CHEMICAL CHARACTERIZATION OF METABOLITES OF MEDICINAL PLANTS USING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC-MS) AND GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS)

GENET MINALE YESHANEW

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in (Pharmaceutical Sciences)
2020

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By GENET MINALE YESHANEW

has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Pharmaceutical Sciences of Naresuan University

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Title	CHEMICAL CHARACTERIZATION OF METABOLITES OF MEDICINAL PLANTS USING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC-MS) AND GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS)
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ABSTRACT

The objectives of this study were to characterize metabolites of medicinal plants using Liquid Chromatography Mass Spectrometry (LC-MS) and Gas Chromatography and Mass Spectrometry (GC-MS). *Centotheca lappacea* (L.) Desv. and *Bacopa monnieri* (L.) Wettst. were selected as model plants for the study.

C. lappacea or "Ya *hee* yum" a grass in Poaceae family that has long been used in Thailand for postpartum care of women. Elemental analysis was undertaken using Wavelength Dispersive X-ray fluorescence, identification of antioxidants was done with online liquid chromatography–electrospray ionization-quadrupole-time of flight-mass (LC-ESI-QTOF-MS) spectrometer coupled with DPPH assay, and the volatile constituents of *C. lappacea* were investigated using headspace GC-MS. The elemental analysis showed the presence of macro and micro elements including silicon, potassium, and calcium. Twelve antioxidants, including *C*-and *O*-glycosides of luteolin and apigenin, were identified from the 50 % ethanolic extract of the aerial parts of the plant. Nineteen compounds were identified in the roots and twenty-five

compounds were found in the aerial parts of *C. lappacea*. The sample material from both the roots and aerial parts were directly burned using a headspace GC-MS in the same process of obtaining smoke during traditional treatment. The antioxidants and volatile constituents in the *C. lappacea* plant material are reported for the first time in this study.

B. monnieri or Brahmi has long been proven to improve memory and intellect. Brahmi essence has been developed for many years from *B. monnirei* standardize extract and mulberry juice to be used as a dietary supplement. In our study, a group of elderly healthy participants were given a course of Brahmi essence over a 12-week period, and their speed of memory was found to have significantly improved over that period. In this study, untargeted LC-MS metabolomics approach was used to characterize metabolites found in plasma, urine and feces that might relate to and contributed to this outcome. From OPLS-DA plots, 15 features (metabolites) were tentatively identified in the plasma, 7 in urine and 17 in feces samples by comparing with data in an on-line data base and in the literature. The metabolites in the plasma samples were in the class of amino acids and their derivatives, acylcarnitine and phospholipids, which were mainly involved in aminoacyl-tRNA biosynthesis, aromatic amino acid biosynthesis and branched chain amino acid biosynthesis pathways. Benzeneactamide-4-O-sulphate and 3-hydroxyhippuric acid together with other metabolites were tentatively identified in urine samples. Amino acids and their derivatives were tentatively identified in fecal samples and the altered metabolic pathways in the fecal samples were similar to those of the plasma samples. Also, jujubogenin/isomer and pseudojujubogenin aglycones were tentatively identified in the feces samples. These metabolites might be directly or indirectly involved with the improvement of speed of memory.

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LIST OF ABBREVIATIONS

BME	=	Standardized Brahmi Extract
CE	=	Capillary electrophoresis
ChEBI	=	Chemical Entities of Biological Interest
DPPH	=	1,1 diphenyl-2-picrylhydrazyl
ESI-MS/MS	=	Electrospray ionizer Tandem mass Spectrometry
FRAP	=	Ferric Reducing Antioxidant Potential
GC-MS	=	Gas Chromatography-Mass Chromatography
HILIC	=	Hydrophilic Interaction Chromatography
HMDB	=	Human Metabolome Database
HPLC	=	High Performance Liquid Chromatography
KEGG	=	Kyoto Encyclopedia of Genes and Genomes
KI	=	Kovates retention indices
LC-ESI-QTOF-MS	=	Liquid Chromatography Electronspray ionizer Quadrapole Time of Flight
LC-MS	=	Liquid Chromatography-Mass Chromatography
LLE	=	Liquid-Liquid Extraction
LTQ-Orbitrap	=	Linear Quadrupole Ion Trap-Orbitrap
MS	=	Mass Spectrometry
MS/MS	=	Tandem mass spectrometry
NIST	=	National Institute of Standard and Technology
NMR	=	Nuclear Magnetic Resonance
OPLS-DA	=	Orthogonal Partial Least Squares Discriminant Analysis
PCA	=	Principal Component Analysis
PLS-DA	=	Partial Least Squares Discriminant Analysis
PP	=	Protein precipitation
SPE	=	Solid-Phase Extraction
TBARS	=	Thiobarbituric acid reactive substance
TIC	=	Total Ion Current
WDXRF	=	wavelength dispersive X - Ray Fluorescence

CHAPTER I

INTRODUCTION

Background and Rationale

The healing power of medicinal plants is as old as mankind and its use has been recorded in writings since as early as 5000 B.C. Recorded evidence also comes from the Indian Rig-Veda which was written during the period 1600 - 3500 B.C, and the Sumerian clay tablets from about 3000 B.C., as well as the Chinese Pen T' Sao, and the Egypt Ebers Papyrus, all of which mentioned the use of medicinal plants and their preparation [1]. The biomedical advantages of medicinal plants and plant preparations, such as safety, efficacy, and cost, have seen these traditional medicines applied in primary healthcare, especially in rural areas of developing countries [2]. Due to the decrease in the effectiveness of synthetic drugs and their increasing negative side effects, herbal medicine usage is increasing worldwide. The contribution of medicinal plants as a curative extends from the raw material through to their final form as a drug in the pharmaceutical sector [3]. They can also be used as a lead compound [4]. Also, traditional medications have played a significant role in the cultural practices of many civilizations.

Phytoconstituents (metabolites) which are present at various levels play a major role in their power of healing and their use in different biomedical applications. Metabolites are small molecules that usually have a molecular weight less than 1500 Da and are produced as either the intermediate or end product of metabolism [5]. Plants synthesize vast and diverse organic compounds containing metabolites that are important for the plant's survival. These are referred to as primary metabolites. However, the majority of metabolites, known as secondary metabolites, do not have a direct role in plant growth and development [6] but are responsible, importantly, for the biological activities of medicinal plants.

Metabolites are very complex in terms of both chemical diversity and quantities. Currently available analytical technologies are not able to detect all metabolites in one single analysis. However, the advance of those technologies and

their integration with data analysis techniques provide a solution in the analysis of metabolites. Mass spectrometry (MS), for example, is now one of the most popular analytical technologies for the analysis of small molecules [7]. The power and separation efficiency of MS is increased when it is coupled with other spectroscopic techniques such as gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE). In our research, we focused on applying these combined techniques (GC-MS and LC-MS) for the analysis of metabolites found in two model medicinal plants: *Centhotheca lappacea* (L.) Desv. and *Bacopa monnieri* (L.) Wettst.

GC-MS is used to analyze volatile compounds such as fatty acids, phenolic compounds, organic acids, sterols, catecholamines, drugs etc. It is highly sensitive, reproducible and specific [8]. The sample preparation techniques prior to GC-MS analysis are important. Direct injection of the sample and headspace sampling are the methods for introducing samples into the GC-MS system. Headspace sampling enables the extraction of volatile compounds from mixtures. In this sampling technique, the sample is held in a sealed vial which is directly heated to a high temperature and the resulting vapor is injected into the GC-MS system. This sampling technique has the advantage of avoiding the extraction step and reducing impurities which improves separation and identification of the volatile compounds [9, 10].

The other combined analytical technique is LC-MS, a popular technique that can cover a wide mass range with high sensitivity [11-14]. It provides separation of components from the sample by their interaction between the liquid mobile phase and the stationary phase. Reversed-phase columns, C8 or C18, are used for both polar and non-polar analytes such as lipids whereas Hydrophilic Interaction Chromatography (HILIC) is used mostly for polar metabolites [15]. LC-MS is widely applied in different fields of research such as drug development, quality control of drugs, and metabolomics.

Metabolomics is a non-selective comprehensive approach that is used to identify and quantify metabolites (low molecular weight compounds) in a biological system. It is classified as a targeted and untargeted metabolomics approach [16]. The targeted metabolomics approach is used to quantify or semi-quantify known metabolites in given samples whereas a nontargeted metabolomics approach relies on

the identification of as many unknown metabolites as possible in a biological sample [17] Due to the complexity of metabolites, both in terms of their chemical diversity and quantity, up to now no single analytical techniques has been available to analyze them. The two most widely used analytical tools that are used in metabolomics are nuclear magnetic resonance (NMR) and MS [18, 19].

C. lappacea, commonly known as ya hee yum or repair grass, is a plant in the Poaceae (Gramineae) family which has been used in Thai traditional medicine to treat women after labor. Traditionally, repair grass is placed in a hot pot and put under a chair with the hole on which the postpartum woman sits. The rising fumes are believed to heal the wounds in the labia and strengthen the respective muscles. It is also claimed to be an energy restorant when it is prepared as a herbal tea. A previous report showed that *C. lappaceae* demonstrates antioxidant activity and has antiaging properties [20]. Phenolic compounds, fatty acids, phytosterols, and flavonoids have been reported from these plants. 4-coumaric acid and 5, 7, 4'-trimethoxyflavone were also isolated from the aerial part of the plant. [21]. However, there have been no reports published on the analysis of repair grass when directly heated, as has been a traditional method of use. In our study, we used headspace GC-MS to analyze the volatile constituents of repair grass which are responsible for the biological activity of this plant.

B. monnieri is a medicinal plant, a creeper, in the family of Plantaginaceae with the common name Brahmi or Bacopa which has been known for a long time for its effect on the central nervous system [22]. Its effect on the central nervous system (CNS) was discussed in ancient Ayurvedic texts, such as Charaka Samhita (2500 B.C.) and Sushruta Samhita (2300 B.C.), and it was included in many preparations [23, 24]. Brahmi has been on the list of drugs for the treatment of anxiety, poor cognition, and lack of concentration [25], and has shown numerous pharmacological activities as an antioxidant [26], neuroprotective [27, 28], with hypotensive effect [29], anti-cholinesterase [30], antihyperglycemic [31-33], antiepileptic [34], hepatoprotective [35, 36] and an anti-inflammatory [37-39]. Brahmi exhibits its pharmacological activities with fewer signs of toxicity as reported in both animal and human studies [40-42]. The chemical constituents of Brahmi are responsible for the pharmacological activities.

There are many classes of compounds from *B. monnieri* reported to date, but the biological activity mainly stems from the dammarane triterpenoid saponins glycosides; bacosides with jujubogenin, and pseudojujubogenin as aglycone [43, 44]. The jujubogenin glycosides (bacoside A3, bacopaside X) and pseudojujubogenin glycosides (bacopaside I, bacopaside II, bacopasaponin C) are the five major saponins in these plants [45]. Moreover, bacosides I-XIII, bacoside A1-A6, bacopasaponin A-H, bacopaside N1, and N2 were reported to be in the ethanolic extract of this plant by Nuengchamnong, et al. (2016) [44]. Flavonoids: apigenin, luteolin, and quercetin, alkaloids: brahmine, nicotine, and herpestine with D-mannitol, hersaponin, monnierasides I–III, cucurbitacins, and plantainoside B are also present in the plants [46, 47]. Bacoside A, a mixture of four saponins: bacoside A3, bacopaside X, bacopaside II, bacopasaponin C with bacopaside I, showed antidepressant activity in mice model [48]. The metabolites of bacoside A, aglycone and their derivatives, are well absorbed by the intestine and able to pass through the blood-brain barrier (BBB) and exert a cognitive effect by binding to the muscarinic (M1) and serotonin (5-HT2A) receptors [49].

There have been various clinical studies on the effect of Brahmi on cognitive functions. A recent randomized clinical, double-blind placebo-controlled clinical trial by Peth-Nui et al. (2012) [50] showed that one Brahmi tablet per day, containing 300 mg of standardized Bacopa extract, improved working memory in elderly healthy human volunteers. Also, 600 mg of Brahmi Tablet reduces depression symptoms [50]. In Thailand, Brahmi Tablets that contain 300 mg of standardized Brahmi extract were licensed and launched into the market by the Thai Governmental Pharmaceutical Organization (GPO) as a dietary supplement.

As an alternative for users, Brahmi concentrated essence has been formulated from Brahmi standardized extract and mulberry juice. The amount of Bacopa extract was the same as in the Brahmi Tablet. The Randomized clinical trial on human elderly healthy volunteers by Assoc. Prof. Dr. Krongkarn Chootip and her research team from the Faculty of Medical Science at Naresuan University, Thailand. showed that, the Brahmi essence increased speed of memory and improved both carotid and cerebral blood flow without showing any sign of toxicity [51]. Previously, there have been no reports on the effect of the metabolite content of Brahmi essence on humans.

Therefore, the objective of our research was to analyze the metabolites found in the plasma, urine, and feces collected from healthy volunteers after consumption of Brahmi essence over 12 weeks, by using the LC-ESI-QTOF-MS metabolomics approach. The purpose was to identify the metabolic biomarker of Brahmi essence which support the clinical use of the essence and to provide a better understanding of its physiological effect

Objectives of the study

To chemically characterize the metabolites of medicinal plants using LC-MS and GC-MS. The model plants were *C. lappacea* and *B. monnieri*.

The objectives for each plant were

C. lappacea

1. To identify the chemical constituents of *C. lappacea* using headspace GC-MS and to also identify the antioxidant compounds in this plant using online LC-MS coupled to DPPH assay.

B. monnieri

1. To characterize the metabolites in the plasma, urine, and feces samples from healthy volunteers after taking Brahmi essence over 12 weeks using the LC-ESI-QTOF-MS metabolomic approach.
2. To identify the biomarkers and identify the metabolic pathways.

Research Scope

This study was focused on using the hyphenated MS techniques (GC-MS and LC-MS) for the characterization of metabolites in plant extract and in biological samples. The volatile constituents of *C. lappacea* were identified by headspace sampling of GC-MS and the antioxidant compounds were identified using online DPPH assay coupled with LC-ESI-QTOF-MS (**Figure 1**). The metabolites in plasma, urine, and feces samples of healthy volunteers after taking Brahmi essence were analyzed using the LC-ESI-QTOF-MS metabolomic approach. The metabolic pathways were predicted by using the Metaboanlyst 4.0 software (**Figure 2**).

Keywords

GC-MS, LC-MS, *Centotheca lappacea*, *Bacopa monnieri*, metabolites, metabolomics, Brahmi essence.

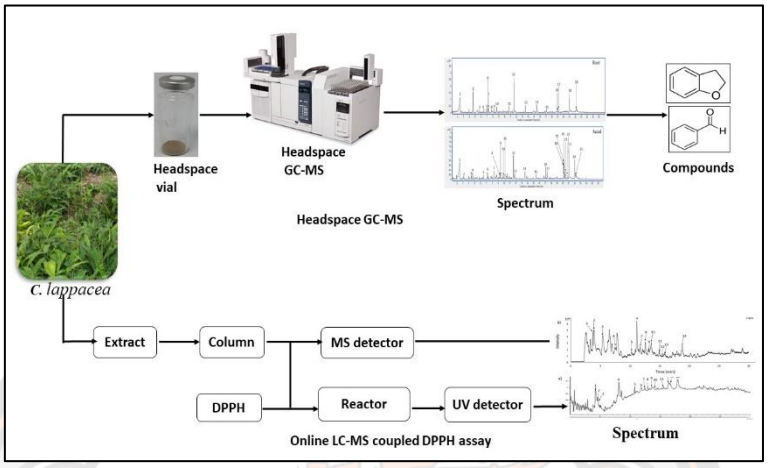

Figure 1 Headspace GC-MS analysis and online LC-MS coupled DPPH assay of *C. lappacea*

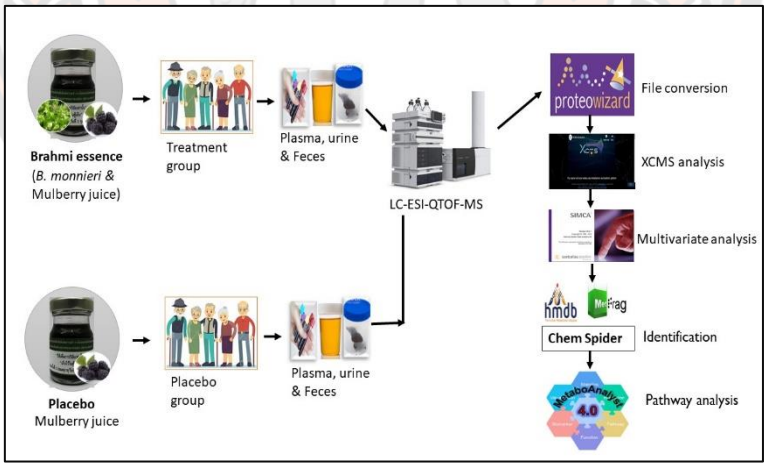

Figure 2 LC-ESI-QTOF-MS analysis of Brahmi essence in human biological samples using a metabolomics approach

Research Hypothesis

1. Direct analysis of *C. lappacea* using headspace GC-MS able to detect compounds which represent chemical constituents in the fume of the plant in traditional use.

2. Antioxidant compounds from *C. lappacea* can be identified using online LC-MS coupled with DPPH assay

3. LC-MS metabolomics approach can be able to detect the metabolites in plasma, urine, and feces

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CHAPTER II

LITERATURE REVIEW

Natural products, which includes plants, animals, and microorganisms, have a long history of use in traditional remedies. In medicine and medical science, natural products play a crucial role, especially as raw materials, lead compounds, or as markers in new drug discoveries [1]. According to the Food and Drug Administration (FDA), 9.1% of the 1562 approved drugs are derived from medicinal plants [2]. The chemical compounds found in many plants (phytoconstituents), for example, demonstrate various biological activities that have led to drug discoveries and development. Importantly, a robust and sensitive analytical technique that can characterize and quantify those compounds (metabolites) is required. Spectroscopic techniques such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) are foremost techniques in drug development.

