ± 1.62	76.01 ± 1.23
Part ethanol	2.04 ± 0.02	4.70 ± 0.28
Part water	> 100	78.37 ± 1.12
Ascorbic acid[3]	1.53 ± 0.02	2.92 ± 0.02

Note [1] DPPH (IC_{50}) represents the extract concentration scavenging 50% of DPPH radical, [2] ABTS (IC_{50}) represents the extract concentration scavenging 50% of ABTS radical, [3] represent the positive control; Results are expressed as means ± SD, n=3.

2.2.3 Nitric Oxide Production Inhibitory Assay

Nitric oxide (NO) is a highly reactive free radical however overproduction of NO cause many diseases in pathogenesis [44-45]. In this study, nitric oxide production, the crude extract of *L. ruthenicum* from microwave extraction showed %NO inhibition at 56.50 ± 0.84% (concentration at 200 µg/mL) while fraction part ethanol and water exhibited inactive (%NO inhibition < 50%). The IC_{50} value of crude extract showed 161.4 µg/mL more than the positive control, indomethacin at 34.67 µg/mL (Table 2.3). A previous study of significant compounds from *L. ruthenicum* is polysaccharides for extract not found in this group [13, 15]. Because, the column in analysis cannot be used to separate the mixture of polysaccharides [46-47].

Therefore, it is necessary to test the quantity of polysaccharides content. Due to, polysaccharide content of crude extract, water, and ethanolic fraction were evaluated as 80.63 ± 3.82, 70.42 ± 2.02, and 67.32 ± 1.23 glucose equivalent/100 µg of the sample, respectively. Moreover, the polysaccharide content of the crude extract was significantly higher than the other fractions. This study confirmed that the polysaccharide-contained extract presented the potential to inhibit the production of nitric oxide [48-49].

Table 2.3 Nitric oxide production inhibitory (NO) of crude extracts from *L. ruthenicum* fruit

Samples	**Concentration (µg/mL)**	**% NO inhibition**	**IC_{50} (µg/mL)**	**% cell viability**
Crude extract	12.5	2.19 ± 0.69		98.62 ± 0.95
	25	5.74 ± 0.47		97.17 ± 1.21
	50	10.93 ± 1.22	161.40	97.10 ± 0.55
	100	28.87 ± 2.98		95.80 ± 1.60
	200	56.50 ± 0.84		95.32 ± 0.96
Indomethacin	6.25	3.95 ± 2.69		99.61 ± 2.24
	12.5	17.24 ± 3.48		92.66 ± 1.18
	25	38.36 ± 276	34.67	88.43 ± 1.30
	50	62.15 ± 3.28		85.36 ± 1.13
	100	96.07 ± 1.83		81.37 ± 0.72

CHAPTER 3

COSMETIC PRODUCT FORMULATION FROM *L. ruthenicum*

3.1 Materials and Methods

3.1.1 Raw Materials

The dried fruit of *L. ruthenicum* (brown and purple color) was purchased from China in May 2018 and Thailand in April 2021.

3.1.2 Reagents and Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ), ascorbic acid, torlox, and resazurin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was purchased from Kemaus (New South Wales, Australia). Iron(II) sulfate heptahydrat ($FeSO_4 \cdot 7H_2O$) and potassium persulphate ($K_2S_2O_8$) were purchased from Ajax Finechem (Australia). Water was reverse osmosis Milli-Q water (Millipore, USA). Ethanol AR grade was purchased from Merck (Darmstadt, Germany). Nutrient Agar, Nutrient Broth, and Mueller-Hinton broth were purchased from HiMedia Laboratories (Mumbai, India). Vancomycin hydrochloride, and Gentamycin sulfate were purchased from Bio Basic Canada (Ontario, Canada).

Aloe barbadensis leaf extract, carbopol 940, phenoxy ethanol and triethanolamine (TEA) were purchased from Chemipan Corporation Co., Ltd. (Bangkok, Thailand). Bis-PEG-18 methyl ether dimethyl silane, propanediol, disodium EDTA and sodium hyaluronic acid were purchased Chanjao Longevity Co., Ltd. (Bangkok, Thailand).

3.1.3 Sample Preparation

The dried fruit of *L. ruthenicum* brown (LRB) and purple (LRP) color (10.0 g) were ground and further extracted by maceration with 100%, 80%, 50%, and 20% ethanol/water (1:10, w:v) and 100% water. The solvent was removed under reduce pressure, and residue afforded a crude extract after freeze-drying.

3.1.4 DPPH Radical Scavenging Assay

Free radical scavenging activity of the plant extract was performed according to the methodology modified [37]. The reaction of the DPPH radical scavenging activity was determined by the hydrogen atom donating ability of the extract to react with a solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH showed violet purple color in ethanol solution when the presence of antioxidants performed yellow color. A solution of 60 μM DPPH radical solution in ethanol (100 μL) was mixed with 100 μL of extract in ethanol at different concentrations. The reaction was incubated for 30 min at room temperature in the dark, and the absorbance was measured at 517 nm (TECAN, infinite 200 PRO). Ascorbic acid and trolox were used as a positive control. The percentage of inhibitory concentration (IC_{50}) of crude extract was calculated by plotting inhibition percentages against the concentrations of extracts. Percentage DPPH radical scavenging activity was calculated as follow:

$$\% \text{ DPPH radical scavenging activity} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of DPPH radical solution, and A_1 is the absorbance of DPPH radical solution for the extractives/standard. The experiment was done in triplicate.

3.1.5 ABTS Radical Scavenging Assay

The ABTS radical scavenging assay of samples was evaluated by modification [37]. The radical cations were prepared by mixing 7 mM aqueous ABTS with 2.45 mM potassium persulphate (1:1) in the dark at room temperature for 16-18 h before use. Dilute $ABTS^{\bullet+}$ solution was mixed with distilled water to an absorbance of 0.700 ± 0.020 at 734 nm. The reaction of $ABTS^{\bullet+}$ solution (160 μL) was added to 40 μL of sample in

50% ethanol at different concentrations, and the absorbance was recorded after 5 min at 734 nm (TECAN, infinite 200 PRO). Ascorbic acid was used as a standard substance. The percentage inhibition and IC_{50} were calculated using the formula:

$$\% \text{ ABTS radical scavenging assay} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of $ABTS^{\circ+}$ solution, and A_1 is the absorbance of $ABTS^{\circ+}$ solution for tested samples/standard. All the measurements were performed in triplicates.

3.1.6 Ferric Reducing Antioxidant Power Assay (FRAP)

FRAP reagent was prepared according to previously described [50]. Briefly, the FRAP reagent was prepared by mixing 100 mL of 300 mM acetate buffer pH 3.6 with 10 mL of 10 mM 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ) dissolved in 40 mM HCl and 10 mL of 20 mM $FeCl_3 \cdot {}_6H_2O$. The freshly prepared reagent was warmed at 37 °C for 15 min before being used. 25 µL of each sample (1 mg/mL) were mixed with 175 µL of the FRAP reagent and then left for 30 min under dark conditions at room temperature. The absorbance was measured at 593 nm (TECAN, infinite 200 PRO). $FeSO_4 \cdot 7H_2O$ was used as standard reference, and different concentrations in the range of 0.1 - 1.0 mM were used for the calibration curve. Results were expressed in mM Fe (II)/1 mg of crude extract. In order to make a comparison, ascorbic acid and trolox were also tested under the same conditions as the standard antioxidant compound. All samples were performed in triplicate.

3.1.7 Antibacterial Activity

Cosmetics must meet strict microbial safety requirements, and preservatives are required to avoid product deterioration and harm consumers' health [51] Reservations over the safety of some cosmetic components, as well as growing demand for more natural beauty products, have encouraged cosmetic makers and formulators to search out natural alternatives to synthetic preservatives [51]. Cosmetic antimicrobial agents help prevent the growth of unwanted microorganisms in skincare products and enhance product preservation.