Analytical techniques that used in the characterization of metabolites

Nuclear magnetic resonance (NMR)

NMR is an analytical platform that has been used in the search for drugs from natural sources as it can provide a great amount of chemical information [3]. The spinning of the atomic nuclei in a magnetic field is the basis of this technique which is a reproducible and non-destructive technique that provides quantitative information about the compounds. The drawback of this technique is poor sensitivity and poor spectral resolution (minimal spectral distance between two peaks) that might hamper the identification of metabolites from a sample matrix [4, 5]. However, sample preparation in NMR analysis is relatively easy and simple which can be used in the analysis of bulk samples [6].

Mass spectrometry (MS)

Mass spectrometry is the analytical technique that is mostly used for the detection, quantification, and structural elucidation of compounds [7, 8]. The high sensitivity and reproducibility, with a wide dynamic range, make these techniques the

best choice for qualitative and quantitative measurements of compounds (metabolites) at the molecular level [9].

The first step in the analysis of samples by mass spectrometry is the conversion of the components of the sample to ions by appropriate ionization techniques in an ionizer (Figure 3). The ions are formed from neutral molecules through electron ejection, electron capture, protonation, deprotonation, or adduct formation. Electrospray ionization (ESI) and electron impact (EI) ionization are the most widely techniques used in mass spectrometry. ESI ionizes small (metabolites) to large molecules (proteins, peptides) with less fragmentation of the molecular ion in the liquid phase whereas EI ionizes molecules in the gaseous phase and causes significant fragmentation [10].

The ionized molecules are transferred to the mass analyzer where ions are separated based on their mass to charge ratio (m/z) in time or space. Linear quadrupole, quadrupole ion trap, Time of Flight, Fourier transform ion cyclotron resonance and Orbitrap are the different types of mass analyzers [11, 12]. Tandem mass spectrometry (MS/MS) is a method that incorporates a two-step mass analysis. The first analyzer is used to separate the precursor ion followed by the second analyzer which analyzes this ion to product ions and neutral fragments. Quadrupole ion trap (QTrap), triple quadrupole (TQ), quadrupole-Time of Flight (Q-TOF), and linear quadrupole ion trap-Orbitrap (LTQ-Orbitrap) are configured in MS/MS [13].

Finally, the detector creates an electric current that is proportional to the ions that strike it and the current is measured as a signal. The plot, the spectrum, shows the mass to charge ratio (m/z) versus the abundance or intensity of each ion obtained from the recorder [14].

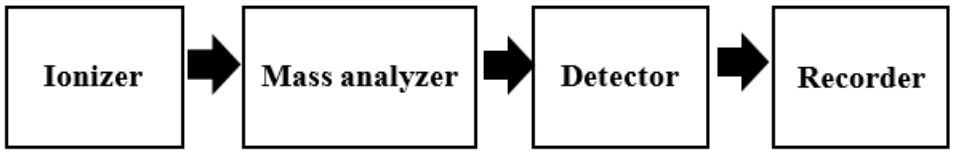


Figure 3 Scheme of mass spectrometry

A sample is introduced to the MS system by direct injection or through coupling with chromatography techniques such as gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) [12]. The coupling can also enhance the sensitivity of the mass spectrometry by separating the components before they are eluted to the mass spectrometry which reduces the complexity of identification and quantification of compounds.

Gas chromatography Mass Spectrometry (GC-MS)

Gas chromatography mass spectrometry is a powerful technique that couples gas chromatography with mass spectrometry (**Figure 4**) which is simple, sensitive, and effective in analyzing volatile components directly from a mixture [15]. The sample should be volatile or should be converted to be volatile by a derivatization technique to enable analysis by GC-MS.

The GC-MS instrument consists of a carrier gas, usually hydrogen or helium, that transfer the sample from the injection port through a column, ionizer, and mass analyzer to the detector. The column is usually a capillary column that is placed inside an oven which is used to separate the components from the mixtures. The partitioning of the components in the mobile phase and the stationary phase governs the separation process. There is an interface that connects the gas chromatogram to the mass spectrometry. The open interface is usually a deactivated fused silica capillary tube and the closed interface is a vacuum sealed joint, both of which are commonly used interfaces in GC-MS [16].

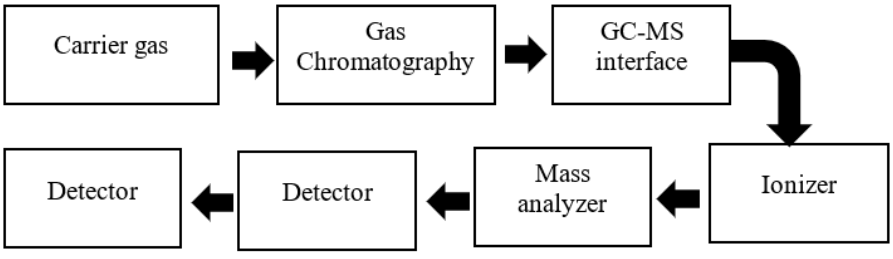


Figure 4 Block diagram of gas chromatography mass spectrometry

The sample preparation techniques are very important in GC-MS analysis. Careful sample preparation can eliminate the interference of polar (ionic) or high molecular weight components. Distillation, extraction, and headspace are some of the methods of sample preparation. Distillation separates the various components of the mixture based on the difference in volatility or vapor pressure of their components. Extraction techniques are based on the solubility of components in a solvent or their adsorption properties on materials such as activated carbon or silica gel [17]

Headspace sample preparation techniques can extract volatile components by partitioning between nonvolatile liquid or solid phase and the vapor phase (headspace) [18]. Static (vapor phase extraction) and dynamic (purge and trap) headspace are the two types that are widely used. In the static headspace, the vapor directly transfers to GC-MS whereas in the dynamic headspace preparation, the carrier gas passes through the sample and the vapor is trapped by a sorbent such as charcoal or silica gel. This method is suitable for concentrating low amounts of analyte in a matrix [19].

GC-MS has wide applications in different fields such as environmental science, toxicology, forensic science, food sciences, and pharmaceutical sciences. Al-Mansur et al, 2017 [20] characterized the volatile constituents of the ethyl acetate extract from the stem and seeds of *Anethum sowa* Linn. using GC-MS. The result showed that 26 compounds were identified from the stem of the plant and 23 were identified from the seed, which were compared against the NIST library. The GC-MS study conducted on plants in the family of Compositae, by Yiguang et al.,2017 [21] revealed 213 volatile constituents that might be used in chemotaxonomy studies. The volatile constituents of *Dendranthema nankingense* and *Borago officinalis* seeds were analyzed using GC-MS and a total of 127 compounds were identified in the class of terpenes, aldehydes, alcohols, and ketones [22]. In another study, headspace GC-MS was used in the analysis of the aerial parts of *Sideritis pisidica* Boiss. & Heldr, commonly known as mountain tea. The analysis identified 104 volatile constituents from the aerial part where the majority being terpenes [23].

Liquid Chromatography-Mass Spectroscopy (LC-MS)

LC-MS is a coupling of two powerful techniques: liquid chromatography, and mass spectrometry. Liquid chromatography separates the components from the

mixture and their structural information (identification) is then obtained from mass spectrometry. After the sample is injected into the column by applying high or ultra-high pressure, separation occurs inside the column (the heart of chromatography). The interaction (ionic, hydrogen bonding etc.) of components with the stationary phase and the mobile phase governs the separation process. Then the eluent enters the MS system i.e., ionizer, a mass analyzer, detector, and is displayed as a spectrum (**Figure 5**).

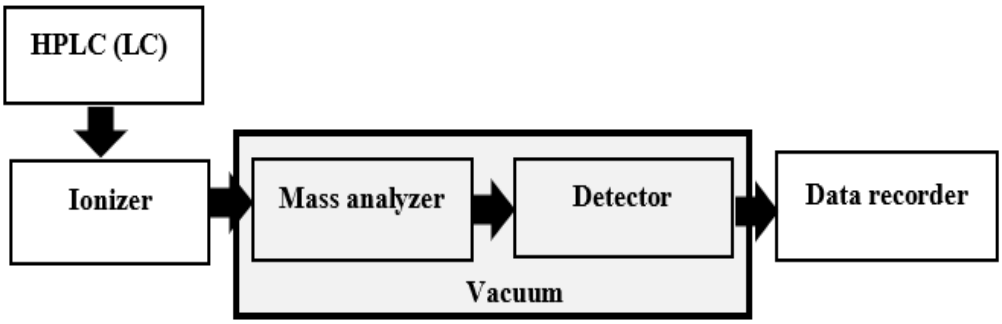


Figure 5 Block diagram of liquid chromatography mass spectrometry

Liquid chromatography uses high pressure (HPLC) or ultra-high pressure (UPLC) to deliver the sample from the injection port into the column. The column is usually 50-300 mm long with a 3-4.6 mm internal diameter and is packed with silica or bonded silica such as C18 and C8. The reversed phase (C18), which is bonded silica, is mostly used in LC-MS analysis of inorganic analytes and samples with range of polarities. Hydrophilic interaction chromatography (HILIC), or normal phase chromatography, uses non-modified silica (hydrophilic or charged) stationary phase that can separate highly polar and hydrophilic analytes. The order of elution is the opposite of reversed-phase chromatography. In general, the efficiency of the column depends on the particle size of the stationary phase and the length of the column [24]. The interface is usually a capillary inlet that is used to transfer the eluent from the LC column to mass spectrometry.

When the components reach the ionizer, they are converted to ions which then enter the mass analyzer where the ions are separated based on the *m/z* ratio. Finally, the ions are transferred to the detector. The ions that strike the detector are recorded as a signal and displayed as a spectrum. The mass spectrum is a plot of abundance as a function of *m/z*.

The samples used in LC-MS are prepared using protein precipitation, liquid-liquid extraction, and solid-phase extraction.

The application of the LC-MS technique is versatile. It is applied in both qualitative and quantitative analysis of different samples and is a widely used analytical technique in drug development starting from the analysis of the raw material to the final product. In the preclinical and clinical phases of drug development, LC-MS is used as a technique for drug discovery, in food science, and metabolomics, among other applications. [24].

LC-MS can be coupled together with a biological assay such as antioxidant activities and used for rapid screening, qualitative and quantitative analysis of active compounds [25-27]. In this analysis, the eluent from the HPLC column is split into two. One portion is analyzed for its biological activities and the other portion is analyzed by mass spectrometry. DPPH assay coupling with LC-MS is one example of this method. In this assay, one portion of the eluent from the HPLC column reacts with DPPH reagents and its antioxidant activities are detected by a UV detector. The other portion of eluent goes to the mass spectrum for detection of antioxidant compounds [27-31]. The retention time gap between the two detectors can be adjusted by addition of standard compounds during sample preparation.

Metabolomics

Metabolomics or metabonomics is a non-selective and universally applicable comprehensive analytical approach that is used to identify and quantify metabolites (low molecular weight compounds) in a biological sample [32]. Metabolites are representative of phenotypes and they are present in downstream of genomics, transcriptomics, and proteomics [33]. They are complex and diverse in terms of chemical classes, physical properties, and concentrations. Analytical technology that can be able to give full information is crucial. Currently, there is no

single analytical tool available that provides a snapshot of all metabolites. Hyphenated spectroscopic techniques such as LC-MS, GC-MS are commonly [34].

There are two approaches in metabolomics: targeted approach and untargeted approach. Targeted metabolomics approach focuses on the identification and quantification of selected or specific metabolites. It involves the quantitation of metabolites related to a specific pathway and can be applied in pharmacokinetic studies of drug metabolism as well as for measuring the influence of therapeutics or genetic modifications on a specific enzyme. On the other hand, untargeted metabolomics is a global approach that used to analyze as many metabolites as possible from biological samples [35-37].

Metabolomics workflow

Metabolomics study follows a series of workflow steps (**Figure 6**) that enables us to meet our analysis objectives.

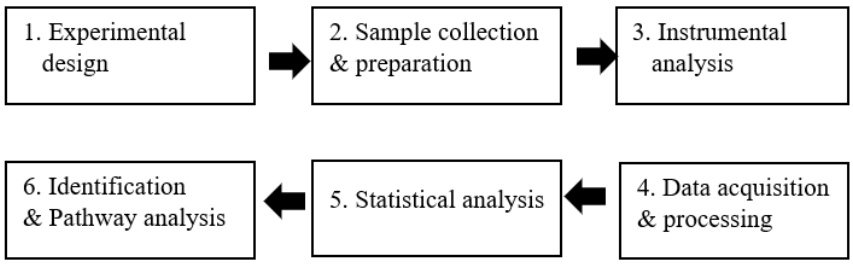


Figure 6 Workflow in metabolomics

1. **Experimental design**

A metabolomics study starts from the design of the experiment. An appropriate experimental design is the first and most important step to answer the research question. The design depends on the goal of the experiment and the experimental limitations of the study [37]. The experimental type (E.g., treatment vs control) and experimental factors (dose, genetic variations) should be considered during the design of the experiment. There are protocols in the literature that help to set up appropriate experimental processes and how to report on a metabolomics study [38].

2. Sample collection and preparation

In metabolomics, sample collection and its preparation (metabolite extractions) are critical steps. Metabolite extraction depends on the sample type (biological fluid, cell, or tissue) and the metabolomic approach [5, 39]. There are different sample extraction techniques such as protein precipitation, solid-phase extraction (SPE), and liquid-liquid extraction (LLE). Protein precipitation (PP) is the simple method of protein removal by using organic solvents such as methanol, acetonitrile [40]. Methanol precipitation is the usually recommended method for large-scale metabolite profiling studies due to its straightforward preparation, low protein interference, comprehensive metabolite profile, and reproducible results. Solid-phase extraction (SPE) enables protein removal together with fractionation of compounds based on their polarity. The liquid-liquid extraction (LLE) method is also a common sample preparation method that uses two immiscible solvents. Chloroform or dichloromethane together with methanol are used to extract polar and nonpolar metabolites. Supported liquid extraction (SLE), phospholipid removal plates, magnetic beads, Turboflow, immunosorbents are also some additional methods for sample preparation [34, 41, 42].

3. Instrumental analysis

The next step in the metabolomics study is sample analysis. The major analytical techniques that are used in metabolomics studies are NMR and MS (coupled with LC and GC). NMR is a widely used analytical technique due to its fast analysis, minimal sample preparation, and reproducibility. Poor sensitivity and spectral resolution are drawbacks of this analysis [6].

GC-MS and LC-MS are the two analytical tools used in metabolomics due to high sensitivity, wider dynamic range, and reproducibility [11]. GC-MS is mainly used to analyze volatile and thermally stable metabolites. For non-volatile metabolites, derivatization techniques such as alkylation, oximation are included in sample preparation. The NIST library databases are available for the identification of metabolites [43]. LC-MS is the first choice for analyzing metabolites that have different polarity ranges and are present in low concentrations in a sample matrix [8]. The separation of metabolites is based on the interaction between the mobile and stationary phases. Highly polar metabolites are well separated using the HILIC column whereas

medium polar to non-polar metabolites are analyzed using reversed phase (C8 and C18) column [38, 44]. The analysis of metabolites by LC-MS can be optimized by modifying the polarity of the column, the mobile phase, and the temperature of the column.

4. Data acquisition and processing

The data generated from analytical techniques contains hundreds to thousands of variables that need appropriate software to extract meaningful information. In data processing, the first step is the conversion of data format to an appropriate file format such as mzXML or mzML using available software such as ProteoWizard. These data formats are suitable as inputs to data processing software such as XCMS (https://xcmsonline.scripps.edu), MzMine (http://mzmine.github/) and Metaboanalyst (http://www.metaboanalyst.ca) [32, 45]. In preprocessing using the above software, every detected peak is assigned by its *m/z*-Rt value, sample, and intensity. Peak alignment is the next step in which the peaks of the same metabolites found in different samples are aligned together to correct the retention time and *m/z* shift between runs. Due to the low intensity of peaks and irregular peak distribution, there might be missed peaks. Gap filling steps enable the finding of such peaks.

Experimental variations that bring noise can be removed by data normalization. The method of normalization can be statistical (mean, median, quartile), scaling (auto, pareto, unit variance), and data transformation (log, power) which will be applied based on the data type [46].

5. Statistical analysis

The normalized three-dimensional data (*m/z*-Rt, sample, abundance) produced by the data processing software are further analyzed statistically to obtain interrelationships within the data set. Due to many variables being present in metabolomics, univariate, and multivariate data analysis are applied to extract information from the data. In univariate analysis, a single variable is analyzed at a time using Student t-tests or ANOVA [47].

Multivariate analysis is usually applied in many metabolomics studies. It is divided into unsupervised and supervised. An unsupervised method such as Principal Component Analysis (PCA) does not need prior knowledge about the sample or the data but shows trends or variations in the samples. Supervised methods such as Partial

Least Squares Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) need prior knowledge about the data [48].

6. Identification and pathway analysis

The final step is feature identification that shows differences between groups or samples. This is achieved by taking the molecular weight, retention time and MS/MS fingerprint of the selected metabolites and comparing the data with a database. Common databases and libraries include the Human Metabolome Database (HMDB), Metlin, MassBank, PubChem, MetFrag, Chemical Entities of Biological Interest (ChEBI), and Kyoto Encyclopedia of Genes and Genomes (KEGG). The databases Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc. and MetaboAnalyst, can be used for visualization and identification of pathways associated with the biological response or regulations [38].

***Centotheca lappacea* (L.) Desv.**

C. lappacea, commonly called Ya hee yum grass or repair grass, (**Figure 7**) in the family of Poaceae (Gramineae), is a perennial grass, 30-60 cm tall and grows in normal soil. It is one of the species in the tribe *Centotheceae* which consists of 11 genera and 33 species. *C. lappacea* is widely distributed in Thailand, India, China, and Africa [49]. Poaceae, the fifth largest family of flowering plants, consists of approximately 660 genera and 10,000 species [50]. It has been used in Thai traditional medicine to treat women after giving birth. The soreness and weakness of pelvic muscles during post-labor were treated by the smoke of this plant as it is believed to tighten the respective muscles. Traditional treatment includes the women sitting on a chair which has a hole, and her genital area is fumed by the smoke from the burned repair grass. In addition, the herbal tea that is prepared by boiling the plant is also another alternative traditional treatment [51, 52].

The phytochemical studies on the aerial part of *C. lappacea* have revealed the presence of phenolic compounds, flavonoids, fatty acids, and phytosterols. 4-coumaric acid and 5, 7, 4’- trimethoxyflavone which were isolated using column chromatography. As well, vitamins and minerals, especially silicon, which contribute to collagen synthesis, have been found in *C. lappaceae* in remarkable quantities [52].

Figure 7 *Centotheca lappacea* (L.) Desv

Kamoltham, et al. (2018) [51] reported that the ethanolic extract of *C. lappacea* showed antioxidant activities in DPPH, metal chelating, and lipid peroxidation inhibition assays and also showed tyrosinase enzyme inhibition, collagenase enzyme inhibition, and gelatinolytic activity. The extract also showed estrogenic activity by the proliferation of the human breast cancer line (MCF-7 cell).

***Bacopa monnieri* (L.) Wettst.**

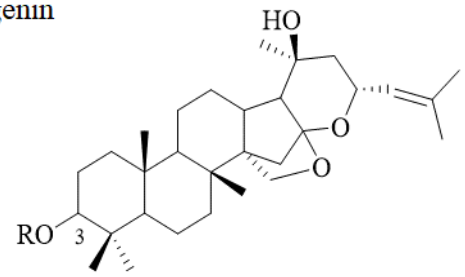
*B. monnieri is a medicinal plan*t with the common name Brahmi or Bacopa which belongs to the family Plantaginaceae (**Figure 8**). It is a creeping plant, 2-3 feet in height, with purple flowers, that grows in damp and marshy areas and is widely distributed in Thailand, India, Africa, and Australia [53-55]. It was referred to in the ancient Ayruvedic texts Charaka Samhita (2500 B.C.) and Sushruta Samhita (2300 B.C.) and has been claimed as a treatment for anxiety, cognitive impairment, and lack of concentration [53]. The biological activities of Brahmi are due to the various chemical constituents present at various levels.

Figure 8 *Bacopa monnieri* (L.) Wettst.

Chemical constituents of *B. monnieri*

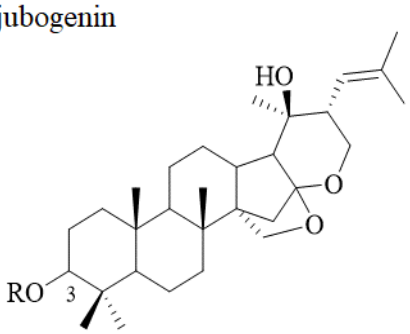
Different pharmacological active compounds have been reported from the extract of *B. monnieri*. The principal compounds are the dammarane steroidal saponin glycosides namely jujubogenin (bacoside A_3 and bacopaside X) and pseudojujubogenin, (bacopaside I, bacopaside II and bacopasaponin C) (**Figure 9**) which differ in the nature of the sugar unit attached to the aglycone as well as the position of the olefinic side chain on the aglycone [55-57]. Other saponin glycosides that have closely related structures, namely bacoside A_4-A_5 [58], bacopaside A_2, and A_6, bacopasaponin B-H, bacopaside III, bacopaside N_1-N_2, bacopaside IV-XIII, have been identified from the ethanolic extract of Bacopa [56, 58, 59]. Bacoside A, which is a mixture of bacoside A3, bacopaside, jujubogenin, bacoposaponin C, and bacoside B are reported as neuroprotective compounds in Brahmi [53]. Moreover, flavonoids (apigenin, luteolin and quercetin), sterols (β-sitosterol, stigma-sterol), D-mannitol, acid A, and betulinic acid have been also identified from *B. monniera* [53, 60].

A) Jujubogenin



Bacoside A3: R= α-L-ara (1→ 2)-[β-D-glc-(1 →3)]-β-D-glc
Bacopaside X: R= α-L-ara (1→ 2)- [β-D-glc-(1→3)]-α-L-ara

B) Psudojujubogenin



Bacopaside I: R = α-L-ara (1 →2)- [6-O-sulfonyl-β-D-glc-(1 →3)]-α-L-ara
Bacopaside II: R = α-L-ara (1 →2)- [β-D-glc-(1 →3)]-β-D-glc
Bacopasaponin C: R = α-L-ara (1 →2)-[β-D-glc-(1 →3)]-α-L-ara

Figure 9 The chemical structure of jujubogenin and pseudojujubogenin glycosides

Biological activities

The pharmacological activities of Brahmi have been reported by many researchers. It includes neuroprotective activities [61] , antiepileptic activities [62], antioxidant activities [63], anti-inflammatory activities [64, 65], increases blood flow [66, 67], and hepatoprotective activities [68]. It shows all activities with low signs of toxicity [69, 70].

1. Neuroprotective effect

Brahmi has been shown to improve various neurological conditions in *in-vitro* models, animal models, and in human studies. A study was conducted by Limpeanchob et al. (2008) [71] on the neuroprotective effect against beta-amyloid protein and neurotoxicity induced by glutamate in primary cortical cultured neurons.

Their results revealed that Brahmi extract protects neurons from toxicity by suppressing cellular acetylcholinesterase activity and neuronal oxidative stress. In Alzheimer's disease, a Wistar rat model of neurodegeneration induced by ethylcholine aziridinium ion and in a Morris water maze test, *B. monnieri* was shown to improve cognitive function [72]. The effect of Brahmi on endogenous oxidative stress markers of prepubertal male mice was studied by Shinomol and Muralidhara (2011) [73] and the result revealed that Brahmi can modulate oxidative stress markers in the brain region.

In a double-blind, randomized, placebo-controlled trial conducted by Peth-Nui et al. (2012) [74], healthy subjects with a mean age of 62 were tested for working memory, attention, and cognitive processing. The result showed significant improvement in working memory, attention, and cognitive effect after 12 weeks of Brahmi treatments when compared to the placebo group. The cognitive effect of a special extract of Brahmi (KeenMind) was studied in a double-blind placebo-controlled trial on volunteers between age 18 and 60 years and the result showed that groups who were given KeenMind over a 12 week period showed improvement in working memory when compared with group administered with the placebo [75]. In another study, Brahmi essence containing standardized Brahmi extract and mulberry juice, showed improvement in cognitive function, reactive hyperemia, and cerebral blood flow without any serious adverse effects after the volunteers took the essence for 12 weeks [76].

2. Antioxidant activities

The antioxidant activities of Brahmi have been studied by many researchers. Limpeanchob, et al. (2008) [71], reported antioxidant activities of Brahmi using Thiobarbituric acid reactive substance (TBARS) and Ferric Reducing Antioxidant Potential (FRAP) assay. The result showed inhibition of lipid peroxidation and reduction of the metal ion in a dose-dependent manner. Bhattacharya et al. (2000) [77], studied the antioxidant effect of standardized *B. monnieri* extract on rat brain frontal cortical, striatal, and hippocampal superoxidedismutasse(SOD), catalase (CAT), and glutathione peroxidase (GPX) activities and the result showed that the extract increased the level of antioxidant enzymes (SOD, CAT, and GPX) following after 14 and 21 days of administration. Moreover, Brahmi increased

antioxidant enzymes: SOD, CAT, and GPX of cognitively impaired rats induced by intracerebroventricular injection of streptozotocin [63].

3. Anti-inflammatory activities

The anti-neuroinflammatory activities of Brahmi have been studied so far. The tea, infusion, and alkaloid extract of Brahmi with that of Bacoside A inhibit the release of proinflammatory cytokines such as TNF-α and IL-6 by the microglial cell which are involved in inflammation in the brain. It showed also inhibitory activity on caspase 1 and 3 and matrix metalloproteinase-3 which is mainly involved in apoptosis and inflammation [78]. Additionally, Brahmi showed anti-inflammatory activities in carrageenan-induced rat paw edema and inhibit the activities of 5-lipoxygenase (5-LOX), 15-LOX, and cyclooxygenase-2 (COX-2) in rat monocyte. Bacoside A and EtOAc fraction of Brahmi inhibit TNF-α [65].

4. Effect on blood flow

Brahmi showed an effect on blood flow by vasodilator activities on basilar arteries through stimulating the release of NO, activation of Ca^{2+} influx, and decrease of the release of Ca^{2+} from sarcoplasmic reticulum [66]. In another study, the alcoholic extract of Brahmi shown an effect on blood flow by its relaxant effect on pulmonary arteries, aorta, and tracheal arteries of guinea-pigs and rabbits [79]. Brahmi extract exhibited calcium channel blocking activity in guinea-pig tracheal smooth muscles which might be one mechanism of the extract on blood flow [80]. The study done by Kamkaew, et al. (2013) [67] on rats showed that chronic administration of Brahmi extract remarkably increases cerebral blood flow compared with the standard drug, donepezil. This might be one mechanism for the neuroprotective activities of Brahmi. The same author studied the vasorelaxant effect of Brahmi and its constituent's, bacoside A, bacopaside I, luteolin and apigenin, on rat mesenteric arteries. The result revealed that the Brahmi extract and all the constituents cause the relaxation of both the endothelial intact and denuded vessels. This demonstrates that the active compounds might be responsible for these effects of Brahmi on blood flow [81].

5. Hepatoprotective activities

Bacoside A and B were reported to have hepatoprotective activities on galantamine induced liver toxicity on male albino Wistar strain rats. It increases the

level of antioxidant enzyme vitamins C and E and decreases the levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), 5-nucleotidase (5-ND), lactate dehydrogenase (LDH) and ϒ-glutamyl transferase (ϒ-GT) [68].

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CHAPTER III

PHYTOCHEMICAL CONSTITUENTS OF CENTOTHECA LAPPACEA (L.) DESV. USING ONLINE DPPH ASSAY COUPLED TO LC-ESI-QTOF-MS AND HEADSPACE GC-MS

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Abstract

Centotheca lappacea (L.) Desv. or “hee yum” grass has been used in Thailand for postpartum care. This study focused on analyses of elements, antioxidants, and volatile compounds from *C. lappacea*. Wavelength dispersive X-ray fluorescence showed the presence of silicon in this plant. Liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometer coupled with DPPH assay was used for separation, detection, and identification of antioxidants in *C. lappacea* in one run. Twelve antioxidants including *C*-and *O*-glycosides of luteolin and apigenin were identified. Moreover, a headspace gas chromatography-mass

spectrometer was used for analyses of volatile compounds by direct burning of the plant material which was the same process of obtaining smoke during traditional treatment. Nineteen and twenty-five compounds were found from the roots and the aerial parts of *C. lappacea*, respectively. In conclusion, antioxidants, and volatile constituents after *C. lappacea* was burnt were reported for the first time in this study.

Key words: *Centotheca lappacea,* antioxidants, LC-ESI-QTOF-MS, DPPH, headspace GC-MS

Introduction

Centotheca lappacea (L.) Desv. is found in the family of Poaceae (Gramineae). It is a perennial plant mainly distributed in Thailand, India, China, and Africa. In Thailand, it is known as ya hee yum or repair grass, which has been used in traditional medicine for postpartum care. It has helped to heal wounds and tears on the labia and tighten the respective muscles. Traditionally, the dried plant material is burned in a smoke pot to fumigate the patient's labia area for 10-30 minutes during the postpartum treatment. They also drink *C. lappacea* tea because of the belief that it creates "balance" inside and outside of their body. The plant powder is also used to disinfect and heal open wounds [1, 2].

A phytochemical study on the aerial part of *C. lappacea* revealed the presence of phenolic compounds, flavonoids, fatty acids and phytosterols. Also, 4-coumaric acid and 5,7,4'-trimethoxyflavone were isolated from the aerial part using column chromatography [2]. In addition, vitamins, and minerals, in particular, silicon, which contributes to collagen synthesis [3, 4] were found in significant amounts in *C. lappaceae*. According to Kamoltham, et al. (2018) [1], the ethanolic extract of *C. lappacea* showed antioxidant activity on DPPH assay, metal chelation, lipid peroxidation and tyrosinase inhibitory activity. It also showed strong collagenase inhibition and estrogenic activity by increasing MCF-7 cell proliferation [1]. However, the compounds responsible for such activities have not previously been reported.

High performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) is a recent method applied to the identification of biologically active compounds from complex samples. Liquid chromatography–electrospray

ionization-quadrupole-time of flight-mass spectrometry (LC-ESI-QTOF-MS) coupled with a DPPH assay has been developed and demonstrated as a useful technique to characterize antioxidants in plant extracts [5]. The HPLC system provided information on individual compounds, while the molecular structure and molecular weight of target compounds were obtained using mass spectrometry. This technique was applied for separation and identification of antioxidants from *C. lappacea*. Moreover, headspace gas chromatography-mass spectrometer (GC-MS) was used to analyze the chemical constituents of *C. lappacea* after directly heating at high temperature which would provide information about the chemical constituents of the smoke as used during traditional treatment.

Materials and Methods

Plant materials and chemicals

Dried *C. lappacea* was obtained from ChaoPhraya Abhaibhubejhr Hospital, Prachin Buri, Thailand. The voucher specimen (Collection no. 004358) was kept at Faculty of Science, PNU herbarium at Naresuan University. Gallic acid, quercetin, Trolox and 1,1 diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldric (St. Louis, MO, USA). Acetonitrile was purchased from Labscan, (Bangkok, Thailand). Ultrapure water prepared by using a Millipore MilliQ Integral 3 Water Purification System (Millipore, Bedford, MA). Formic acid (analytical grade) was obtained from Merck (Darmstadt, Germany).

Sample preparation

The dried aerial part and roots of *C. lappacea* were powdered using a grinder and passed through a 60-mesh size sieve prior to elemental analysis. For the antioxidant evaluation, the plant powder was macerated with 50% and 95% ethanol at room temperature. The extracts were filtered and dried under reduced pressure. The water extract was prepared by infused the plant powder in hot water and dried by lyophilization.

For LC-ESI-QTOF-MS coupled with DPPH assay, 50% ethanolic extract of aerial part was diluted with methanol to obtain a concentration of 20 mg/ml and filtered by 0.2 µm syringe before injection.

Elemental analysis using X-ray florescence

Elemental analysis of the powder of the aerial part and roots of *C. lappacea* were conducted using Bruker S8 Tiger model wavelength dispersive X-ray fluorescence (WDXRF), at the Thailand Institute of Nuclear Technology, Thailand.

Antioxidant activity by DPPH assay

The antioxidant activity of *C. lappacea* was determined using a method adopted from Brand-Williams et al. (1995) [6]. Serial dilutions at a concentration of 0.1 - 10,000 µg/ml of 50% ethanolic extracts were mixed with 0.1 mM DPPH and incubated for 30 min in a dark area at room temperature. The absorbance was read at 517 nm. Antioxidant activity was calculated as percentage inhibition using equation 1, while IC_{50} values were determined from the percentage inhibition versus the log concentration plot. The data was presented as mean values ± standard deviation of three replicates. Trolox was used as a positive control.

$$\% \text{ inhibition} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100 \ldots\ldots \quad 1$$

LC–ESI–QTOF-MS coupled with DPPH assay

The online LC-ESI-QTOF-MS coupled with DPPH assay was conducted as reported previously by Nuengchamnong et al. (2011) [7] . The instrument was composed of HPLC coupled to MS and a continuous flow of DPPH solution where the eluent from the analytical column was split into the MS detector (Line 1) and the reactor coil where DPPH reaction took place (Line 2). Line 1 specifications were HPLC system 1260 infinity model (Agilent Technologies, Waldbronn, Germany) coupled with an Agilent 6540 QTOF-MS spectrometer (Agilent Technologies, Singapore) equipped with electrospray ionization interface (ESI). The chromatographic separation was achieved using a Phenomenex Luna C18 (2) column 150 x 4.6 mm i.d. (Phenomenex, Torrance, USA) protected by a C18 guard column. The mobile phase consisted of solvent A (0.1% v/v formic acid in water) and solvent B (0.1% v/v formic acid in acetonitrile). The linear gradient elution was started by 5% B then increased to 90% over 30 min with a post run of 5 min. The column temperature was set at 35°C and the injection volume was 20 µl with a flow rate of

500 µl/min. The MS operating condition was set as: drying gas (N_2) temperature 350°C with flow rate 10 l/min, nebulizer pressure 30 psig, capillary 3500, fragmenter voltage 100, skimmer 65 V and octapole RFPeak 750 V. The full scan mass spectra from *m/z* 100 –1000 amu were acquired in negative ion modes.