The antibacterial activity was tested both Gram-positive and Gram-negative bacteria by Broth micro dilution methods [52]. The Gram-positive bacteria tested were *Micrococcus luteus* (DMST 15503), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (DMST5040), *Listeria monocytogenes* (F2369), and *Bacillus subtilis* (TISTR1248). The Gram-negative bacteria tested were *Shigella flexneri* (DMST4423), *Pseudomonas aeruginosa* (ATCC 10145), *Salmonella Typhi* (DMST 22842), and *Salmonella typhimurium* (DMST S62). The Minimum Inhibitory Concentrations (MICs) were performed with 2-fold dilution series. All samples were dissolved in DMSO. Gentamicin sulfate and vancomycin were used as a positive control.

3.1.8 Serum Formulation

Carbopol 940 polymer for gel formulation is a white powder, cross-linked polyacrylic acid polymer. It's a highly effective rheology modifier that produces sparkling clear gels or hydro-alcoholic gels and creams to high viscosity. The acidic pH of the carbopol polymeric solution was neutralized by adding enough triethanolamine [53].

The formation of *L. ruthenicum* serum used ingredient in Table 3.1 by mixed disodium EDTA with water. Firstly, carbopol 940 and sodium hyaluronic acid was dissolved in water. After, bis-PEG-18 methyl ether dimethyl silane, propanediol, aloe barbadensis leaf extract, and phenoxyethanol were combined in another ingredient. Lastly, triethanolamine (TEA) was slowly dropped for the happening gel serum.

Table 3.1 Ingredient for a serum formulation

No	Ingredient	Percentage	Function
1	Water	Up to 100	Soluble
2	Propanediol	3.4	emollient, humectant, and solvent
3	Bis-PEG-18 methyl ether dimethyl silane	1.5	Silicone oil
4	Aloe barbadensis leaf extract	1.0	Moisture and irritations
5	Carbopol 940	0.5	Gel maker
6	Phenoxy ethanol	0.4	Preservative
7	Disodium EDTA	0.1	chelating agent
8	Sodium hyaluronic acid	0.1	Moisture, emollient, humectant, and solvent
9	*L. ruthenicum* extract (LPR extract with 80% EtOH)	0.5-1.0	Antioxidant
10	Triethanolamine (TEA)	0.10	Neutralize

3.1.9 Stability Test [54]

3.1.9.1 After preparation, the stability test observes color, smell, viscosity, texture, pH, and centrifuge test at 3,000 rpm for 30 minutes.

3.1.9.2 Heating cooling cycle method by kept at 4 °C for 24 h and 45 °C for 24 h counted as one cycle of testing cycles. Then, the stability test observes color, smell, viscosity, texture, pH, and centrifuge test.

3.1.10 Viscosity Measurement

The viscosity of the gels was determined using a Brookfield viscometer (Brookfield DV-II + Pro viscometer) by using spindle number RV0.5(5). The gel serum was subjected to a torque ranging from 10 to 100 %.

3.1.11 Skin Irritation

The overall test technique followed the rules of the Cosmetic, Toiletry, and Fragrance Association (CTFA) [55]. Skin irritation with 30 volunteers was tested allergic contract dermatitis by closed patch test on the inside part of the arm for 48 h. All samples were dropped 20 μL at Finn chambers on scanpor 8 mm (SmartPractice, Phoenix, USA). After 48 hours, then check skin irritation by Mean Irritation Index [55] as follows:

Mean Irritation Index (M.I.I) = Sum of grade/ Total of volunteers

Table 3.2 Grading criteria of skin reaction by CTFA Guideline

Symbol	Grade	Clinical description
-	0	Negative reaction
+	1	Slight erythema, either spotty or diffuse
++	2	Moderate uniform erythema
+++	3	Intense erythema with oedema
++++	4	Intense erythema with oedema and vesicles

Source [56]

3.2 Result and Discussion

3.2.1 Antioxidative Activities Assays

Antioxidants are commonly used as active components in many cosmetics on the market today [57]. They can protect cells from the damage caused by unstable molecules or free radicals [27-28]. Plant extracts are used in various patents and commercial cosmetic goods [57].

The antioxidative activities were compared using 2,2- diphenyl- 1-picrylhydrazyl (DPPH), ferric reducing ability of plasma (FRAP), and 2,2'-azinobis-(3-ethyl-benzthiazoline-6-sulphonate) (ABTS) assays. *L. ruthenicum* brown (LRB)

and purple (LRP) were extracted by maceration with 100% EtOH, 80% EtOH, 50% EtOH, 20% EtOH and 0% EtOH. The antioxidant assay showed that LRP extract with 80% EtOH obtained the most excellent value of IC_{50} at 18.90 ± 0.82 µg/mL, 84.40 ± 1.03 µg/mL and 0.4825 mM Fe(II)/mg of sample in DPPH, ABTS, and FRAP assay, respectively (Table 3.3). The result corresponded to previous report [58] that of 70% ethanol-water crude extract of *L. ruthenicum* fruit showed a strong ability to scavenge free radicals in DPPH assay. This extract showed good antioxidant activity when compared with other extracts. Therefore, LRP extract with 80% ethanol has been used ingredient for formulating cosmetic products.

3.2.2 Antibacterial Activity

Cosmetics of high quality are subjected to strict quality control. Detection of particular organisms that thrive in air and at moderate temperatures and are forbidden from being discovered in cosmetics, including *P. aeruginosa*, *S. aureus*, and *Candida albicans*, which can cause diseases of the skin mucous membranes, and eyes [59]. *Pseudomonas aeruginosa* is a gram-negative bacillus that thrives in moist conditions. As a result, some cosmetics with high water content are vulnerable to housing these germs, allowing them to multiply and infect end users. [60]

The antioxidative activities of LRP extract with 80% ethanol have been used as an ingredient for formulating cosmetic products. The antibacterial activity of LRP extract with 80% ethanol inhibited *P. aeruginosa* at 1280 µg/mL, but *S. aureus* showed inactive (Table 3.4).

Table 3.3 Antioxidant activities result of LRB and LRP extracts

Sample	Condition for maceration	Antioxidant activities		
		DPPH (IC_{50}, µg/mL)[1]	ABTS (IC_{50}, µg/mL)[2]	FRAP (mM Fe(II)/mg of sample)
LRB	100% EtOH	67.67 ± 1.20	inactive	0.2486
	80% EtOH	31.56 ± 2.08	inactive	0.3632
	50% EtOH	35.48 ± 1.06	102.31 ± 1.24	0.3387
	20% EtOH	42.03 ± 2.25	103.79 ± 0.29	0.3632
	0% EtOH	46.26 ± 2.13	inactive	0.2015
LRP	100% EtOH	22.98 ± 0.38	104.09 ± 2.45	0.4270
	80% EtOH	18.90 ± 0.82	84.40 ± 1.03	0.4825
	50% EtOH	27.56 ± 0.56	90.84 ± 2.00	0.4045
	20% EtOH	35.40 ± 1.52	107.26 ± 0.41	0.3361
	0% EtOH	27.98 ± 1.12	104.47 ± 2.99	0.3107
Ascorbic acid[3]		1.71 ± 0.039	3.87 ± 0.12	10.9685
Trolox[3]		2.26 ± 0.054	4.90 ± 0.08	7.8630

Note [1] DPPH (IC_{50}) represents the extract concentration scavenging 50% of DPPH radical

[2] ABTS (IC_{50}) represents the extract concentration scavenging 50% of ABTS radical

[3] represent the positive control; Results are expressed as means ± SD, n=3.