The on-line antioxidant activity (line 2) was performed using an HPLC equipped with a 1260 infinity cap pump (Agilent Technologies, Waldbronn, Germany), and home-made knitted reaction coil PEEK tubing with an inner diameter of 180 µm and volume of 100 µL. 0.1 mM DPPH set at a flow rate of 200 µl/ min and the change in color recorded as positive peak at wavelength 515 nm using a UV–vis detector (1260 VWP Agilent Technologies, Waldbronn, Germany). The system was operated at 25°C. The split ratio of the eluent between the MS and DPPH line was 8:2. Agilent Chem station for UV-vis Rev.B.04.03 was used for data analysis. 50% ethanolic extract of the aerial part of *C. lappacea* was used for analysis and quercetin and gallic acid at concentrations of 100 µg/ml were added to the sample to mark an antioxidant peak with the MS peak. Tentative identifications of active compounds were obtained by comparing the retention time and mass spectra of the sample with a public data base i.e. Chemspider and previous reports.

Headspace GC-MS analysis

The analysis was done using Agilent 7890 B gas chromatography coupled to an Agilent 5977A mass spectrometer with an Agilent Model 7697 headspace auto-sampler equipped with a 1 ml sample loop. A 0.1 g sample of the dried powders of the aerial parts and roots of *C. lappacea* were placed in 20 ml vials which were tightly closed. An Agilent 19091S-433 HP 5 MS (30 m x 0.25 mm ID, 0.25 µm film thickness) capillary column was used for separation. The temperatures of the headspace oven, loop and transfer line were set at 180°C, 190°C and 225°C, respectively. The vial was heated to 180°C, pressurized for 0.1 min at 15 psi and equilibrated for 20 min until injection. Loop equilibrium time and injection duration were set as 0.05 and 0.5 min.

The injector temperature was set at 250°C and the injection split mode ratio was 1:10. The GC oven temperature was initially programmed at 40°C and held for 3 min, then increased by 10°C/min to 120°C, which was held for 3 min and then increased again at the rate of 15°C/min to 240°C which was held for a period of 5

min. This gave a total GC run time of 27 min. Helium (99.995%) was used as the carrier gas at a flow rate of 2.0 ml/min during the analysis. A series of n-alkane (C_{10} - C_{40}) obtained from CPAchem (Stara Zagora, Bulgaria) was used at the same condition mentioned above to calculate Kovates retention indices (KI).

The MS operating condition was set in positive electron ionization mode with 70 eV of electron energy. It was operated in the SIM /SCAN mode from *m/z* 50 to 500 amu. The mass (MS) source and quadrupole temperature were set at 230°C and 150°C. Each compound was identified by comparing the retention time and *m/z* to the reference in the National Institute of Standards and Technology (NIST) mass spectral database and retention index matching.

Results and Discussion

Elemental analysis of *C. lappacea*

The trace elements, including silicon in the aerial parts and roots of *C. lappacea,* were determined. As shown in **Table 1**, *C. lappacea* contained a high number of essential elements such as potassium (5.5% w/w), calcium (2.8% w/w) and silicon (3.14% w/w). These elements play important role in maintenance of human health [8]. Silicon which is most abundant element in this plant and its family increases collagen synthesis and promotes wound healing [3] and has anti-inflammatory activity [9]. Therefore, the presence of this element in higher amount in water extract (0.72 % w/w) and the powder might explain the effect of *C. lappacea* in postpartum care. Moreover, higher percentages (w/w) of macro-elements were found in the aerial part, as compared to the roots. This was the first report for the elemental analysis of the roots while that of aerial part was comparable with the study of Kamoltham, et al. (2017) [2].

Antioxidant activity by DPPH assay

Ethanolic extract of the aerial part of *C. lappacea* showed antioxidant activity using DPPH assay [2] but the active compounds have not previously been reported. In our study, the antioxidant activity of the water extract and the 50% and 95% ethanolic extract of the aerial part and roots of *C. lappacea* were analyzed using DPPH assay.

Table 1 Elemental analysis of aerial part, root, and water extracts of *C. lappacea* by wavelength dispersive X - Ray Fluorescence (WDXRF)

Element	Aerial part (% w/w)	Root Part (% w/w)	Water extracts (% w/w)
Potassium	5.05	0.12	22.05
Silicon	3.14	0.44	0.72
Calcium	2.80	0.99	4.96
Chlorine	0.86	ND	3.68
Sulfur	0.38	0.05	1.05
Manganese	0.09	< 0.01	0.1
Phosphorous	0.56	0.10	1.33
Magnesium	0.14	0.03	0.54
Iron	0.03	0.02	ND
Zinc	0.08	<0.01	0.04
Rubidium	0.03	ND	0.07
Strontium	0.03	ND	0.03
Titanium	0.02	ND	ND

ND: not detected

*Remark: - Light elements such as carbon (C) and hydrogen (H) not detected by XRF

Determination of antioxidant compounds by LC-ESI-QTOF-MS coupled with DPPH assay

50 % ethanolic extract of the aerial part of *C. lappacea* was injected into online LC-ESI-QTOF-MS coupled with DPPH assay**.** As a result, 12 compounds that exhibited antioxidant activities were identified by comparing the retention time of each peak from the antioxidant activity detector (line 2) with the retention times of peaks from a mass detector (line 1). Gallic acid and quercetin were added to the extract to mark the delay times of the two detectors. The delay time between retention times of the peak from the MS detector and the corresponding peak from antioxidant activity detector was calculated as 0.6 min. A negative ionization mode was used as it

gave more fragmentation when compared to the positive mode. The online antioxidant activity trace and the total ion current (TIC) chromatogram are shown in **Figure 10**. The negative molecular ions, together with their fragmented ions and the tentative identification of the compounds were listed in **Table 2**. The ESI-MS/MS spectra of the antioxidant compounds can be seen in the supplementary data.

Compound 1, at a retention time of 4.2 min on antioxidant activity detector showed molecular ions $[M-H]^-$ at *m/z* 133.0196 corresponding to the molecular formula of $C_4H_6O_5$. It fragmented at *m/z* 115 and 71 by losing 18 ($-H_2O$) and 44 ($-CO_2$) Da, respectively. From the fragmentation pattern and the report of Fernandez-Fernandez et al. (2010) [10], compound 1 was tentatively identified as malic acid. Compound **2** (Rt= 4.5 min) and **3** (Rt=4.6 min) showed the same molecular ions at *m/z* 191.0269 $[M-H]^-$ and fragmentation ions at *m/z* 111 but different retention time. These suggested that the two compounds were isomers with the molecular formula of $C_6H_8O_7$. Based on these results, and from the database [11] , compound **2** and **3** might tentatively identified as isocitric and citric acid. Peak **4** (Rt =8.0 min) showed *m/z* 169.0198 $[M-H]^-$ which typically represents gallic acid ($C_7H_6O_5$) that was the internal standard added to the sample to mark the delay time between the MS and Uv-vis detectors.

Compound **5** (Rt =10.6 min) showed $[M-H]^-$ ions at *m/z* 579.1417 corresponding to the molecular formula of $C_{26}H_{28}O_{15}$. Fragment ions at *m/z* 369 [luteolin + $C_4H_3O_2]^-$ and 399 (luteolin + $C_5H_5O_3$) were similar with the pattern of di-substituted (deoxy pentose and hexose) luteolin glycosides reported by Geng et al. (2016) [12] and Wang et al. (2019) [13]. Additional ions at *m/z* 519 [M-H-60 ($-C_2H_4O_2$)]$^-$, *m/z* 489 [M-H-90 ($-C_3H_6O_3$)]$^-$ and *m/z* 459 [M-H-120 ($-C_4H_8O_4$)]$^-$ (**Figure 11a**) might gave information about the position of the substituents on luteolin [14-16]. Based on the above information and by comparing with the literature, compound **5** was tentatively assigned as luteolin 6*C*-deoxyarabinoside-8*C*-glucosides.

Compound 6 (Rt =11.8 min) showed $[M-H]^-$ ions at *m/z* 593.1673 which corresponds to the molecular formula of $C_{27}H_{30}O_{15}$. Fragmented ions at *m/z* 309 [luteolin+C_3H_5-$H_2O]^-$, *m/z* 327 [luteolin + $C_3H_5]^-$ and *m/z* 357 [luteolin + $C_4H_7O]^-$ together with ions at *m/z* 503 $[M-H-90]^-$ and *m/z* 473 $[M-H-120]^-$ (**Figure 11b**) revealed the presence of mono C-hexose substituent on luteolin which is in

agreement with the literature report [16]. Additional fragment ion at *m/z* 429 [M-H-164 (-$C_6H_{12}O_5$)]$^-$ revealed the presence of *O*-substituted rhamnose on this molecule [14, 17]. However, we could not confirm the position of rhamnose linkage from the data. Based on above information and literature, compound **6** could be tentatively identified as luteolin *O*-rhamnoside-8*C*-glucoside.

Compound **7** (Rt = 12.3 min) showed [M-H]$^-$ ions at *m/z* 533.1351 corresponding to the molecular formula of $C_{25}H_{26}O_{13}$. The fragmentation pattern fitted to that of apigenin di-*C* substituted glycoside with prominent fragment ions at *m/z* 353 [apigenin + $C_4H_3O_2$] and 383 [apigenin + $C_5H_5O_3$]. The fragment ions at *m/z* 473 [M-H-60]$^-$, *m/z* 443 [M-H-90]$^-$, and *m/z* 413 [M-H-120]$^-$ (**Figure 11c**) might be from pentose sugar (arabinose) substituent on 6*C* and 8*C* position of the aglycon [16]. Therefore, compound **7** could be tentatively identified as apigenin 6,8-di-*C*-arabinoside.

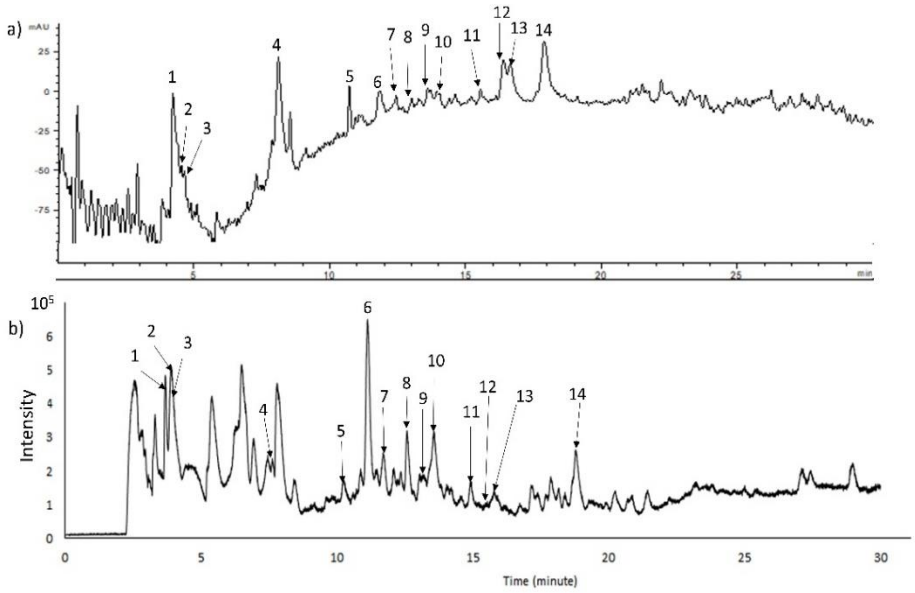


Figure 10 Separation of antioxidant compounds from 50% ethanol extract of *C. lappacea* performed by LC-ESI-QTOF-MS coupled with DPPH assay (a) Chromatogram from antioxidant activity detector set at 515 nm. (b) The total ion current (TIC) output from the LC-ESI-QTOF-MS in negative mode

Compound **8** (Rt = 13.5) showed $[M-H]^-$ ions at *m/z* 563.1533 which was 30 Da less than compound **6**. Fragment ions at *m/z* 309 [luteolin + C_3H_5-$H_2O]^-$ and *m/z* 327 [luteolin + $C_3H_5]^-$ revealed compound 8 might be mono-*C* substituted glycosides of luteolin. Fragment ions at *m/z* 503 $[M-H-60]^-$ and *m/z* 473 $[M-H-90]^-$ (**Figure 11d**) indicated the presence of pentose sugar substituent on the 6*C* position of the parent compound. Moreover, the presence of a prominent peak at *m/z* 399 $[M-H-164]^-$ probably resulted from the cleavage of deoxy hexose sugar (rhamnose) from the molecule. By comparing the above information with the literature, compound **8** could be tentatively identified as luteolin *O*-rhamnoside-6*C*-arabinoside. The position of rhamnose on arabinose could not be confirmed from this study.

Compound **9** (Rt =13.9) showed $[M-H]^-$ ions at *m/z* 547.1516 where 17 Da (-OH) was less than compound **8** which revealed apigenin as aglycone. A fragment ion at *m/z* 293 [apigenin + C_3H_5-$H_2O]^-$ and *m/z* 311 [apigenin + $C_3H_5]^-$ indicated that the molecule might be mono substituted apigenin glycoside. Fragment ions at *m/z* 457 $[M-H-90]^-$ and *m/z* 383 $[M-H-164]^-$ (**Figure 11e**) might have resulted from the loss of $C_3H_6O_3$ and $C_6H_{12}O_5$ groups from the molecule, respectively. Therefore, from the above spectral data, compound **9** could be tentatively assigned as apigenin-*O*-rhamnoside-6*C*-arabinoside.

Compound **10** (Rt=14.2) which showed $[M-H]^-$ ions at *m/z* 163.0458 and intense peak at *m/z* 119 $[M-H-44]^-$ which revealed the loss of CO_2 from the parent ion. The fragmentation pattern was similar to that of Ferreres, et al. (2011) [15] , so compound **10** was tentatively identified as *p*-coumaric acid.

Compound **11** (Rt =15.5) showed $[M-H]^-$ions at *m/z* 577.1663 which were 30 Da greater than compound **9** were fragmented at *m/z* 311 [aglycone+ $C_3H_5]^-$, *m/z* 473 [M-H-104 ($-C_4H_8O_3$)]$^-$, *m/z* 415 [M-H-162 ($-C_6H_{10}O_5$)]$^-$ and *m/z* 353 [M-H-(104+120)]$^-$ (**Figure 11f**). The molecular and fragment ions were similar with literature report [17]. Therefore, compound **11** were tentatively identified as apigenin-*O*-rhamnoside-6*C*-glucoside.

Compounds **12** (Rt=16.2) and **13** (Rt=16.5) showed molecular ions at *m/z* 571.2073 and *m/z* 549.2125, respectively. Compound **12** fragmented at *m/z* 469 and *m/z* 427 whereas compound **13** fragmented at *m/z* 447 and *m/z* 405 where there were 22 Da difference between their fragment ions. However, it was difficult to elucidate

their structure only by the above spectral information. More information acquired by other spectroscopic techniques is needed.

Compound **14** (Rt=17.7) with molecular ion at *m/z* 301.0409 and fragmented as prominent peak at *m/z* 178 and *m/z* 151 revealed the compound was quercetin that was mixed in a sample as a marker for the two detectors.

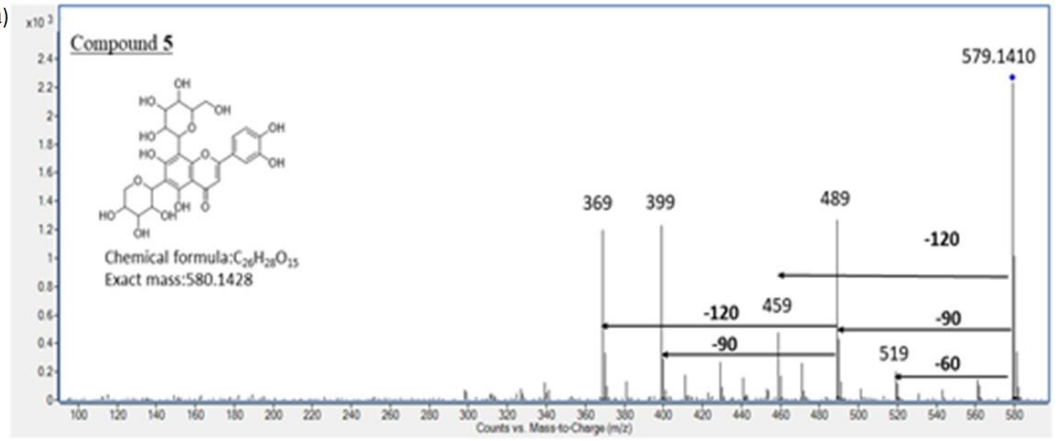
The antioxidant activities of flavonoid glycosides have been well-documented. Their activities depend on the type of aglycone, type of glycosylation and number of sugars attached to their structures which also affect their solubility and stability [18, 19]. The *C* and *O* flavonoid glycosides present in *C. lappacea* might be used as marker for further study on this plant.

Table 2 Tentative identification of compounds from the 50% ethanolic extract of *C. lappacea* using LC-ESI-QTOF-MS coupled to DPPH assay

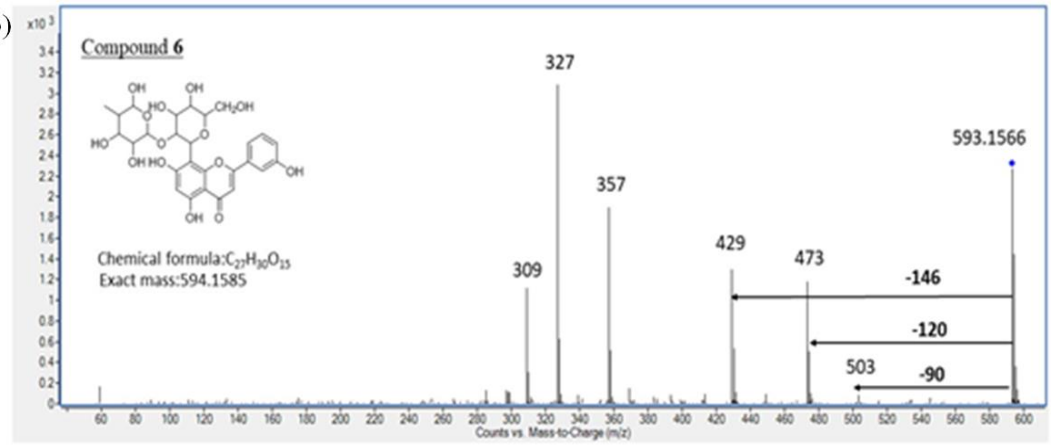
Compound no	Rt (min)	$[M-H]^-$ (*m/z*)	Error (ppm)	ESI-MS (*m/z*) MS/MS	Formula	Tentative Identification
1	4.2	133.0196	-4.16	115, 71	$C_4H_6O_5$	Malic acid
2	4.5	191.0269	-	173, 111	$C_6H_8O_7$	Isocitric acid/ citric acid
3	4.6	191.0261	-	-	$C_6H_8O_7$	Isocitric acid/ citric acid
4	8.0	169.0198*	-2.68	125	$C_7H_6O_5$	Gallic acid
5	10.6	579.1410	9.42	561, 519, 489, 459, 429, 399, 369	$C_{26}H_{28}O_{15}$	Luteolin 6*C*-deoxyarabinoside-8*C*-glucoside
6	11.8	593.1566	-9.11	503, 473, 429, 357, 327, 309	$C_{27}H_{30}O_{15}$	Luteolin -*O*-rhamnoside-8*C*-glucoside
7	12.3	533.1351	-9.5	503, 515,473, 443, 413, 383, 353	$C_{25}H_{26}O_{13}$	Apigenin-6, 8- di-C-arabinoside
8	13.5	563.1533	-9.9	503, 473, 399, 381, 357, 327, 309	$C_{26}H_{28}O_{14}$	Luteolin -*O*-rahmnoside-6*C*-arabinoside
9	13.9	547.1516	-10.8	457, 383, 311, 293, 117, 59	$C_{26}H_{28}O_{13}$	Apigenin-*O*-rahmnoside-6*C*-arabinoside
10	14.2	163.0458	-7.0	119, 93	$C_9H_8O_3$	*p*-Coumaric acid
11	15.5	577.1633	-12.2	515, 473, 457,413, 311, 293	$C_{27}H_{30}O_{14}$	Apigenin-*O*-rhamnoside-6*C*-glucoside
12	16.2	571.2073	-11.0	469, 427, 325, 221, 179, 99, 57	$C_{26}H_{36}O_{14}$	unidentified
13	16.5	549.2125	-2.5	447, 405, 343, 303, 261, 179, 125, 99, 57	$C_{26}H_{30}O_{13}$	unidentified
14	17.7	301.0409*	-9.8	273, 245, 227, 201, 178, 151, 121, 93, 65	$C_{15}H_{10}O_7$	Quercetin

*Compounds used as delay time markers.

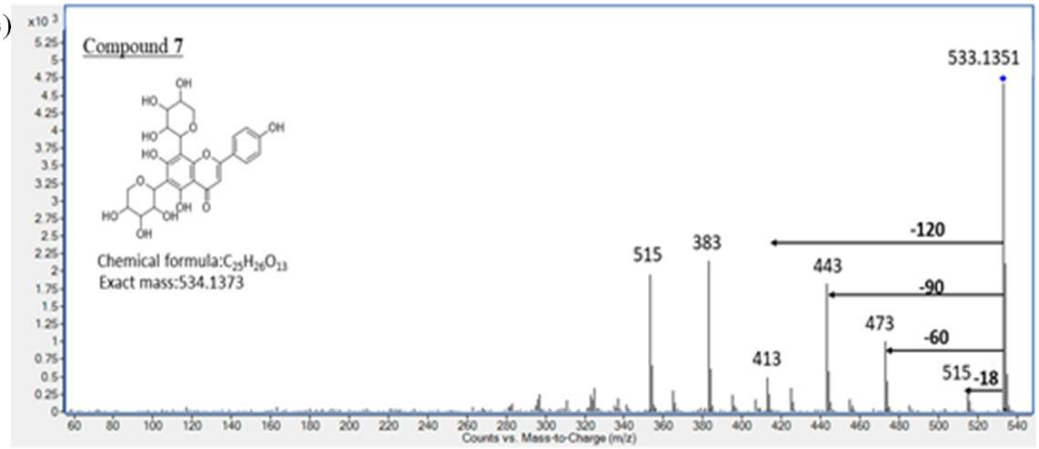
a)


Compound 5
579.1410
369
399
489
-120
-120
459
-90
-90
519
-60
Chemical formula:C26H28O15
Exact mass:580.1428
Counts vs. Mass-to-Charge (m/z)

b)


Compound 6
327
593.1566
357
429
309
473
-146
-120
503
-90
Chemical formula:C27H30O15
Exact mass:594.1585
Counts vs. Mass-to-Charge (m/z)

c)


Compound 7
533.1351
-120
515
383
443
-90
473
-60
413
515
-18
Chemical formula:C25H26O13
Exact mass:534.1373
Counts vs. Mass-to-Charge (m/z)

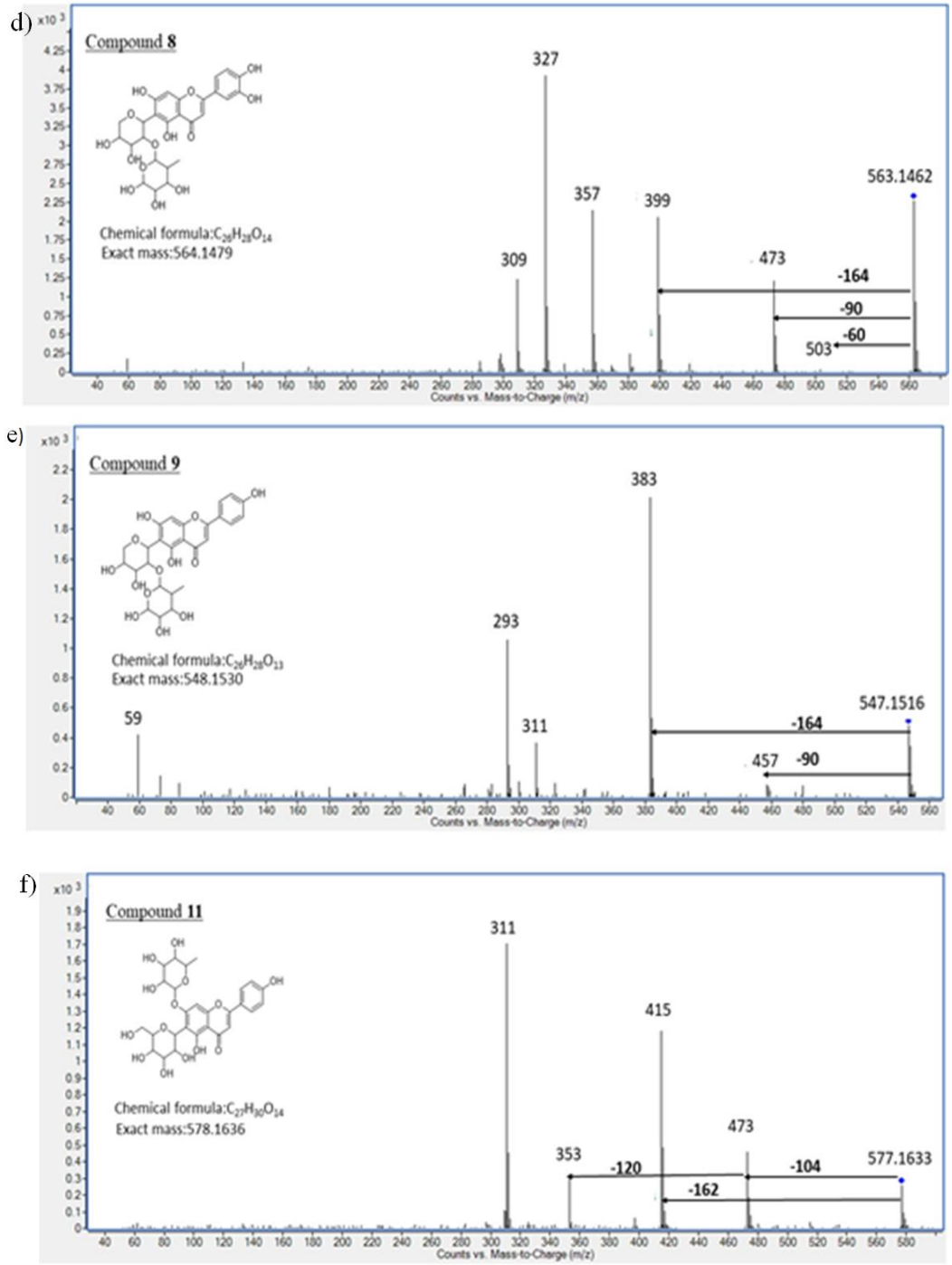


Figure 11 ESI-MS/MS spectra of antioxidant compounds: a) compound 5; b) compound 6; c) compound 7; d) compound 8; e) compound 9 and f) compound 11

Headspace GC-MS analysis of *C. lappacea*

C. lappacea has long been used in Thailand to treat women after giving birth. The post-partum women were fumed by *C. lappacea* smoke during the treatment. It is believed that it will heal wounds and tighten stretched muscles after delivery [1]. Therefore, in this study the volatile compounds from the burnt raw materials were studied using headspace GC-MS at high temperature.

As shown in **Tables 3** and **4** and **Figures 12A** and **12B**, 19 compounds from the roots and 25 compounds from the aerial part were detected and tentatively identified. The identification was performed by comparing retention times and fragmentation patterns with that of the database of the National Institute of Standards and Technology (NIST) library together with Kovats index. The matching score with that of the NIST library was taken from 650 - 990 and the peaks showed more than 0.5% area of the total area of the whole chromatogram were identified.

A shown in Table 3, Furfural, 2- methoxy-4-vinylphenol, vanillin, coumran and palmitic acid were the most abundant compounds in the roots of *C. lappacea*. There have been no reports previously on the chemical constituents of the roots. Coumaran and palmitic acid were also found in the aerial part of these plants (**Table 4**). The presence of phytol, palmitic acid, vanillin, and oleic acid in the aerial part were in agreement with the report by [2]. Other compounds to found in this plant were identified for the first time

Coumaran and its derivatives have shown anticancer, antitubercular and anti-HIV activities [20] and have been reported in many plants [21]. These compounds were found in both the aerial part and roots and might play an important role in the biological activities of this plant. Palmitic acid showed antitumor activities in-vivo in mice. Phytol, the other abundant compound in the aerial part showed antioxidant anti-inflammatory, immune modulating, and antimicrobial effects [22].,

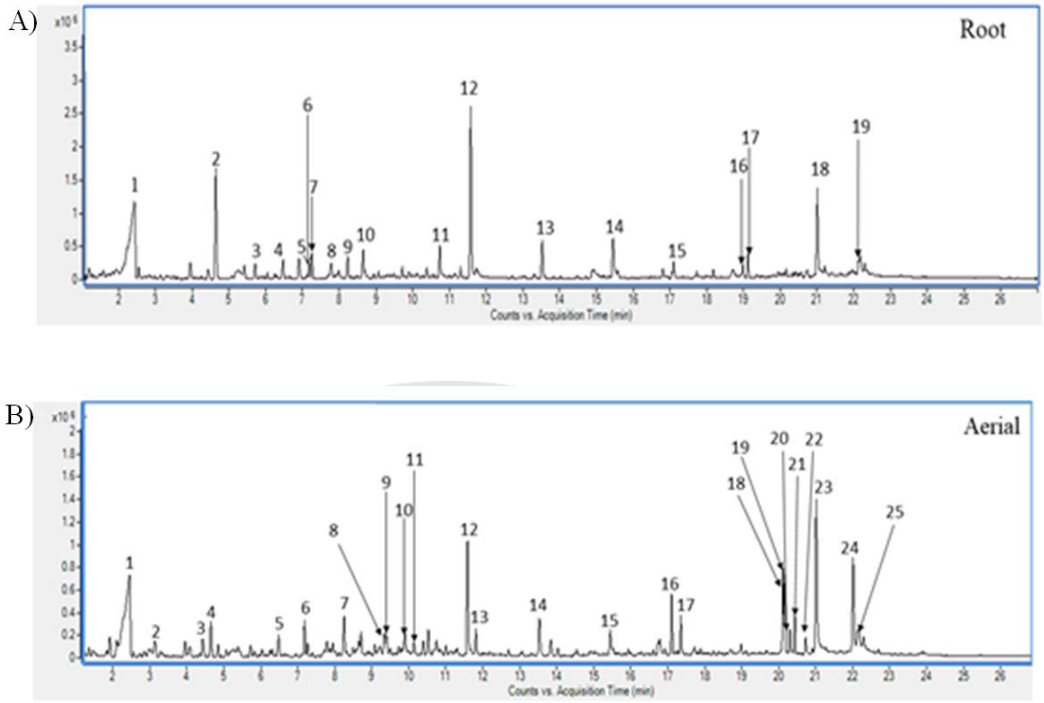


Figure 12 TIC from headspace GC-MS analysis of A) root part and B) aerial part of *C. lappaceae.* The peak numbers correspond to that of Tables 3-4

Table 3 Tentative identification of volatile compounds from the root of *C. lappacea* using headspace GC-MS.

No.	**Compounds**	Rt (min)	KI	Theoretical index	Match	Molecular weight	Formula	Fragmentation	% area
1	Acetic acid	2.47	-	662	972	60.0	CH_3COOH	-	19.6
2	Furfural	4.65	-	835	910	96.0	$C_5H_4O_2$	94.8	7.6
3	4-Cyclopentene-1,3-dione	5.71	-	880	882	96.0	$C_5H_4O_2$	67.8, 53.8	1.0
4	2(5H)-Furanone	6.49	-	916	939	84.0	$C_4H_4O_2$	54.9	1.3
5	2,5-Furandione, 3-methyl-	6.91	-	NF	933	112.0	$C_5H_4O_3$	-	1.6
6	Benzaldehyde	7.19	-	961	926	106.0	C_7H_6O	77.0, 51.0	0.8
7	2-Furancarboxaldehyde, 5-methyl-	7.26	-	966	944	110.0	$C_6H_6O_2$	80.8, 52.8	1.4
8	Furan, 2-pentyl-	7.79	-	996	728	138.1	$C_9H_{14}O$	80.8, 72.8, 59.8	1.7
9	1H-Pyrrole-2-carboxaldehyde	8.24	1020	1018	821	95.0	C_5H_5NO	94.8, 83.9, 65.8	1.5
10	2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (+/-)-	8.66	1045	1032	898	130.0	$C_6H_{10}O_3$	70.8, 56.8	2.0
11	Endo-Borneol	10.74	1172	1166	937	154.1	$C_{10}H_{18}O$	121.0, 110.0, 94.9, 66.9	2.1
12	Coumaran	11.59	1218	1219	861	120.0	C_8H_8O	90.8, 64.8	11.0
13	2-Methoxy-4-vinylphenol	13.54	1311	1315	904	150.0	$C_9H_{10}O_2$	134.9, 106.8, 76.9	2.8
14	Vanillin	15.45	1403	1394	923	152.0	$C_8H_8O_3$	136.9, 108.9, 80.8	3.4
15	2,4-Di-tert-butylphenol	17.11	1529	1525	927	206.1	$C_{14}H_{22}O$	191.0, 56.9	0.9
16	2-Pentadecanone	18.99	1708	1698	885	226.2	$C_{15}H_{30}O$	84.9, 70.9, 57.9	0.7
17	Pentadecanal	19.13	1723	1715	877	226.2	$C_{15}H_{30}O$	198.9, 109.0, 95.9, 81.9	1.2
18	Palmitic acid	21.02	1968	1964	934	256.2	$C_{16}H_{32}O_2$	213,0, 185.0, 157.0, 129.0	6.4
19	Oleic Acid	22.17	2144	2140	858	282.2	$C_{18}H_{34}O_2$	256.1, 129.0, 96.9, 82.9	1.4

NF- not found

Table 4 Tentative identification of volatile compounds from the aerial part of *C. lappaceae* using headspace gas chromatography.