Table 3.4 Antibacterial activity result of LRB and LRP extracts

Samples	Condition of maceration	MICs (µg/mL)								
		S. aureus	***M. luteus***	***B. subtillis***	***L. monocytogenes***	***S. typhi***	***S. typhimurium***	***S. flexneri***	***P. aeruginosa***	***B. cereus***
LRB	100% EtOH	1280	320	1280	1280	1280	-	-	1280	1280
	80% EtOH	640	160	1280	-	1280	-	-	1280	640
	50% EtOH	-	640	-	-	-	-	-	1280	-
	20% EtOH	-	640	-	-	-	-	-	--	-
	0% EtOH	-	1280	-	-	-	-	-	1280	-
LRP	100% EtOH	-	1280	-	-	-	-	-	-	-
	80% EtOH	-	1280	-	1280	-	-	-	1280	-
	50% EtOH	-	1280	-	-	1280	1280	1280	1280	-
	20% EtOH	-	1280	-	-	-	-	-	1280	-
	0% EtOH	-	-	-	-	-	-	-	-	-
Gentamycin		ND	0.25	0.25	1	1	4	2	1	1
Vancomycin		1	0.25	ND	1	ND	ND	ND	ND	ND

Note - = inactive, ND = Not detect

Gram-positive bacteria: *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus*, *Listeria monocytogenes*, *Bacillus subtilis*

Gram-negative bacteria: *Shigella flexneri*, *Pseudomonas aeruginosa*, *Salmonella Typhi*, *Salmonella typhimurium*

3.2.3 Formulation of Serum Product

Formulation of serum product found the criteria for using the appropriate amount of extract in cosmetics. The extract liquefied as the concentration of the extract was increased. Not as expected, with reduced antibacterial efficacy. It was discovered that when cosmetics containing extracts were left at a temperature, fungi or bacteria could appear. Therefore, *L. ruthenicum* extract was used at 0.5% and 1.0% to formulate the serum product.

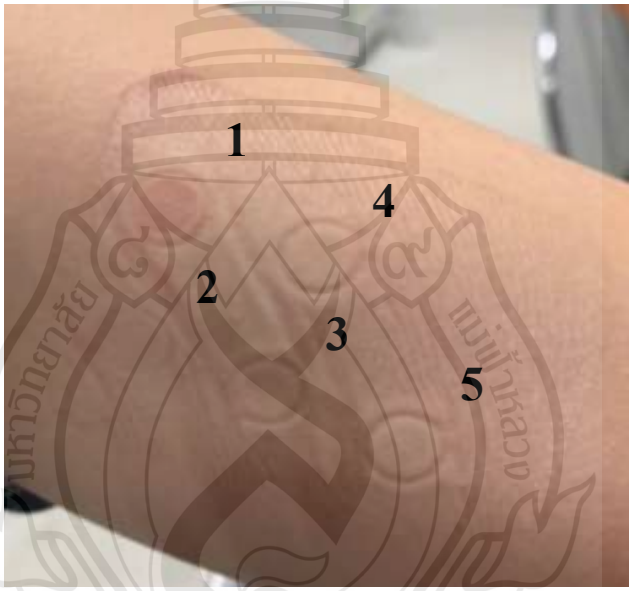
Stability studies are essential to ensure cosmetic formulations' product quality, safety, and efficacy [61]. Stability can be affected by surroundings factors such as pH, temperature light, air, and movements, which can damage the product's constituents [62]. Thus, these studies contribute to developing and improving formulations. The results of stability test in Table 3.5 showed test texture, smell, creaming, viscosity, and pH in serum product. Centrifugation test or creaming did not show any phase separation. The viscosity observed a decrease in viscosity with an increasing gradient of *L. ruthenicum* extract ($p < 0.05$). The pH value of all prepared formulations ranged from 5.36 - 5.65 ($p > 0.05$) that which is suitable to the human skin pH, which generally ranges from 4.50 to 6.00 [63]. We can improve the gradient for formulating serum product based on the result.

Table 3.5 Stability test for serum product of fruits from *L. ruthenicum*

Evaluate	**Base serum**		**extract 0.5%**		**extract 1.0%**	
Texture	fine-grained, very viscosity		fine-grained, very liquid		fine-grained, liquid	
Smell	fragrant		fragrant		fragrant	
Creaming	No		No		No	
Viscosity (cP)	T_0	T_6	T_0	T_6	T_0	T_6
	7,768	7,543	7,808	7,652	1,766	1,475
pH	5.65	5.53	5.36	5.27	5.36	5.24

3.2.4 Skin Irritation

For the study, skin irritation of serum contained *L. ruthenicum* extract at 0.5% and 1.0% by close patch test with 30 volunteers (mean age 25 years). After 48 hours, all volunteers were not showed skin irritation with base serum, serum containing 0.5% of *L. ruthenicum* extract, and serum containing 1.0% of *L. ruthenicum* extract (score of skin irritation = 0) when compared with DI water as a positive control and 1.0% SLS as a negative control (Figure 3.1). The average of the irritation index (Mean irritation index, MII) is 0.00, less than 0.20 (Table 3.6) [55-56]. Show that all two recipes do not irritate the product be safe.



Note **1**: 1.0% SLS as a positive control

2: DI water as a negative control

3: Base serum

4: Serum containing 0.5% of *L. ruthenicum* extract

5: Serum containing 1.0 % of *L. ruthenicum* extract

Figure 3.1 Skin irritation with volunteer test

Table 3.6 Mean irritation index (MII) of the skin by close patch test observed at 48 hr

Samples for test	**Average of mean irritation index (n = 30)**	**SD (n = 30)**
DI water	0.00	0.00
1.0% SLS	0.40	0.68
Base serum	0.00	0.00
Serum containing 0.5% of *L. ruthenicum* extract	0.00	0.00
Serum containing 1.0% of *L. ruthenicum* extract	0.00	0.00

CHAPTER 4

CONCLUSION

The fruits of *L. ruthenicum* were extracted by microwave-assisted extraction (MAE) and C_{18} solid-phase extraction for antioxidant compounds. The study performed ethanolic fraction as the most potent antioxidant activity in DPPH and ABTS assay. Thirty-two compounds have been investigated from ethanol fraction using high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS/MS). Among these thirty compounds were identified as dicaffeoyl spermidines derivatives.

L. ruthenicum purple (LRP) color extract with 80% ethanol has been used to formulate cosmetic products, showing the most significant antioxidant activities (DPPH, ABTS, and FRAP assay). For the study, serum skin irritation did not lead to skin irritation with serum-containing *L. ruthenicum* extract (mean irritation index at 0.00).

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APPENDICES

APPENDIX A

CERTIFICATION OF APPROVAL

The Mae Fah Luang University Ethics Committee on Human Research
333 Moo 1, Thasud, Muang, ChiangRai 57100
Tel (053)917-170 to 71 Fax (053)917-170 E-mail: rec.human@mfu.ac.th

CERTIFICATE OF APPROVAL

COA: 200/2021 Protocol No: EC 19364-11

Title: Bioactive compounds from goji berry for cosmetic products

Principal investigator: Wisanu Maneerat

School: Science

Funding support: Research and Researchers for Industries (RRI)

Approval:

1) Research protocol — Version 3 date May 7, 2020
2) Information sheet for research project participants — Version 3 date May 7, 2020
3) Informed consent form — Version 3 date May 7, 2020
4) Questionnaire — Version 3 date May 7, 2020
5) Research participant recruitment information — Version 3 date May 7, 2020
6) Principal investigator and Co-investigators
   - Wisanu Maneerat
   - Chalinee Janta

The aforementioned documents have been reviewed and approved by the Mae Fah Luang University Ethics Committee on Human Research in compliance with international guidelines such as Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use - Good Clinical Practice (ICH - GCP)

Date of 1st Approval: May 18, 2020

Date of 2nd Approval: May 18, 2021

Date of Expiration: May 17, 2022

Frequency of Continuing Review: 1 year

(Assoc. Prof., Maj. Gen. Sangkae Chamnanvanakij, M.D.)
Chairperson of the Mae Fah Luang Ethics Committee on Human Research

The Mae Fah Luang University Ethics Committee on Human Research
333 Moo 1, Thasud, Muang, ChiangRai 57100
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For all investigators approved by the Mae Fah Luang University Ethics Committee on Human Research (MFU EC) must comply with the followings:

1. Strictly conduct the research as required by the protocol
2. Use only the information sheet, consent form, questionnaire and case record form bearing the MFU EC stamp of approval
3. Send a progress report (AP 05/2019) for continuing review and for renewing the approval at least 30 days before expiration date.
4. When there are changes of the protocol, the investigator must send an amendment report (AP 06/2019) with amended protocol for MFU EC approval before implementing any changes in the research (unless those changes are required urgently for the safety of the research subjects).
5. When there is any unanticipated problem or severe adverse event, the investigator must send a safety report (AP 07/2019) as set forth in the ICH-GCP.
6. When there is any deviation or non-compliance with the approved protocol, the investigator must send a protocol deviation/non-compliance report (AP 08/2019).
7. When the research stops before planned schedule, the investigator must send a premature termination document.
8. When the research finishes, the investigator must send a final report (AP 09/2019).

I, as an investigator, agree to comply with the above obligation.

(Wisanu Maneerat)

Date 8/7/2021

Please go to https://ethic.mfu.ac.th to download MFU EC forms for reporting.

APPENDIX B

RESEARCH PUBLISHED IN CONFERENCE

47th INTERNATIONAL CONGRESS ON SCIENCE, TECHNOLOGY AND TECHNOLOGY-BASED INNOVATION

(STT47)

ON 5-7 OCTOBER 2021

THE ANTIOXIDANT ACTIVITY AND THEIR CHEMICAL PROFILE FROM FRUITS OF *Lycium ruthenicum* BY LC-MS/MS

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Abstract:

The fruits of *Lycium ruthenium* are widely used to be functional foods and traditional medicinal herbs worldwide. This study demonstrated promising results for microwave-assisted extraction (MAE) of antioxidative from the fruits of *L. ruthenium*. The separation of *L. ruthenium* extract by C_{18} solid-phase extraction led to give water and ethanol fractions. The chemical composition of ethanol fraction from *L. ruthenium* has been investigated by high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS/MS). A total of 32 compounds were identified in fruits and the most detected of 30 compounds of dicaffeoyl spermidines derivatives. Spermidine alkaloids were the most compounds that showed the most potent antioxidant activity in *L. ruthenium* fruits. For antioxidant activities, their ethanol fraction showed the powerful antioxidant in terms of DPPH and ABTS assays.

Introduction:

Microwave-assisted extraction (MAE) is an environmentally friendly technique known as "green extraction". MAE is the technique extraction of natural products by absorption of microwave energy through heating the water or alcohol as a solvent, hence accelerate the solvent dissipation into the material.[1,2] This extraction uses the MAE method because the advantages are more rapid, less solvent, more compact produces, and energy consumption compared to conventional extraction processes.[3,4] This method can extract bioactive compounds, including phenolic, flavonoid, and antioxidant compounds, that have the rich electron-donating ability of crude extract.[5] Therefore, the MAE process has been used to extract antioxidants or bioactive compounds from natural products.

Antioxidants are molecules or compounds that can be inhibitors in oxidation processes.[6] They can protect cells from the damage caused by unstable molecules or free radicals.[7,8] An effect from free radicals can occur in the human body or from outside factors such as exposure to X-rays, ultraviolet light, air pollutants, smoking, alcohol, industrial chemicals, and cooking (burned meat, fried food).[9,10] Free radicals can produce cardiovascular disease, inflammatory disease, diabetes mellitus, neurodegenerative diseases, cancer, cataract, asthma, autism, and rheumatoid arthritis.[10-12] However, many foods or plants contain chemical constituents for eliminating free radical scavenging molecules that have hydroxyl group and a double bond, e.g., phenolic compound (phenolic acid, etc.) nitrogen compounds (alkaloids, amines, etc.) which enhance antioxidants.[12,13]

Plants in the *Lycium* genus, especially *Lycium ruthenicum*, are wildly utilized for medicines and dietary supplements in Southeast Asia, such as in China.[14,15] This plant, known as "black goji berry," belong to the Solanaceae family, a wild perennial thorny shrub.[16] The fruit of this plant was used for traditional medicine in northwestern China to treat hypotension, diabetes, abnormal menstruation, heart disease, and menopause.[16-20] Previously reported the pharmacological activities of goji berries shown immunomodulation, anti-tumor activity, anti-aging, antioxidant, anti-fatigue, anti-cardiovascular disease, and hepatoprotective activity.[19-21] The fruits of *L. ruthenicum* have been found a rich source of polysaccharides, anthocyanins, alkaloids, amides, flavonoids, and organic acid.[20,22] Chemical constituents as the bioactive compound in the Solanaceae family are dicaffeoylspermidine and dicaffeoyl spermine derivative (spermidine alkaloids), a type of alkaloid group acylated hydroxycinnamic acid amides.[20,22,23] These secondary metabolites compounds in plants are connected with caffeoyl group or caffic acid derivative and spermidine or spermine via amide bonds.[24-26] This alkaloid has been outstanding for its antioxidant properties and low toxicity.[22,27] Also, it has been treated for cardiovascular disease such as obesity, dilates, and renal abnormalities.[22]

This study aims to establish chemical profiling of spermidine alkaloids from *L. ruthenicum* by HPLC-ESI-QTOF-MS coupled with MAE and SPE methods, along with their antioxidant properties.

Methodology:

Materials and Reagents

The dried fruits of *L. ruthenicum* were purchased from China. HPLC-grade acetonitrile, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). SPE cartridge Sep-Pak Vac 35cc C18 10g obtained from Water™ (St. Milford, MA, USA). Water for LC-MS mobile phase was reverse osmosis Milli-Q water (Millipore, USA). Ethanol AR grade was purchased from Merck (Darmstadt, Germany).

Sample Preparation

The dried fruits of *L. ruthenicum* (5.0 g) were grounded and extracted under a microwave-assisted extractor with 50% methanol/water (1:10, w:v). In brief, The program was performed at 1000 W for 5 min, extraction temperature 85 ℃ to give *L. ruthenicum* extract (1.64 g). The solvent was removed under vacuum, and residue afforded a crude extract after freeze-drying. The crude extract was future separated by solid-phase extraction (SPE, 10g C_{18} cartridge, Water™, Milford, MA, USA) in a portion of 5 g dissolved in 2 mL of distilled water. Two fractions were collected in each separation eluent with 200 mL of distilled water and ethanol to obtain F1 (1.4 g) and F2 (190 mg), respectively. The solvents of each fraction were removed under vacuum distillation, and the residues were dissolved in distilled water and freeze-dried. HPLC-ESI-QTOF-MS analysis (1 mg of the sample) was dissolved with methanol and filtered with a 0.22 mm filter membrane.

Chromatographic Conditions

Agilent 1290 Infinity LC instrument (Agilent, USA) coupled to an Agilent 6540 series QTOF-MS (Agilent, USA) equipped with an ESI source, a diode-array detector (DAD), an automatic sample injector, a degasser, and a column Agilent ZORBAX Eclipse XDB column (100 x 2.1 mm i.d.; particle size 1.7 µm; Palo Alto, CA, USA) maintained at 35 ℃ was finally chosen for separation of these extracts. The mobile phase was water (0.1% formic acid, A) mixed with acetonitrile (B) at a 0.2 mL/min flow rate. The elution gradient mode was following: 0-5 min, 5% to 17% B; 5-6 min, 17% B; 6-30 min, 17% to 100% B; 30-35 min, 100% B; 35-40 min, 100% to 5% B; 40-45 min, 5% B. The injection volume was 1.0 µL and samples were set at 4 ℃.