No	Compounds	Rt (min)	KI	Theoretical index	Match	Molecular weight	Formula	Fragmentation	% area
1	Acetic acid	2.47	-	662	981	60.0	CH_3COOH	-	11.6
2	Propanoic acid	3.14	-	740	908	73.9	$C_3H_6O_2$	66.9, 56.9	1.2
3	Methylpyrazine	4.43	-	826	916	94.0	$C_5H_6N_2$	85.9, 66.9, 52.9	1.1
4	Furfural	4.65	-	835	930	96.0	$C_5H_4O_2$	-	1.9
5	2(5H)-Furanone	6.48	-	916	944	84.0	$C_4H_4O_2$	54.9	1.1
6	Benzaldehyde	7.19	-	961	941	106.0	C_7H_6O	76.8, 50.8	1.5
7	Benzene acetaldehyde	8.71	1048	831	876	120.0	C_8H_8O	90.9	1.1
8	2,6-Dihydroxypyridine	9.35	1087	NF	742	111.0	$C_5H_5NO_2$	82.9, 67.9	1.2
9	1-Pyrrolidinecarboxaldehyde	9.40	1090	NF	794	99.0	C_5H_9NO	70.9, 59.9	1.2
10	Methyl imidazole-4-carboxylate	9.90	1121	NF	692	126.0	$C_5H_6N_2O_2$	94.9	1.6
11	Camphor	10.40	1151	1145	883	152.1	$C_{10}H_{16}O$	107.9, 94.9, 80.9, 67.9	0.7
12	Coumaran	11.59	1219	1219	831	120.0	C_8H_8O	90.9, 64.6	5.5
13	Methylethylmaleimide	11.82	1229	1265	875	139.0	$C_7H_9NO_2$	123.8, 95.9, 66.9, 52.9	1.1
14	2-Methoxy-4-vinylphenol	13.54	1311	1315	858	150.0	$C_9H_{10}O_2$	134.9, 106.9, 76.9	2.2
15	Vanillin	15.45	1403	1394	897	152.0	$C_8H_8O_3$	136.9, 108.9, 80.8	1.3
16	2,4-Di-tert-butylphenol	17.11	1529	1525	903	206.1	$C_{14}H_{22}O$	191.0	2.5
17	Dihydroactinidiolide	17.36	1548	1532	922	180.1	$C_{11}H_{16}O_2$	152.0, 136.9, 110.9	1.6
18	Neophytadiene	20.12	1845	1840	909	278.2	$C_{20}H_{38}$	137.0, 122.9, 108.9, 94.9, 81.9, 67.9	3.2
19	2-Pentadecanone,6,10,14 trimethyl	20.17	1851	1847	936	268.2	$C_{18}H_{36}O$	123.9, 108.9, 94.9, 84.9, 70.9, 57.9	1.0

No	Compounds	Rt (min)	KI	Theoretical index	Match	Molecular weight	Formula	Fragmentation	% area
20	Neophytadiene	20.30	1869	1840	853	278.2	$C_{20}H_{38}$	136.9, 122.9, 108.9, 94.9, 81.9, 67.9	1.5
21	Neophytadiene	20.43	1888	1840	869	278.2	$C_{20}H_{38}$	136.9, 122.9, 108.9. 94.9, 81.9, 67.9	-
22	3-Methyl-2-(3,7,11-trimethyldodecyl) furan	20.73	1929	1931	725	292.1	$C_{20}H_{36}O$	135.9, 108.9, 94.9, 80.9, 68.9	0.9
23	Palmitic acid	21.02	1969	1964	936	256.2	$C_{16}H_{32}O_2$	213.1, 185.1, 157.0,129.0	8.2
24	Phytol	22.01	2118	2122	889	296.3	$C_{20}H_{40}O$	136.9, 122.9, 110.9, 94.9, 81.9	4.9
25	Oleic acid	22.17	2141	2140	764	282.2	$C_{18}H_{34}O_2$	256.2, 129.0, 96.9, 82.9	1.3

Conclusion

An on-line LC-ESI-QTOF-MS coupled to a DPPH assay led to the identification of 12 antioxidant compounds from the aerial part of *C. lappacea*. The results support the use of this herb as a food supplement. In addition, headspace GC-MS analysis revealed that the volatile constituents of *C. lappacea* after burnt which might imply to the constituents of the smoke of *C. lappacea* when used as in Thai traditional fashion.

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CHAPTER IV

CHARACTERIZATION OF METABOLITES IN PLASMA, URINE AND FECES FROM HEALTHY VOLUNTEERS AFTER TAKING BRAHMI ESSENCE FOR TWELVE WEEKS USING LC-ESI-QTOF-MS METABOLOMIC APPROACH

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Abstract

Brahmi essence developed from *Bacopa monnieri* (L) Wettst. standardized extract and mulberry juice, was proven to improve the speed of memory of elderly healthy participants, following a 12-week dietary course of Brahmi essence. In the present study, an untargeted LC-MS metabolomics approach was followed to characterize metabolites in plasma, urine, and feces. From OPLS-DA plots of plasma,

urine, and feces samples, 15 features (metabolites) were tentatively identified in the plasma samples, 7 in the urine samples, and 17 in the feces samples by comparing our data with on-line databases and the literature. The metabolites in the plasma samples were in a class of amino acids and their derivatives, and acylcarnitine, and phospholipids. These were all involved in aminoacyl-tRNA biosynthesis, aromatic amino acid biosynthesis, and branched chain amino acid biosynthesis pathways. Benzeneactamide-4-O-sulphate and 3-hydroxyhippuric acid and other metabolites were tentatively identified in urine samples. The amino acids and their derivatives were tentatively identified in fecal samples in which the altered metabolic pathways were similar to that of the plasma samples. Jujubogenin/isomer and pseudojujubogenin aglycones were also tentatively identified in the feces samples. It is these metabolites that might be involved with improvements in speed of memory.

Keywords**:** *Bacopa monnieri*, metabolomics, Brahmi essence, LC-ESI-QTOF-MS, OPLS-DA

Introduction

Bacopa monnieri (L.) Wettst. (Bacopa or Brahmi) is an Ayurvedic medicinal plant in the family of Plantaginaceae which grows in wet soils near to water bodies and is mainly distributed in Southeast Asia, tropical Asia, sub-tropical United States, tropical Africa, and tropical Australia [1, 2]. Besides its traditional use for memory and intellect, Brahmi showed neuroprotective effects through antioxidant [3-6], antiepileptic [7] anti-inflammatory [8, 9], anti-cholinesterase [10] activities, and by increasing cerebral blood flow [11]. Brahmi exhibits a low sign of toxicity as it is reported in both animal and human studies [12-15].

There are various chemical classes of compounds reported in Brahmi including saponins glycosides, phenolic compounds, flavonoids, and steroids, etc. The major active compounds are the dammarane steroidal saponin glycosides: jujubogenin glycosides (bacoside A3 and bacopaside X) and pseudojujubogenin glycosides (bacopaside I, bacopaside II and bacopasaponin C) [16-18]. These compounds have been used as a marker in the preparation of standardized Brahmi extract. The phytosterols: stigmasterol, β-sitosterol, flavonoids: apigenin and luteolin, were also reported from this plant [16, 19]. Bacoside A, which is a mixture of four saponin

glycosides: bacoside A3, bacopaside II, bacopasaponin C, and bacopaside X, has been reported for nootropic and neuroprotective activities [9, 18].

Brahmi extract is used as an ingredient in dietary supplements. In Thailand, a tablet containing 300 mg of standardized Brahmi extract (BME) was developed and marketted by the Government Pharmaceutical Organization (GPO) of Thailand with a recommended dose of one tablet per day [15]. As an alternative preparation, Brahmi essence was also prepared from BME and mulberry juice. A randomized, double-blind, placebo-controlled study on healthy participants has shown that Brahmi essence improves the speed of memory and blood flow [20]. However, the metabolites responsible for these effects have not been reported thus far. Our objective was to identify the metabolites in the plasma, urine, and feces of healthy participants after 12 weeks consumption of Brahmi essence, by using a LC-MS based- metabolomics approach.

Metabolomics is a platform that is used to analyze metabolites in biological samples by combining instrumental techniques and bioinformatics [21, 22]. It can be applied to monitor the change of metabolites in complex biological matrixes in response to interventions or disturbances of either chemicals or disease process [23]. It can detect and identify hundreds of small molecules in a biological sample such as blood, urine, and feces

Materials and Methods

Chemicals and reagents

Acetonitrile and methanol (LC-MS reagents) were purchased from RCI Labscan Thailand. Ultrapure water was obtained from Millipore MilliQ Integral 3 Water purification system (Millipore, Bedford, MA). Formic acid (analytical grade) was obtained from Merck (Darmstadt, Germany). Digitoxin and prednisolone were purchased from Sigma-Aldric.

Brahmi essence and mulberry preparation

Mulberry fruit was obtained from The Queen Sirikit Department of Sericulture, Thailand. Brahmi was provided by GPO (Bangkok, Thailand). BME was prepared and standardized as described previously by Phrompittayarat et al. (2007) [24], Kamkaew et al. (2011) [25]. Of this extract, 16.03 ± 0.07% (w/w) was total

saponins comprising bacoside A3 (2.22 ± 0.01%), bacopaside I (3.54 ± 0.01%), bacopaside II (4.68 ± 0.02%), bacopaside X (3.25 ± 0.01%) and bacopasaponin C (2.34 ± 0.01). Brahmi essence was prepared from BME equivalent to 15 mg of total saponins, and mulberry juice, together being 40 ml per dose. The placebo was prepared in the same way except for the absence of BME. The color, odor, and taste of the Brahmi essence were identical with that of the placebo. All the products were pasteurized and kept in the refrigerator (4˚C) until used.

Human ethical statement

The study protocol was approved by the Naresuan University Ethical Committee for Human Research (NU-IRB) with the certificate number of 197/2018.

Subjects and study protocol

The participants were healthy participants from the age of 55-80 years old who were free from any diseases such as schizophrenia, dementia, depression, liver disease, kidney disease, diabetes, cancer, stroke, hypertension, and hyperlipidemia and who did not take any drugs or herbal supplements that might interfere with the study. The participants were also selected based on their Thai ethnicity, ability to listen and write Thai language, educational level (at least greater than 4th year of primary school) and could sign the consent form.

The study was conducted in a double-blind, placebo-controlled project. The participants were randomly divided into 2 groups i.e., treatment (A) and placebo (B). In every visit, the participants were asked to fast overnight before biological samples (blood, urine, and feces) were collected. At the first visit, the subjects were evaluated for their memory efficiency before the intervention. All participants (n=45) were instructed to take one bottle of placebo per day for two weeks, 30 minutes after their evening meal. At the second visit (after two weeks of taking the placebo), the participants were evaluated for all study parameters. The participants then received either Brahmi essence (group A, n=21) and placebo (group B, n=24) for 12 weeks. At the end of the 12 weeks, the participants asked a 3rd visit to meet the researcher and were evaluated for all study parameters again.

Blood samples were collected from each participant in an EDTA tube by medical technicians. The plasma was separated immediately by centrifugation at 1,700 RPM at 4˚C for 13 minutes, and the supernatants then stored at -80 ˚C until

required for analysis. Urine and fecal samples were collected and stored immediately at -80 ˚C until required for analysis.

Assessment of speed of memory

A computerized test was used to measure the speed of memory of participants. The time taken to complete word recognition, picture recognition, spatial working memory, and numeric working memory were summed and taken as the speed of memory [20]. The % change of response time from the 2nd visit to the 3rd visit was calculated to measure the speed of memory increments of the participants, in both group A and group B, over the 12-week period.

Sample preparations

Preparation of Brahmi essence and placebo for LC-MS analysis

1 ml of Brahmi essence or placebo was mixed with 2 ml of methanol in 0.1%formic acid. The samples were sonicated for 15 minutes and centrifuged at 4000 RPM for 5 minutes. The supernatants were filtered through a 0.2 µm syringe filter and transferred to a vial for LC-MS injection.

Preparation of plasma, urine, and feces samples for LC-MS analysis

Plasma preparation

200 µl of frozen plasma samples were thawed on ice and vortexed for 1 minute. The sample was mixed with two internal standards i.e., 20 µg/ml of prednisolone and 50 µg/ml of digitoxin in cold methanol with 0.1% formic acid in the ratio of 1:3, sample: solvent, to precipitate the protein. The samples were then kept at 4˚C for 1 hr. to facilitate protein precipitation and then centrifuged at 14,000 RPM at 4˚C for 20 minutes. The supernatant was dried by SpeedVac and reconstituted by methanol in a 10:1 ratio and injected into LC-MS. Pooled plasma samples (QC sample) were prepared by taking a 10 µl sample from each participant into a micro centrifuged tube

Urine preparation

Urine samples were thawed on ice and centrifuged at 14,000 RPM for 15 minutes at 4˚C to get a clear solution. Protein precipitations were done by adding cold methanol with 0.1% formic acid with an internal standard solution (20 µg/ml of prednisolone and 50 µg/ml of digitoxin) in a 1:2 ratio. The sample was vortexed for 1

min and centrifuged at 14,000 RPM at 4˚C for 20 minutes. The supernatants were transferred to the HPLC vial and stored at -20 ˚C until analysis.

Feces preparation

Feces samples were dried by SpeedVac at 40˚C and 10 mg of each sample was taken and weighed. The sample was dissolved by 1 ml of 80% cold methanol mix with 0.1% formic acid and standard compounds (20 µg/ml of prednisolone and 50 µg/ml of digitoxin) to precipitate the protein. The solutions were vortexed for 1 min and centrifuged at 14,000 RPM for 20 minutes. The supernatant was transferred to the HPLC vial and stored at -20 ˚C until injection.

LC-ESI-QTOF-MS analysis conditions

The chromatography conditions consisted of Agilent 1260 Infinity LC-QTOF-MS dual ESI 6540B model (Agilent Technologies, Singapore) and Agilent 1260 Infinity Series HPLC system (Agilent, Waldbronn, Germany). Zorbax, Eclipse plus C18 column 4.6×100 mm, 3.5 µm with 5 µm guard cartridges (Agilent Technologies, USA) which were used for separation of the plasma samples and Luna 5u C18 (2) 100 A 150 x 4.6 mm column which were used for the urine and feces samples. The mobile phase composed of 0.1% formic acid in water (v/v) (A) and 0.1% formic acid in acetonitrile (v/v) (B). The linear gradient elution of the mobile phase was started at 5% of solvent B and ended with 95% of B. The run time was 40 min with post-run 5 min. The flow rate was set as 0.5 ml/min and column temperature set as 35 °C with injection volume 10 µl.

The mass detections were composed of an electrospray ionization (ESI) source in positive ionization mode with the following detection parameters: drying gas (N2) flow rate 10 L/min, drying gas temperature 350 °C, nebulizer pressure 30 psig, capillary, 3500 V; skimmer, 65 V; octapole RFV, 750 V; and fragmentor voltage, 250 V in positive ionization mode. The mass range was set as at m/z 100 - 1000. For MS/MS analysis, the collision energy was set as 10, 20, and 40 V.

Data analysis

The data obtained from the LC-MS (. d file) were converted to mzXML file format using ProteoWizard software (MSConvert). This format was suitable for further analysis using the XCMS online platform (https://xcmsonline.scripps.edu). The data generated from the XCMS analysis consisted of retention time (Rt), mass to

charge ratio (*m/z*) and peak intensity. These data were then normalized and exported to SIMCA-P software (version 13.0, Umetrics, Umea, Sweden) for supervised orthogonal partial least squares discriminant analysis (OPLS-DA). The OPLS-DA, score, and S-plot were used to discriminate the features in the Brahmi essence treated group from the other group using the mass spectrometric data as X-variables and the speed of memory as Y-variables. All variables were pareto scaled before analysis. The contribution/covariance (p) and reliability/ correlation (p(corr)) cut off values from the S-plot were taken to identify the differential features which were tentatively identified by comparing their *m/z* and fragmentation pattern (MS/MS data) with on-line databases such as the Human metabolomic database (HMDB;http//:hmdb.ca), METLIN (http://metlin.scripps.edu), ChemSpider (http:// chemspider.com), Metfrag (http://metFrag), and Massbank (http://www.masinterpertation sbank.jp),. The possible metabolic pathways of the identified metabolites were predicted by using MetaboAnalyst 4.0 software (http://www.metaboanalyst.ca).

Results and Discussion

Analysis of metabolites in plasma samples from healthy volunteers after taking Brahmi essence for 12 weeks

Untargeted metabolomic analysis of whole metabolites in plasma samples from four different groups of participants was undertaken. The groups were coded as 2PA (Group A) where plasma samples were taken from 21 participants after taking a placebo for 2 weeks, 2PB (Group B) where plasma samples were taken from 24 participants who took a placebo for 2 weeks. Following this 2-week period, Group A, coded this time as 3PA, took Brahmi essence for 12 weeks and Group B, coded as 3PB, continued taking the placebo for 12 weeks. Plasma samples were taken from both groups and analyzed by using LC-ESI-QTOF-MS analysis. The mass spectrometric data was processed by XCMS online and the output of 2513 molecular features was then analyzed by multivariate analysis to derive an OPLS-DA score plot and an S-plot. The data matrix (retention time, *m/z*, and intensity) of all features was combined with the memory assessment data (**Table 5**) which was then employed to construct these plots. This process helps to discriminate groups and also to find those metabolites responsible for the separation of the groups. As shown in

Figures 13a, the OPLS-DA score plot separates 3PA from 2PA, 2PB and 3PB with $R^2(X) = 0.36$, $R^2(Y) = 0.87$, and $Q^2 = -0.16$. The metabolites that might contribute to the separation were selected from the upper side of the S-plot (**Figures 13b**) based on p (1) (≥ 0.5) and p(corr) (≥ 0.2) values. As shown in **Table 6**, 18 features (**1**-**18**) were selected for further identification, and **15** of them were tentatively identified by comparing with the on-line databases. More spectroscopic data is needed to identify other features.

Branched chain amino acids: valine; *m/z* 114.0863, Rt=3.1 min **(2)**; leucine/isoleucine; *m/z* 132.1019, Rt=3.9 min **(5)**, and aromatic amino acids; tyrosine; m/z 182.0810, Rt=3.5 min **(4)**, phenylalanine; *m/z* 166.0861, Rt=5.3min **(6)**, and tryptophan; *m/z* 205.0972, Rt=6.7 min **(7)** were tentatively identified in plasma samples. Both amino acids were present in both Brahmi essence and placebo (**Appendix B and C)** but their presence in plasma samples of volunteers may or may not be from the Brahmi essence or placebo. However, those amino acids were abundant (**Appendix D**) after the participants took Brahmi essence which might imply the regulatory effect of the essence.

The acylcarnitines; acetylcarnitine; *m/z* 204.1241, Rt=3.2min **(3)**; octanoylcarnitine; *m/z* 288.2169, Rt=13.1min **(8)**, decanoylcarnitine; *m/z* 316.2480, Rt=15.7min (**9**), palmitoylcarnitines; *m/z* 400.3415, Rt=22.7min **(13)** and oleoylcarnitines; *m/z* 426.3572, Rt=23.3 min (**14**) showed same characteristic fragment ions at *m/z* 85 ($-C_4H_5O_2^+$) and *m/z* 144 ($-C_7H_{14}NO_2^+$). Acylcarnitine has a role in neuroprotection by stabilizes membrane composition by modulation of synthesis of lipids, improving mitochondrial function, increasing antioxidant activity, and stimulate cholinergic neurotransmission [26]. Also, carnitines and their derivatives have a role in improving neurological functions by normalizing brain energy metabolites and reversing the metabolic change of ageing [27].