For identification of the compounds, both positive and negative ion modes within the mass/charge (*m/z*) ratio range of 50–1000 at a resolution of 4,000 were used to confirm fragment ions in MS/MS data by energy collision dissociation (HCD, collision energy: 40, 20, 10 eV). The other parameters were as follows: gas temperature 350 ℃, drying gas flow rate 12 L min^{-1}, nebulizer gas pursuer 45 psi, sheath gas temperature 250 ℃, and sheath gas flow rate 12 Arb. Agilent mass Hunter workstation software B.08.00 was used analysis components for LC-MS/MS control and data handling.

Antioxidant Assay

DPPH radical scavenging assay

Free radical scavenging activity of the plant extract was performed according to the methodology modified.[28] The reaction of the DPPH radical scavenging activity was determined by the hydrogen atom donating ability of the extract to react with a solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH showed violet purple color in ethanol solution when the presence of antioxidants performed yellow color. A solution of 60 µM DPPH radical solution in ethanol (100 µL) was mixed with 100 µL of extract in ethanol at different concentrations. The reaction was incubated for 30 min at room temperature in the dark, and the absorbance was measured at 517 nm. Ascorbic acid was used as a positive control. The percentage of inhibitory concentration (IC_{50}) of crude extract was calculated by plotting inhibition percentages against the concentrations of extracts. Percentage DPPH radical scavenging activity was calculated as follow:

$$\% \text{ DPPH radical scavenging activity} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of DPPH radical solution, and A_1 is the absorbance of DPPH radical solution for the extractives/standard. The experiment was done in triplicate.

ABTS radical scavenging assay

The ABTS radical scavenging assay of samples was evaluated by modification.[26] The radical cations were prepared by mix seven mM aqueous ABTS with 2.45 mM potassium persulphate (1:1) in the dark at room temperature for 16-18 h before use. Dilute $ABTS^{\bullet+}$ solution was mixed with distilled water to an absorbance of 0.700 ± 0.020 at 734 nm. The reaction of $ABTS^{\bullet+}$ solution (160 µL) was added to 40 µL of sample in 50% ethanol at different concentrations, and the absorbance was recorded after 5 min at 734 nm. Ascorbic acid was used as a standard substance. The percentage inhibition and IC_{50} were calculated using the formula:

$$\% \text{ ABTS radical scavenging assay} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of $ABTS^{\bullet+}$ solution, and A_1 is the absorbance of $ABTS^{\bullet+}$ solution for tested samples/standard. All the measurements were performed in triplicates.

Total polysaccharide content

Total polysaccharide content was evaluated according to previous studies.[29] Dissolve solution of sample and glucose was prepared in distilled water. For reaction, 200 µL of 100 ug/mL sample was mixed 200 µL of 5% phenol solution and 1 mL of concentrated H_2SO_4. The absorbance was measured 10 min at 488 nm against a calibration curve with glucose standards (100-10 ug/mL).

Nitric oxide production inhibitory assay

The nitric oxide production inhibitory assay was previously described.[30] RAW 264.7 were seeded at 4 x 10^4 cell/wells in 96-well plates and incubated at 37°C under 5% CO_2 for 24 hr. Then cells were stimulated with 1 µg/mL of LPS for 1 hr and treated with different concentrations of samples for 24 h. After 24 hours, the samples were added 50 µl of Griess reagent for 10 min. The determination of nitric oxide was measured at 570 nm with a microplate reader.

Cell viability assay

The cell viability was used to determine by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Millipore Sigma) assay, as previously described.[30]

Statistical Analysis

Significant differences were analyzed using Graph-Pad Prism (GraphPad Software Inc.) by one-way ANOVA. The level of statistical significance was $p<0.05$. Agilent mass Hunter workstation software version B.08.00 (Agilent Technologies, Palo Alto, CA, USA) was used analysis components. Parameters for the program were using compound discovery workflow, molecular feature function, m/z range 50–1000, retention time (RT) range 0.1–2 min, with a minimum absolute abundance of 2000 counts. For identification of the compounds was performed by comparing specific fragments from databases including Mass Hunter PCDL Manager (Version B.08.00), Pubmed, Chemspider, MassBank, and literature data.[19,22,25]

Result and discussion:

The extraction of *L. ruthenicum* fruits was analyzed chemical constituents profile by comparison mass fragment in negative and positive ions. The MS conditions were optimized sensitivity for analysis components that key parameter is collision energies. CID represented an attractive approach for selectively yielding fragment ions. Different collision energies 10, 20 ,40 eV was tested for extraction. Abundant fragmentation ions were generated in the MS/MS spectra when the collision energy was raised to 40 eV. The result in the MS/MS spectra showed that dicaffeoylspermidine derivatives (spermidine alkaloids) were the most detected compounds in positive ion mode. According to the fragment ions and fragmentation analysis, some of the peaks in ethanol fraction (**Figure 1**) were identified as spermidine alkaloids that shown the greatest antioxidant activity. The detailed information of the 32 compounds found in fruits of *L. ruthenicum* is performed in **Table 1**.

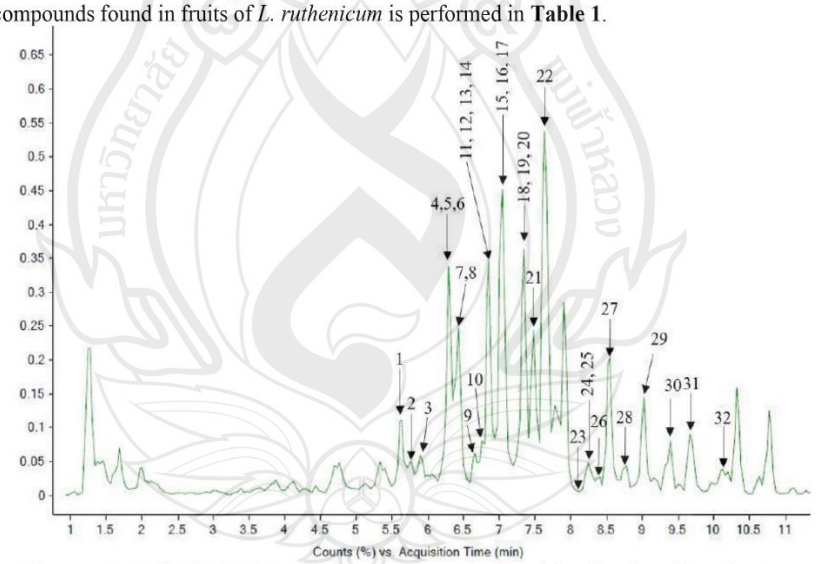

Figure 1. HPLC-ESI-QTOF-MS chromatogram of ethanol fraction from *L. ruthenicum* (positive ion)

Table 1. Compound identification from ethanol fraction of *I. ruthenicum* fruits by HPLC-QTOF-MS/MS (positive ion)