Table 5 The % change of response time for speed of memory from the 2nd visit of subjects in Brahmi essence group (Group A) and the placebo group (Group B)

Group A		Group B	
Subjects	% change of response time	Subjects	% change of response time
1	6.37	1	20.36
2	-24.87	2	-4.30
3	-31.91	3	13.04
4	8.19	4	-4.27
5	1.36	5	-0.67
6	-2.71	6	-4.02
7	0.02	7	-9.87
8	-3.92	8	-16.86
9	4.27	9	6.97
10	-19.82	10	7.32
11	-22.22	11	8.03
12	4.24	12	-8.18
13	-4.32	13	7.93
14	-4.71	14	5.88
15	-30.72	15	-24.42
16	-3.31	16	-7.31
17	5.61	17	-9.37
18	-24.28	18	-13.28
19	-23.76	19	-0.35
20	0.50	20	-13.34
21	0.25	21	4.12
		22	17.46
		23	-12.03
		24	-7.70

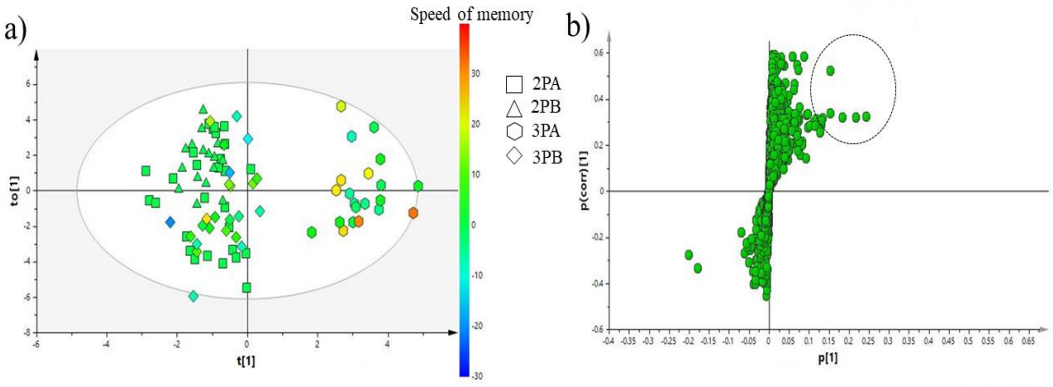

Figure 13 OPLS-DA score plot (a) and S-plot (b) of plasma samples of those volunteers who took placebo for 2 weeks (2PA and 2PB), after taking of Brahmi essence for 12 weeks (3PA) and after taking of placebo for 12 weeks (3PB) where speed of memory was used as a y-variable

Lysophosphatidylcholines (LysoPC), the group of compounds that are derived from partial hydrolysis of phosphatidylcholines, were also found in the plasma samples. LysoPC(16:1 (9Z)/0:0); *m/*z 494.3238, Rt=22.6 min **(12)**, lysoPC(20:4 (8Z, 11Z, 14Z, 17Z)/0:0); *m/*z 544.3398, Rt=23.7 min **(15)**, lysoPC(18:2 (9Z, 12Z)/0:0); *m/*z 520.3392, Rt=23.8 min **(16)**, lysoPC(16:0/0:0); *m/*z 496.3398, Rt=25.5 min **(17)** and lysoPC(18:1); *m/*z 522.3556, Rt=26.6 min **(18)** were abundant in the plasma samples from the group who took Brahmi essence for the 12 weeks. These compounds were characterized by showing a unique LysoPC fragmentation pattern at *m/*z 104 ($-C_4H_8O_3$) and *m/*z 184 ($-C_5H_{15}NO_4P^+$). Plasma lysoPCs are components of the neuronal cell membrane that enhance cognitive function by inhibiting oxidative stress [28]. They are also involved in the reuptake of docosahexaenoic acid which reduces the risk of Alzheimer's disease [29, 30]. **Figure 14** showed 10 possible metabolic pathways might be linked to the above metabolites which were predicted by using Metaboanalyst 4.0.

The major altered pathways were the amino acid metabolic pathways; namely 1) aminoacyl-tRNA biosynthesis, 2) phenylalanine, tyrosine and tryptophan biosynthesis, valine, and 3) leucine and isoleucine biosynthesis. These pathways play

a role in the production of neurotransmitters and protein synthesis [31-34]. They catabolize acetyl CoA and succinyl CoA and enter into the TCA cycle and modulate immune function [35, 36]. Also, those metabolites and their pathways are involved in the production of neurotransmitters, such as L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine, epinephrine, and norepinephrine from phenylalanine and tyrosine, all of which are important for the normal functioning of the brain [37, 38]. This supports the role of Brahmi essence in the prevention of neuronal damage, by its antioxidant activity, acetylcholinesterase inhibitory activity, and its effect on effect on cerebral blood flow [4, 11, 39].

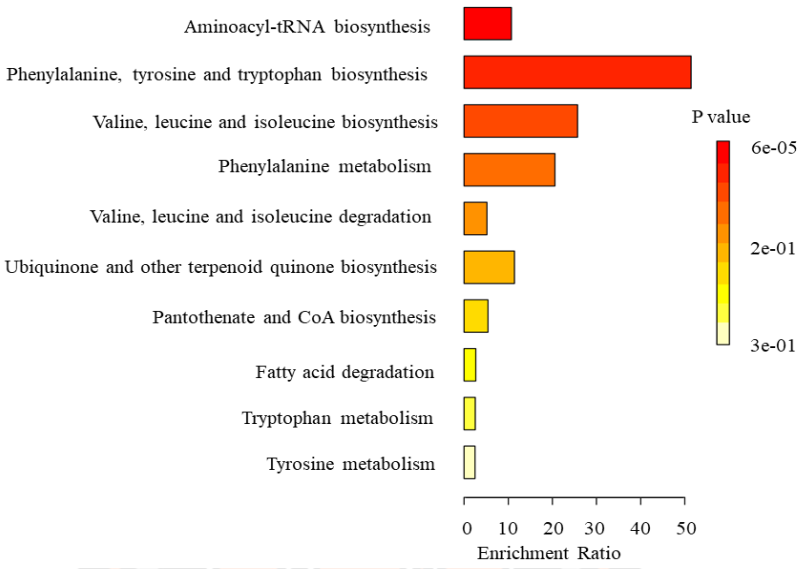

Figure 14 Summary plot of over representation analysis (ORA) for plasma samples (the color represents: red- lower *p* values and yellow- higher *p* value)

Analysis of metabolites in urine samples from healthy volunteers after taking Brahmi essence for 12 weeks.

Four groups of urine samples 2UA (Group A): where urine samples were taken from 21 participants after taking a placebo for 2 weeks, 2UB (Group B): where urine samples were taken from 24 participants who took a placebo for 2 weeks, 3UA: participants of Group A who took Brahmi essence for 12 weeks, and 3UB:

participants of group B who took a placebo for 12 weeks were subjected to LC-MS, XCMS online, and multivariate analysis to investigate the metabolites after taking Brahmi essence. The three data matrix (Rt, *m/z,* and intensity) of 6170 molecular features, obtained from XCMS, considered together with the data of speed of memory (**Table 5**) of each volunteer, were employed to construct the OPLS-DA score plot and the S-plot. As shown in **Figures 15a**, the 3UA group were separated from the other groups in the OPLS-DA score plot with $R^2(X) = 0.42$, $R^2(Y) = 0.7$, and $Q^2 = -0.51$. The discriminating metabolites were selected from the S-plot of the OPLS-DA (**Figures 15b**) based on p (1) (≥ 0.5) and p (corr) (≥ 0.2) values. As shown in **Table 6**, 12 features **(19-30)** were selected from the right top quadrant of the S-plot for identification, and 7 of them were tentatively identified by comparison with the on-line databases and the literature. Further information is needed from other spectroscopic techniques for the unidentified metabolites.

One of the tentatively identified metabolites that identified tentatively after taking Brahmi essence was creatinine; *m/z* = 114.0675, Rt= 3.1 min (**19**). It is a common metabolite in urine [40]. Proline betaine (stachydrine); *m/z* =144.1027, Rt= 3.4 min (**20**) was another abundant metabolite in the Brahmi essence treated group. This compound is used as a urinary biomarker for the consumption of citrus fruit [41]. The aromatic amino acid, phenylalanine; *m/z* =166.0870, Rt= 7.4 min (**23**) were also detected in urine samples of Brahmi essence treated groups. The metabolites at *m/z* = 330.2272 with Rt= 10.1 min (**25**) were tentatively identified as an acylcarnitine-6 keto decanoylcarnitine.

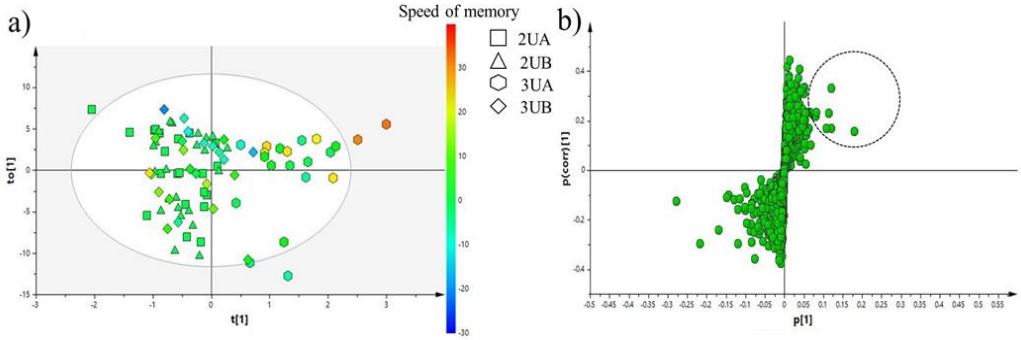

Figure 15 OPLS-DA score plot (a) and S-plot (b) of urine samples of those volunteers who took placebo for 2 weeks (2UA and 2UB), after taking of Brahmi essence for 12 weeks (3UA) and after taking of placebo for 12 weeks (3UB) where speed of memory was used as a y-variable

These might be linked with beta oxidation of fatty acid and energy metabolism. The feature at *m/z* =196.0611 and Rt= 13.58 min (**26**) was tentatively identified as 3-hydroxy hippuric acid which is a microbial metabolite derived from polyphenols and flavonoids in human urine [42].

The tentatively identified metabolites in urine were subjected to metabolic pathway analysis by Metaboanalyst 4.0. The result showed that phenylalanine tyrosine, and tryptophan pathway, phenylalanine metabolism, and aminoacyl-t-RNA biosynthesis were the major pathways (Figure 16), which is in agreement with the results from the analysis of plasma samples.

Table 6 Tentative identification of metabolites in plasma, urine and feces samples in Brahmi essence treated groups

Metabolite ID	Rt (min)	Detected *m/z* $[M+H]^+$	Detected MS/MS	Error (ppm)	Tentative identification
I.	**Metabolites in plasma**				
1	3.0	101.003	56	-	UI
2	3.1	118.0863	102,72,61	-0.4	Valine
3	3.2	204.1241	144,129,98,85	-6.7	Acetylcarnitine
4	3.5	182.0810	136,123,91,77,65,51	0.9	Tyrosine
5	3.9	132.1019	115, 86	0.8	Leucine/isoleucine
6	5.3	166.0861	149,131,120,103,77	1.5	Phenylalanine
7	6.7	205.0972	188,159,146,132,118,91,84,65	0.3	Tryptophan
8	13.1	288.2169	229,144,129,85,57	0.1	Octanoylcarnitine
9	15.7	316.248	257,214,144,129,95,85,57	0.7	Decanoylcarnitine
10	16.5	332.0700	164, 121,95	-	UI
11	18.8	219.1872	191,163,78	-	UI
12	22.6	494.3238	311,258,184,104	0.8	LysoPC(16:1(9Z)/0:0)
13	22.7	400.3415	341,239,144,85	1.6	L-palmitoylcarnitine
14	23.3	426.3572	367,297,144,129,95,85,60	1.4	O-oleoylcarnitine
15	23.7	544.3398	527,485,441,339,184,104,86,60	-0.1	LysoPC(20:4(8Z,11Z,14Z,17Z)
16	23.8	520.3392	443,337,258,184,104	1.1	LysoPC(18:2(9Z,12Z)/0:0)
17	25.5	496.3398	419,184,104	-0.1	LysoPC(16:0/0:0)
18	26.6	522.3556	445,339,258,184,104	0.7	LysoPC(18:1(9Z))

Metabolite ID	Rt (min)	Detected *m/z* $[M+H]^+$	Detected MS/MS	Error (ppm)	Tentative identification
II	**Metabolites in urine**				
19	3.1	114.0675	86,72, 59	-12.5	Creatinine
20	3.4	144.1027	116, 84, 72, 58	-5.5	Proline betaine
21	6.9	220.069	203, 142, 130, 120, 112, 112, 88	-	UI
22	7.5	166.087	131, 120, 103, 77, 51	-4.5	Phenylalanine
23	8.8	318.2025	274, 241, 157, 111, 85, 60	-0.5	Val-leu-Ser
24	9.5	232.0283	169, 114, 86, 59	-3.8	Benzeneacetamide-4-O-sulphate
25	10.8	330.2272	312, 253, 198, 123, 85, 60	-	6-Keto-decanoylcarnitine
26	13.58	196.0611	178, 150, 121, 93, 76, 65	-3.4	3-hydroxyhippuric acid
27	13.6	432.2406	253, 144, 85	-	UI
28	13.9	310.2098	251, 196, 157, 144, 131, 121, 93, 85	-	UI
29	14.6	272.1683	254, 225, 208, 147, 130, 84, 69, 55	-	UI
30	14.7	325.2272	267, 233, 183, 91, 86	-	UI
III	**Metabolites in feces**				
31	2.8	147.1143	130, 84, 78, 67,56	-4.0	L-lysine
32	3.7	130.0875	90, 84, 59	-9.6	Cycloleucine
33	3.7	160.1347	125, 101, 83, 70, 60, 55	-9.3	2-aminooctanoic acid
34	4.0	118.0878	103, 91, 72, 59, 55	-13.1	5-amino-pentanoic acid
35	4.1	150.0596	133, 104, 87, 74, 61, 56	-8.5	L-methionine
36	4.2	182.0833	165, 147, 136, 123, 112, 91	-9.0	L-tyrosine
37	4.7	132.1032	91, 86, 69, 57	3.8	L-leuc/isoleu

Metabolite ID	Rt (min)	Detected *m/z* $[M+H]^+$	Detected MS/MS	Error (ppm)	Tentative identification
38	5.6	150.0596	133, 104, 87, 74, 61, 56	-8.5	Racemethionine
39	6.2	182.0828	165, 147, 136, 123, 107, 91	-9.0	L-tyrosine
40	6.9	132.1002	86, 69, 57	12.8	L-leuc/isoleu
41	7.0	264.1457	246, 228, 200, 182, 131, 115, 98, 86	-	UI
42	8.0	166.088	149, 131, 120, 103, 93, 77	-10.5	Phenylalanine
43	8.5	229.1561	186, 116, 86, 70, 57	-6.2	Leucylproline
44	9.2	205.0988	188, 146, 118, 91, 74	-8.0	L-tryptophan
III	**Metabolites in feces**				
45	12.8	588.4142	133, 89	-	UI
46	15.5	398.3456	382, 155, 98, 55	-	UI
47	19.1	348.0668	210, 194, 180, 152, 137, 111, 94, 83, 71	-	UI
48	20.4	455.3547	437, 369, 219, 187, 163, 135, 109	-6.0	Jujubogenin/ jujubogen isomer
49	23.0	373.2754	355, 337, 309, 245, 145, 107, 81, 55	-4.5	Cervonoyl ethanolamide
50	27.7	455.3542	437, 369, 219, 187, 163, 135, 109	-4.9	Jujubogenin/ jujubogen isomer
51	28.2	473.3656	455, 437, 369, 315, 247, 163, 69	-6.5	Psuedojujubogenin

*UI- Unidentified

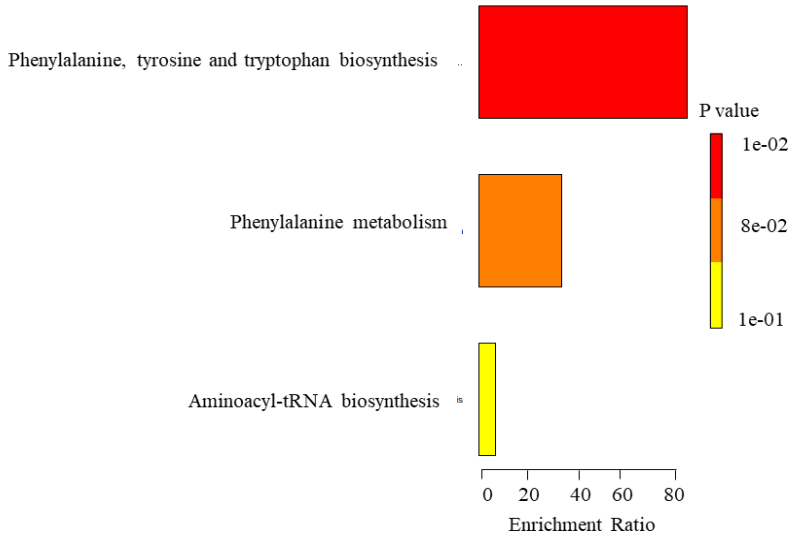

Figure 16 Summary plot of over representation analysis (ORA) for urine samples (the color represents: red- lower *p* values and yellow- higher *p* value)

Analysis of metabolites in feces samples from healthy volunteers after taking Brahmi essence for 12 weeks.