No	RT[1]	Formula	$[M+H]^+$	Mass error	MS/MS fragment ion[3]	Identification[4]
1	5.724	$C_{37}H_{53}O_{16}N_3$	796.3464	1.10	**634.2908**; 472.2582; 310.2221; 220.0947; 163.0371	N^1-caffeoyl-N^3-dhc-spermidine-di-hex
2	5.769	$C_{37}H_{53}O_{16}N_3$	798.3640	0.31	**636.3101**; 474.2568; 384.1652; 236.1263; 222.1131; 100.7360	N^1-N^3-bis-dhc-spermidine-di-hex
3	5.910	$C_{37}H_{53}O_{16}N_3$	796.3507	-0.37	**634.2981**; 472.2686; 384.1626; 310.2120; 293.1858; 234.1111; 222.1093; 163.0380	N^1-caffeoyl-N^3-dhc-spermidine-di-hex
4	6.223	$C_{25}H_{33}O_7N_3$	488.2395	0.35	308.1972; 234.1118; 220.0969; 185.5188; 172.1423; **163.0392**; 72.0811	Dihydrocaffeoyl spermidine derivative
5	6.244	$C_{31}H_{43}O_{11}N_3$	634.2962	-0.17	472.2426; 310.2102; 236.1267; **220.0966**; 163.0388	N^1-caffeoyl-N^3-dhc-spermidine-hex
6	6.325	$C_{31}H_{43}O_{11}N_3$	636.3120	0.77	474.2595; 310.2124; 293.1872; **222.1126**; 165.0550; 123.0430; 100.0752; 72.0808	N^1-N^3-dhc-spermidine-hex
7	6.405	$C_{16}H_{18}O_9$	355.1024	-0.03	**163.0392**; 145.0286; 135.0444; 117.0343	Chlorogenic acid
8	6.430	$C_{31}H_{43}O_{11}N_3$	634.2975	-1.49	474.2620; 310.2123; 293.1919; 236.1254; **222.1111**; 165.0543; 72.0807	N^1-caffeoyl-N^3-dhc-spermidine-hex
9	6.587	$C_{31}H_{39}O_9N_3$	598.2749	0.91	472.2412; 454.2351; 336.1897; 310.2142; 234.1110; **222.1126**; 163.0383	Dihydrocaffeoyl spermidine derivative
10	6.650	$C_{31}H_{43}O_{11}N_3$	634.2972	-0.03	472.2510; 310.2117; 293.1880; 236.1271; **220.0966**; 163.0392; 123.0469	N^1-caffeoyl-N^3-dhc-spermidine-hex
11	6.826	$C_{31}H_{41}O_{11}N_3$	632.2808	0.98	470.2297; 308.1952; 293.1784; 234.1130; **220.0936**; 163.0399	dhc-caffeoyl-cyclic-spermidine-hex
12	6.923	$C_{31}H_{39}O_9N_3$	598.2741	-0.14	472.2412; 455.2176; 310.2093; 23.1122; **222.1118**; 220.0962; 163.0398	Dihydrocaffeoyl spermidine derivative
13	6.930	$C_{31}H_{43}O_{11}N_3$	634.2967	0.07	472.2418; 384.1658; 310.2104; 293.1820; 234.1120; 222.1122; **163.0385**; 145.0281; 72.0807	N^1-caffeoyl-N^3-dhc-spermidine-hex
14	6.970	$C_{34}H_{35}O_7N_{11}$	580.2000	-1.54	455.1494; 293.0994; 275.0982; 231.1096; 147.0473; 129.0543; **126.0549**	Dihydrocaffeoyl spermidine derivative
15	7.033	$C_{25}H_{35}O_6N_3$	474.2564	-0.51	310.2116; 293.1895; 236.1272; **222.1124**; 165.0546; 123.0444; 100.0754; 72.0811	N^1,N^3-bis(dhc)spermidine
16	7.202	$C_{25}H_{35}O_6N_3$	474.2593	1.25	310.2176; 236.1250; **222.1121**; 165.0543; 123.0445; 100.0748	N^1,N^3-bis(dhc)spermidine
17	7.249	$C_{15}H_{22}O_3N_4$	307.1768	-0.95	177.0546; 149.0591; **145.0282**; 117.0332	Feruloylagmatine
18	7.355	$C_{31}H_{43}O_{11}N_3$	634.2963	1.27	472.2441; 310.2118; 293.1853; 291.1679; 236.1271; 234.1094; **222.1105**; 220.1105; 163.0249; 123.0413	N^1-caffeoyl-N^3-dhc-spermidine-hex
19	7.434	$C_{26}H_{33}O_5N_{13}$	576.2906	-0.94	522.2633; 414.2371; 397.2071; 331.0507; 265.1436; 236.1205; **162.0912**; 147.0459	Dihydrocaffeoyl spermidine derivative
20	7.465	$C_{25}H_{33}O_6N_3$	472.2442	-0.02	310.2131; 293.1835; 253.1521; 236.1269; 220.0961; **163.0386**; 145.0287; 72.0812	N^1-dhc-N^3-caffeoyl-spermidine
21	7.525	$C_{26}H_{35}O_6N_3$	486.2598	0.30	237.1310; 222.1114; 193.0527; 165.0540; 147.0452; 124.0460; **123.0432**	Dihydrocaffeoyl spermidine derivative
22	7.639	$C_{25}H_{33}O_6N_3$	472.2443	-0.02	293.1861; 234.1122; **222.1124**; 165.0542; 163.0390; 123.0440; 100.0750; 72.0821	N^1-caffeoyl-N^3-dhc-spermidine
23	8.193	$C_{24}H_{31}ON_3$	518.2848	-0.49	293.1792; **222.1116**; 173.1508; 163.0381; 84.0794; 72.0821	Dihydrocaffeoyl spermidine derivative

No	RT[1]	Formula	$[M+H]^+$	Mass error	MS/MS fragment ion[3]	Identification[4]
24	8.242	$C_{25}H_{31}O_6N_3$	470.2289	-0.70	308.1954; 291.1713; 234.1112; 220.0963; **163.0392**; 145.0280; 72.0813	N^1,N^3-bis(caffeoyl)spermidine
25	8.291	$C_{26}H_{37}O_6N_3$	488.2733	0.29	293.1872; **236.1277**; 222.1133; 179.0701; 165.0548; 137.0593; 123.0436; 100.0753	Dihydrocaffeoyl spermidine derivative
26	8.397	$C_{26}H_{33}O_6N_3$	484.2434	0.09	348.1928; 322.2118; 277.1550; 234.1127; **222.1116**; 163.0386; 84.0805	Dihydrocaffeoyl spermidine derivative
27	8.566	$C_{25}H_{33}O_5N_3$	456.2478	1.05	293.1828; 222.1093; 204.1012; **147.0441**; 119.0506	N^1-dhc-N^{10}-cou-spermidine
28	8.777	$C_{25}H_{33}O_5N_3$	456.2486	-0.35	253.0648; **222.1151**; 165.0671; 147.0449; 123.0495; 100.0741; 84.0804	N^1-dhc-N^{10}-cou-spermidine
29	8.992	$C_{26}H_{35}O_6N_3$	486.2593	0.60	306.0398; 293.1834; 278.0463; 236.1246; 222.1116; **177.0545**; 165.0541; 145.0291; 123.0443	Dihydrocaffeoyl spermidine derivative
30	9.170	$C_{26}H_{35}O_6N_3$	486.2592	1.62	293.1832; 278.0463; 236.1248; 222.1115; **177.0546**; 165.0540; 145.0288	Dihydrocaffeoyl spermidine derivative
31	9.580	$C_{26}H_{35}O_6N_3$	486.2590	1.85	469.2319; 336.1822; 293.1919; **222.1133**; 177.0528; 123.0433	Dihydrocaffeoyl spermidine derivative
32	10.164	$C_{34}H_{46}O_8N_4$	639.3387	0.42	512.2678; 439.2713; 383.1984; 293.1861; 291.1703; 236.1265; **222.1115**; 219.1484; 191.0543; 165.0534; 150.0906; 123.0436	Dihydrocaffeoyl spermidine derivative

[1] RT represent retention time (min)
[2] Mass error (ppm) show chemical formula was determined using a mass difference tolerance of ± 5 ppm
[3] MS/MS fragment show fragmentation ion which the number in **bold** represent the most abundant product ion.
[4] Abbreviations: dhc, dihydrocaffeyl; hex, hexose; cou, *p*-coumaroyl

Compound **2** had the $[M+H]^+$ at *m/z* 798.3640 ($C_{37}H_{55}O_{16}N_3$, cal. 798.3655, mass error 0.31 ppm) and obtained of fragment ion at *m/z* 636.3101, 474.2568, 222.1131 and 100.7360. The ion at *m/z* 636 was observed by the loss hexose unit. The one ion *m/z* 474 is performed after the loss of two glucosyl units. The fragment ion *m/z* 310 and 163 were formed by the cleavage of the caffeoyl unit. The fragment ion *m/z* 310 and 165 were formed by the cleavage of the dihydrocaffeoyl unit. The ion at *m/z* 293 resulted in a loss of the ammonia unit. The ion *m/z* 222 was formed by the cleavage of a rearrangement ion at *m/z* 72. The one ion *m/z* 123 was caused after losing of molecule ion at *m/z* 100 from fragment ion *m/z* 222. So, compound **2** was characterized as N^1-N^3-bis-dihydrocaffeyl-spermidine-di-hexose.

Compound **3** had molecular ion $[M+H]^+$ at *m/z* 796.3507 ($C_{37}H_{53}O_{16}N_3$, cal. 796.3499, mass error -0.37 ppm) and fragment ions at *m/z* 634.2981, 472.2686, 384.1626, 310.2120, 293.1858, 234.1111, 222.1093 and 163.0380. The ion at *m/z* 634 $[M+H-C_6H_{10}O_5]^+$ and *m/z* 472 $[M+H-C_6H_{10}O_5-C_6H_{10}O_5]^+$ were performed by the loss of one and two hexose units, respectively. The fragment ion *m/z* 310 was formed by the cleavage of the caffeoyl unit (163 Da). The ion at *m/z* 293 was detected with loss ammonia unit from the ion at *m/z* 310. The ion at m/z 222 was presented by leaving a rearrangement ion at m/z 72 from molecule ion m/z 293. The ions at m/z 220 and 253 were produced from another pathway after the ion 253 appeared a 234 by the loss of ammonia unit. In the same fragment ion with compound 1. Thus, compounds **1** and **3** were identified as N^1-caffeoyl-N^3-dihydrocaffeyl-spermidine-di-hexose.

Compound **5** had a $[M+H]^+$ at *m/z* 634.2962 ($C_{37}H_{43}O_{11}N_3$, cal. 634.2970, mass error -0.17 ppm) and produced fragment ions at *m/z* 472.2426, 310.2102, 236.1267, 220.0966 and 163.0388. The one ion *m/z* 472 $[M+H-C_6H_{10}O_5-C_6H_{10}O_5]^+$ is performed after the loss of two glucosyls. The fragment ion *m/z* 310 and 163 were resulted from the leave of the caffeoyl unit. The ion at *m/z* 293 was generated of ammonia unit from the ion at *m/z* 310. The ion at *m/z* 222 was produced by leaving a cyclization ion at *m/z* 72 from molecule ion *m/z* 293. The ions at m/z 220 and 253 were observed from another pathway after the ion 253 appeared a 236 by the cleaving of the ammonia unit. Compounds **8**, **10**, **13**, and **18** had the same $[M+H]^+$ at *m/z* 634, and In consequence, these compounds were confirmed to be N^1-caffeoyl-N^3-dihydrocaffeyl-spermidine-hexose.

Compound **6** had a $[M+H]^+$ at *m/z* 636.3120 ($C_{37}H_{45}O_{11}N_3$, cal. 636.3127, mass error 0.77 ppm) and yielded molecule ions at *m/z* 474.2595, 310.2124, 293.1872, 222.1126, 165.0550, 123.0430, 100.0752 and 72.0808. The ion at *m/z* 474 $[M+H-C_6H_{10}O_5]^+$ were detected by the hexose unit from the ion at *m/z* 636. The ion *m/z* 310 appeared after the loss of ion at *m/z* 165. The ion at *m/z* 293 was indicated when losing the ammonia unit from molecule ion at *m/z* 310. The ion at *m/z* 222 was presented by the leaving of a rearrangement ion at *m/z* 72. The fragment ion at *m/z* 123 was generated by losing the substitution rearrangement production ion at *m/z* 100 from the ion at *m/z* 222. Thereby, Compound **6** was assigned as N^1-N^3-dihydrocaffeyl-spermidine-hexose.

Compound **11** had molecular ion $[M+H]^+$ at *m/z* 632.2808 ($C_{37}H_{45}O_{16}N_3$, cal. 632.2893, mass error -0.37 ppm) and seven fragment ions at *m/z* 470.2297, 308.1952, 293.1784, 234.1130; 220.0936, and 163.0399. The ion at *m/z* 470 was formed by the loss of the sugar molecule (162 Da). The fragment ion *m/z* 308 was indicated when loss of caffeoyl unit (163 Da). The ion at *m/z* 293 was detected after the loss ammonia unit from the ion at *m/z* 308. The ion at *m/z* 220 was appeared by the loss of a rearrangement ion at *m/z* 72 from molecule ion *m/z* 291. The ions at m/z 220 and 253 were produced from another pathway after the ion 253 appeared a 234 by the loss of ammonia unit. As a result, compound **11** was determined to be dicaffeoyl-caffeoyl-cyclic-spermidine-hexose.

Compound **15** had molecular ion $[M+H]^+$ at *m/z* 474.2564 ($C_{25}H_{35}O_6N_3$, cal. 474.2599, mass error -0.51 ppm) and obtained of fragment ion at *m/z* 310.2116, 293.1895, 236.1272, 222.1124, 165.0546, 123.0444, 100.0754 and 72.0811. The fragment ion *m/z* 310 and 165 were formed by the cleavage of the dihydrocaffeoyl unit. The ion at *m/z* 293 was generated by the loss of the ammonia unit. The ion at *m/z* 222 was detected when cleavage of a rearrangement ion at *m/z* 72 from molecule ion *m/z* 293. The one ion at *m/z* 123 was produced after losing the substitution cyclization molecule ion at *m/z* 100 from the ion at *m/z* 222. The ions at m/z 220 and 253 occurred from another pathway after the ion 253 presented a 234 by the loss of ammonia unit. Compound **16** had similar $[M+H]^+$ and fragment ions with compound **15**. In this way, compounds **15** and **16** were recognized as N^1,N^3-bis(dicaffeoyl)spermidine.

Compound **20** had a $[M+H]^+$ at *m/z* 472.2442 ($C_{25}H_{33}O_6N_3$, cal. 472.2441, mass error -0.02 ppm) and fragment ions at *m/z* 310.2131, 293.1835, 253.1521, 236.1269, 220.0961, 163.0386, 145.0287 and 72.0812. The ion at *m/z* 310 and 163 was yielded after the loss of the caffeoyl unit from the protonated parent ion. The ion at *m/z* 293 was presented with a loss of ammonia unit from the ion at *m/z* 310. The cleavage of a rearrangement ion caused the ion at m/z 222 and 72 from molecule ion m/z 293. The ions at m/z 220 and 253 were produced from another pathway after the ion 253 appeared a 236 by the loss of ammonia unit. Compound **22** was exhibited a similar fragmentation ion with compound 22. Therefore, Compounds **20** and **22** were proposed to be N^1-dicaffeoyl-N^3-caffeoyl-spermidine.

Compound **24** had molecular ion $[M+H]^+$ at *m/z* 470.2289 ($C_{25}H_{31}O_6N_3$, cal. 470.2283, mass error -0.70 ppm) and produced fragment ions at *m/z* 308.1954, 291.1713, 234.1112, 220.0963, 163.0392, 145.0280 and 72.0813. The fragment ion *m/z* 308 was formed by the cleavage of the caffeoyl unit (163 Da). The ion at *m/z* 291 was observed by the loss ammonia unit from the ion at *m/z* 308. The ion at *m/z* 220 was produced by the loss of a rearrangement ion at *m/z* 72 from molecule ion *m/z* 291. Thus, Compound **24** was suggested as N^1,N^3-bis(caffeoyl)spermidine.

Compound **28** had a $[M+H]^+$ at *m/z* 456.2486 ($C_{25}H_{33}O_6N_3$, cal. 456.2493, mass error -0.35 ppm) and yielded molecule ions at *m/z* 253.0648, 222.1151, 165.0671, 147.0449, 123.0495, 100.0741, 84.0804. The ion at *m/z* 293 appeared after cleavage of the caffeoyl unit (163 Da) from the protonated parent ion. The fragment ion at *m/z* 222 and 123 was indicated by the leaving of rearrangement ion at *m/z* 72 and 100, respectively. From another pathway, the one ion at *m/z* 147 was formed by the loss of a coumaroyl group from the protonated parent ion. In the same manner, compound 27 was showed $[M+H]^+$ similar to compound 28. Hence, Compounds **27** and **28** were presented to be N^1-dicaffeoyl-N^{10}-coumaroyl-spermidine.

In this plant, *L. ruthenicum* can found phenolic amide compounds, which detected fragment ions at *m/z* 177 (feruloyl group), 163 (caffeoyl group), and 147 (coumaroyl group).[26,31] Compound 7 had a $[M+H]^+$ at *m/z* 355.1024 ($C_{16}H_{18}O_9$, cal. 355.1039, mass error -0.03 ppm) and four fragment ions at *m/z* 163.0392, 145.0286, 135.0444 and 117.0343. This compound was identified as Chlorogenic acid. The ion at *m/z* 163 was formed by loss of caffeoyl group from molecule ion. The one ion *m/z* 135 was presented with the leaving of CO (28 Da). The ion at *m/z* 145 and 117 were produced by the loss of a water molecule from the ion at *m/z* 163 and 135, respectively. Compound 17 was confirmed to be feruloylagmatine with $[M+H]^+$ at *m/z* 307.1768 ($C_{15}H_{22}O_3N_4$, cal. 307.1772, mass error -0.95 ppm) and generated fragment ions at *m/z* 177.0546, 149.0591, 145.0282, and 117.0332. The one ion at *m/z* 177 was observed after the loss of the agmatine (130 Da) from the ion at *m/z* 307. The ion at *m/z* 145 and 117 were detected by leaving molecule CH_4O and CO from molecule ion at *m/z* 177 and 145, respectively.[32]

In addition, compound **4**, **9**, **12**, **14**, **19**, **21**, **25**, **26**, **29**, **30**, **32** and **33** were presented to be dicaffeoylspermidine derivative. The dicaffoylspermidine can be performed by abundant fragment ion peak at *m/z* 310/308, 293/291, 222/220, or 165/163.26. The key characteristic for this group of compounds was obtained of fragment ion at *m/z* 222 and 220.[22] Summary, spermidine alkaloids were appeared by fragment ion at *m/z* 310/308 was formed by the cleavage of one caffeoyl unit, 293/291 was detected with loss ammonia unit, 222/220 represented the leaving of a rearrangement ion at *m/z* 72, and 165/163 was formed by further neutral loss of the spermidine unit. The possible fragmentation of dicaffeoylspermidine derivative was proposed in **Figure 2**.

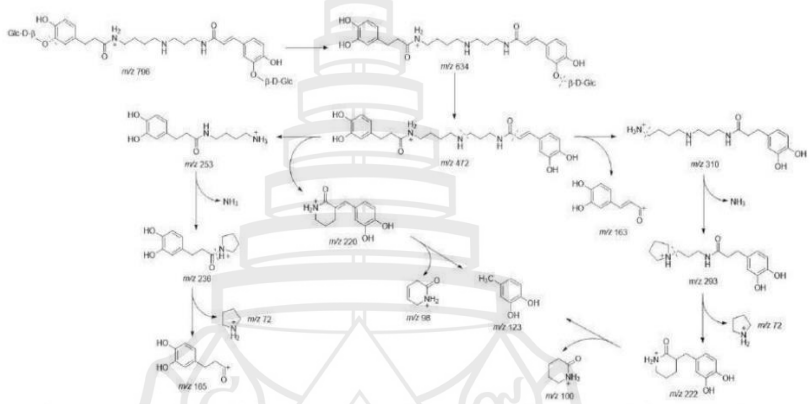

Figure 2. The proposed fragmentation pathway of dicaffeoylspermidine derivatives.

The antioxidant assay showed that ethanol fraction obtained the greatest value of IC_{50} at 2.04 ± 0.02 µg/mL and 4.70 ± 0.28 µg/mL in DPPH and ABTS assay, respectively. Water fraction preformed IC_{50} > 100 µg/mL and 78.37 ± 1.12 µg/mL while ascorbic acid showed 1.53 ± 0.02 µg/mL and 2.92 ± 0.02 µg/mL for DPPH and ABTS assay, respectively (**Table 2**). In addition, the result significant differences in water fraction, which showed low antioxidant properties. From this study was confirmed that the extract which contains a large amount of spermidine alkaloid showed the strongest antioxidant activity.[26]

Table 2. Antioxidant activities of extracts from *L. ruthenicum* fruits

Extracts	DPPH (IC_{50}, µg/mL)[1]	ABTS (IC_{50}, µg/mL)[2]
Crude extract	27.26 ± 1.62	76.01 ± 1.23
Part ethanol	2.04 ± 0.02	4.70 ± 0.28
Part water	> 100	78.37 ± 1.12
Ascorbic acid[3]	1.53 ± 0.02	2.92 ± 0.02

[1] DPPH (IC_{50}) represents the extract concentration scavenging 50% of DPPH radical
[2] ABTS (IC_{50}) represents the extract concentration scavenging 50% of ABTS radical
[3] represent the positive control; Results are expressed as means ± SD, n=3.

Nitric oxide (NO) are a highly reactive free radical however overproduction of NO cause many diseases in pathogenesis.[33,34] In this study, nitric oxide production, the crude extract of *L. ruthenicum* from microwave extraction showed %NO inhibition at 56.50 ± 0.84% (concentration at 200 µg/mL) while fraction part ethanol and water exhibited inactive (%NO inhibition < 50%). The IC_{50} value of crude extract showed 161.4 µg/mL more than the positive control, indomethacin at 34.67 µg/mL (**Table 3**). A previous study of significant

compounds from *L. ruthenicum* is polysaccharides for extract not found in this group.[20,22] Maybe because the column from the analyst cannot separate the mixtures of polysaccharides.[35,36] Therefore, it is necessary to test the quantity of polysaccharides content. Due to, polysaccharide content of crude extract, water, and ethanol fraction was evaluated as 80.63±3.82, 70.42±2.02, and 67.32±1.23 glucose equivalent µg/100µg of the sample, respectively. Moreover, the polysaccharide content of the crude extract was significantly higher than the other fractions. This study confirmed that the polysaccharide-contained extract presented the potential to inhibit the production of nitric oxide.[37,38]

Table 3. Nitric oxide production inhibitory of extracts from *L. ruthenicum* fruits

Samples	Concentration (µg/mL)	% NO inhibition	IC_{50} (µg/mL)	% cell viability
Crude extract	12.5	2.19 ± 0.69	161.4	98.62 ± 0.95
	25	5.74 ± 0.47		97.17 ± 1.21
	50	10.93 ± 1.22		97.10 ± 0.55
	100	28.87 ± 2.98		95.80 ± 1.60
	200	56.50 ± 0.84		95.32 ± 0.96
Indomethacin	6.25	3.95 ± 2.69	34.67	99.61 ± 2.24
	12.5	17.24 ± 3.48		92.66 ± 1.18
	25	38.36 ± 276		88.43 ± 1.30
	50	62.15 ± 3.28		85.36 ± 1.13
	100	96.07 ± 1.83		81.37 ± 0.72

Conclusions:

The fruits of *L. ruthenicum* were extracted by microwave-assisted extraction (MAE) and C_{18} solid-phase extraction for antioxidant compounds. In the study, ethanol fraction was performed as the most potent antioxidant activity in DPPH and ABTS assay. The chemical constituents in this part have been investigated by high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS/MS). Dicaffeoyl spermidines derivatives were identified 30 compounds from 32 compounds in ethanol fraction, for the study was to evaluate the physiological activity of *L. ruthenicum* to investigate their potential as a raw material that can be used in the cosmetic industry.

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