Four groups of fecal samples 2FA (Group A): where fecal samples were taken from 8 participants taking a placebo for 2 weeks, 2FB (Group B): where fecal samples were taken from 7 participants after taking a placebo for 2 weeks, 3FA: participants of samples Group A who took Brahmi essence for 12 weeks, and 3FB: participants of Group B who took a placebo for 12 weeks) were analyzed by LC-MS, XCMS online and multivariate analysis. The data from the memory assessment (**Table 5**) was used as a y-variable. As shown in the OPLS-DA score plot (**Figures 17a**), the fecal samples in 3FA were well separated from 2FA, 2FB, and 3FB with $R^2(X)$ = 0.27, $R^2(Y)$ = 0.96, and Q^2 = -0.07. The responsible features for the separation were obtained on the right top quadrant of the S-plot of OPLS-DA (**Figures 17b**). Twenty-one features **(31-51)** were selected based on the high p (1) (≥ 0.5) and p (corr) (≥ 0.2) values and **17** of them were tentatively identified (**Table 6**) by comparing with the on-line database and the literature. More spectroscopic data is needed to identify other features.

The tentatively identified compounds that are in the class of amino acids were lysine; *m/z* 147.1143, Rt 2.8 min **(31)**, methionine; *m/z* 150.0596, Rt 4.1 min (**35**), tyrosine; *m/z* 182.0833, Rt 4.2 min (**36**), leucine/isoleucine; *m/z* 132.1002, Rt 4.7 min (**37**), Phenylaniline; *m/z* 166.0880, Rt 8.0 min (**42**), Tryptophan; *m/z* 205. 0988, Rt 9.2 min (**44**), cycloleucine; *m/z* 130.0875, Rt 3.7 min (**32**) and amino acids derivatives 2-aminooctanoic acid; *m/z* 160. 1347, Rt 3.7 min (**33**), 5- aminopentanoic acid; *m/z* 118. 0878, Rt 4.0 min (**34**), racemethionine; *m/z* 150.0596, Rt 5.6 min (**38**) and leucyl proline; *m/z* 229.1561, Rt 8.5 min (**43**).

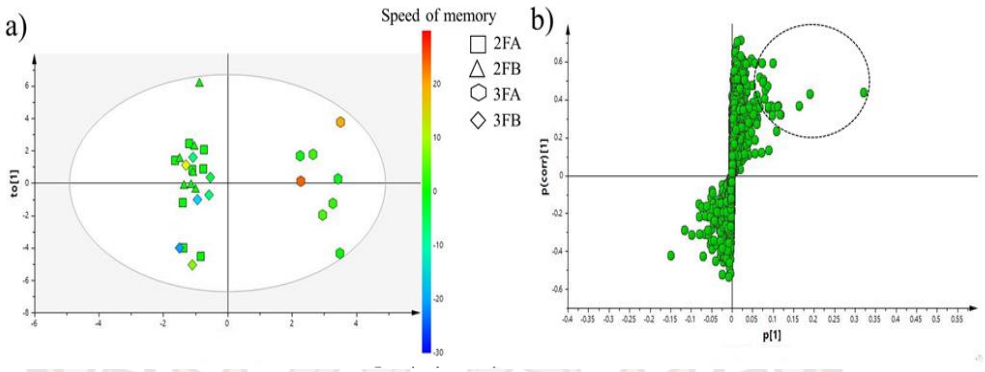

Figure 17 OPLS-DA score plot (a) and S-plot (b) of fecal samples of those volunteers who took placebo for 2 weeks (2FA and 2FB), after taking of Brahmi essence for 12 weeks (3FA) and after taking of placebo for 12 weeks (3FB) where speed of memory was used as a y-variable

In addition, the ions at *m/z* 455.3547, Rt 20.4 min (**48**) and *m/z* 455.3542, Rt 27.7 min (**50**) showed similar fragmentation pattern at *m/z* 437, *m/z* 369, *m/z* 315. These might indicate the two compounds are isomers. From the above data, they were tentatively identified as jujubogenin and its isomer. Other metabolites at *m/z* 473.3656, Rt 27.7 min **(51)** which were fragmented at *m/z* 455, *m/z* 437, *m/z* 369, *m/z* 315 also detected in 3FA. By comparing with the literature [16, 43], **(51)** was tentatively identified as pseudojujobogenin. These metabolites might be gut microbial metabolic products of active compounds, jujubogenin, and pseudojujobogenin glycosides present in the Brahmi essence. The presence of these

metabolites in feces samples might reveal the disposition of the jujubogenin and pseudojujobogenin glycosides from the body.

There were 11 metabolic pathways which might be linked with the metabolites in feces samples after taking Brahmi essence. As shown in **Figure 18**, the majority of the pathways were also seen in plasma and urine samples. The amino acids which were also detected in our samples have been reported as a differential metabolite in fecal samples after taking certain food supplements [44, 45].

The improvement of speed of memory after chronic consumption of Brahmi essence by elderly participants was assessed by Kamkaew (2020) [20]. In our study, we tentatively identify those metabolites in plasma, urine, and feces samples which might be responsible for the above effect by using an untargeted LC-MS metabolomics approach

In all the samples (plasma, urine, and feces) amino acids and their pathways were mainly detected. These metabolites improve brain function by being involved in the production or modulation of neurotransmitters and increasing blood flow by supply oxygen and nutrients to the brain. There are reports that Brahmi extract exhibits antioxidant activities [4] and also increases cerebral blood flow thereby improving brain function [11].

The active compounds in Brahmi essence were not detected in the plasma or urine. This might be due to subsequent exposure to gastric acid, intestinal fluid, bile, and intestinal microflora after oral administration and might opportunistically convert to other forms. The presence of metabolites in low amounts after oral intake, together with the large time gap between intake of the essence and the collection of samples, might be another reason for unable to detect these active compounds. In this study, we did not control the diets or lifestyles of the participants. We assumed that the active compounds in the essence (Bacopa saponins) would not have any interaction.

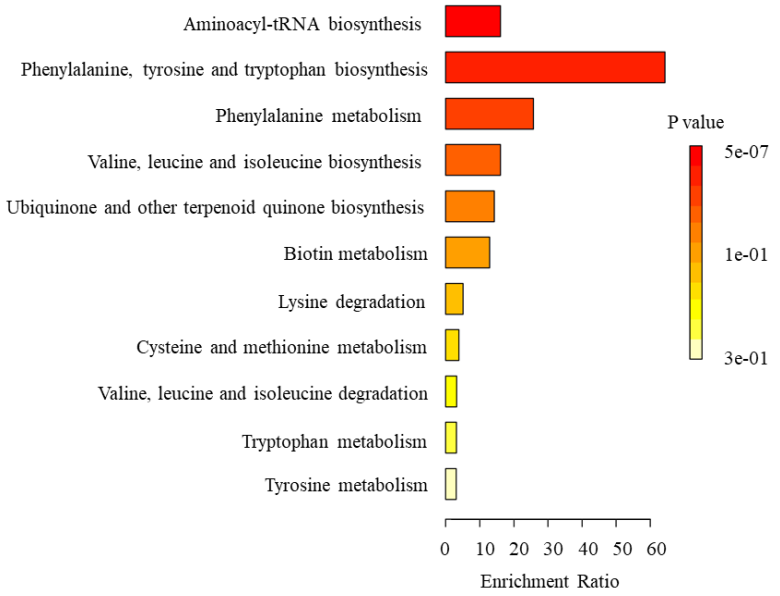

Figure 18 Summary plot of over representation analysis (ORA) for feces samples (the color represents: red- lower *p* values and yellow- higher *p* value)

Conclusion

The LC-MS metabolomics approach was applied to characterize the altered metabolites in plasma, urine, and feces samples after consumption of Brahmi essence by healthy human participants over a 12-week period. The tentatively identified metabolites might be responsible for the effect of Brahmi essence that was found on the improvement of speed of memory of the healthy elderly participants. The untargeted LC-MS metabolic approach can be used to detect metabolic changes in biological samples.

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CHAPTER V

CONCLUSION

In this research work, the analytical techniques of headspace GC-MS and LC-ESI-QTOF-MS were used for the characterization of metabolites found in medicinal plants. *C. lappacea* and *B. monnieri* were used as the model plants.

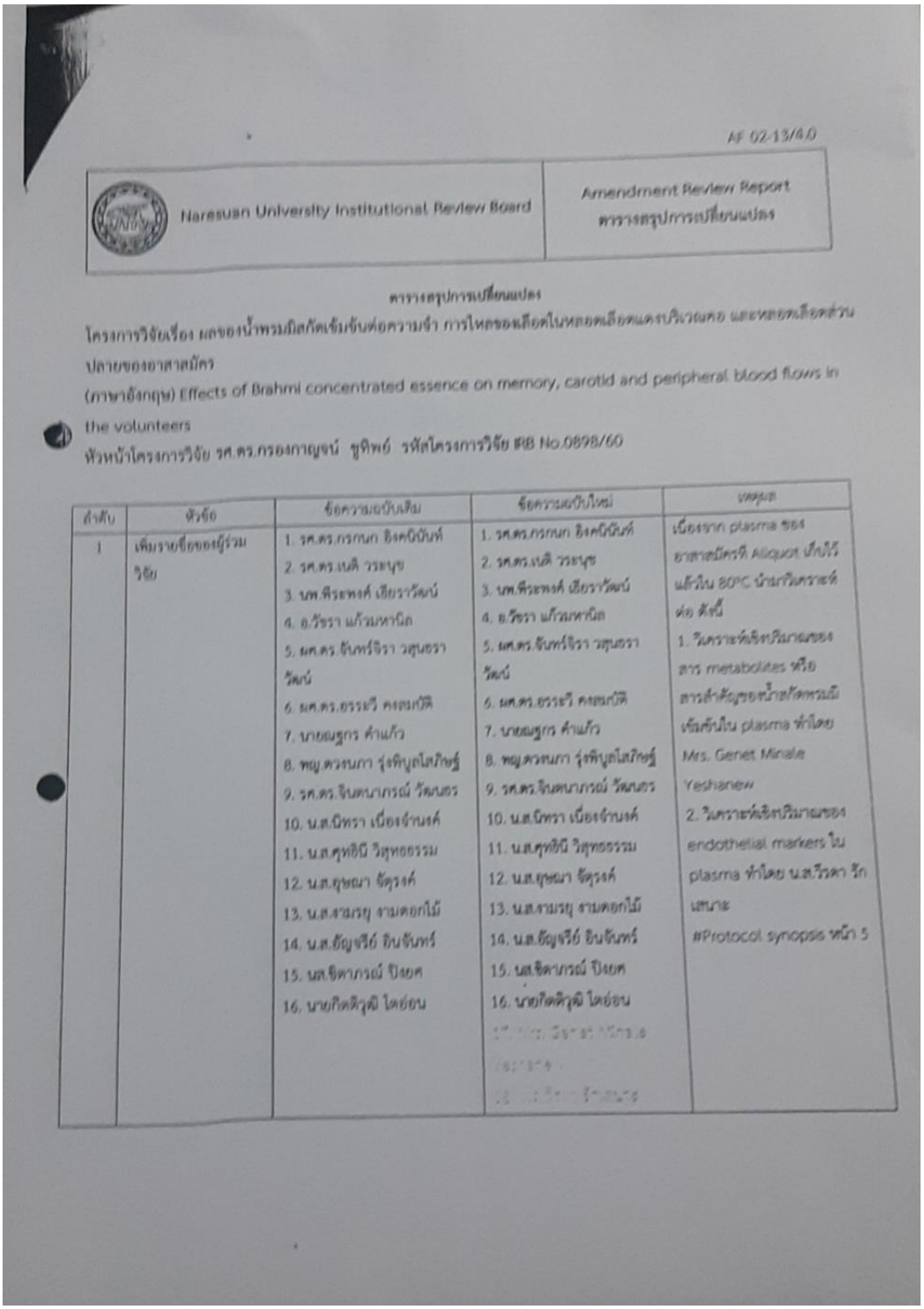
Coupling the techniques of LC-ESI-QTOF-MS with DPPH assays led to the identification of antioxidants from the aerial parts of *C. lappacea* which might be used as markers for the plant material and the extracts, as well as for preparations from this plant. In this study, 12 antioxidant compounds, including *C*- and *O*-glycosides of luteolin and apigenin, were identified from the aerial parts of the *C. lappacea*. We also report the presence of elements such as potassium, calcium, and silicon which might support the traditional use of this plant. Headspace analysis has the advantage of characterizing the chemical constituents as it represents the constituents of the smoke during traditional use (the smoke has been the primary therapeutic in traditional use). Volatile compounds from the aerial parts and the root of *C. lappacea* that were identified by headspace GC-MS might be responsible for the plant's therapeutic effect on the labia of postpartum women.

LC-ESI-QTOF-MS techniques were used to characterize metabolites in biological samples (plasma, urine, and feces) obtained from the participants before and after taking a12-week course of Brahmi essence or placebo. The metabolomic study using OPLS-DA led to the tentative identification of 15 metabolites in the plasma, 7 in the urine, and 17 in the feces. Those metabolites might be involved with the increase of speed of memory observed in the participants who received the Brahmi essence. The pathway that related to those metabolites were tentatively predicted. Also, the information obtained from this research might assist in future research into the clinical application of Brahmi essence and further studies on characterization of metabolites on Alzheimer's Disease.

REFERENCES

APPENDICES

APPENDIX A APPROVED RESEARCH AMENDMENTS



AF 02-13/4.0

Naresuan University Institutional Review Board

Amendment Review Report
ตารางสรุปการเปลี่ยนแปลง

ตารางสรุปการเปลี่ยนแปลง

โครงการวิจัยเรื่อง ผลของน้ำพรมมิสกัดเข้มข้นต่อความจำ การไหลของเลือดในหลอดเลือดแดงบริเวณคอ และหลอดเลือดส่วนปลายของอาสาสมัคร

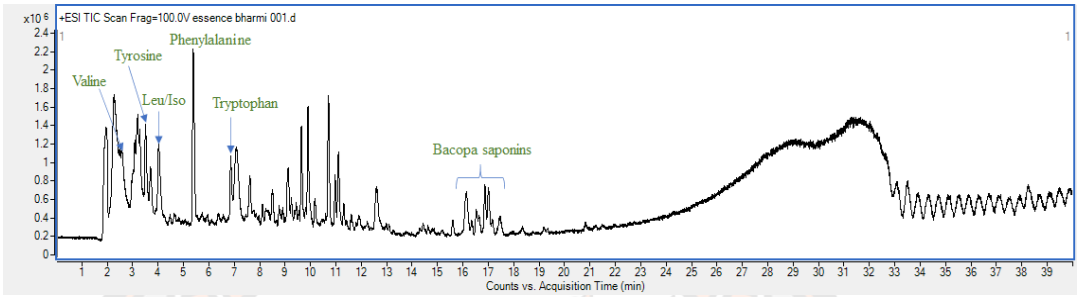
(ภาษาอังกฤษ) Effects of Brahmi concentrated essence on memory, carotid and peripheral blood flows in the volunteers

หัวหน้าโครงการวิจัย รศ.ดร.กรองกาญจน์ ชูทิพย์ รหัสโครงการวิจัย IRB No.0898/60

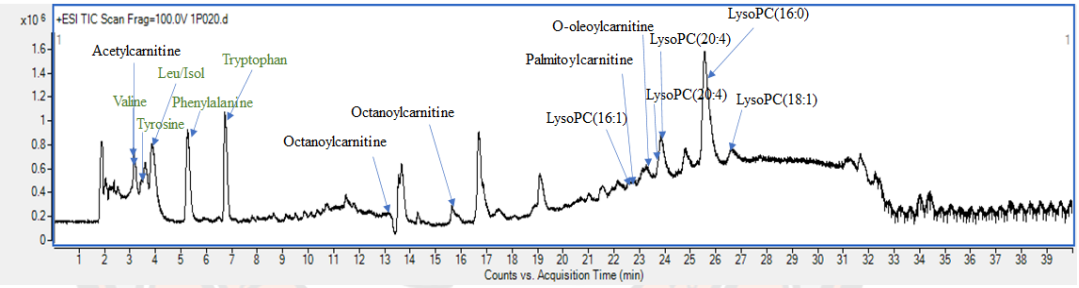
ลำดับ	หัวข้อ	ข้อความฉบับเดิม	ข้อความฉบับใหม่	เหตุผล
1	เพิ่มรายชื่อของผู้ร่วมวิจัย	1. รศ.ดร.กรกนก อิงคนินันท์ 2. รศ.ดร.เนติ วรธนุช 3. นพ.พีระพงศ์ เอียราวัฒน์ 4. อ.วิธรา แก้วมหานิล 5. ผศ.ดร.จันทร์จิรา วสุนธรา วัฒน์ 6. ผศ.ดร.ธรระวี คงธนกิติ 7. นายณฐกร คำแก้ว 8. พญ.ดวงนภา รุ่งพิบูลโสภิษฐ์ 9. รศ.ดร.จินตนาภรณ์ วัฒนธร 10. น.ส.นิพรา เนื่องจำนงค์ 11. น.ส.ศุทธินี วิสุทธธรรม 12. น.ส.ยุพผา จัตุรงค์ 13. น.ส.รามรยุ รามดอกไม้ 14. น.ส.ธัญจิรีย์ ยินจันทร์ 15. นส.ชิดาภรณ์ ปิยะศ 16. นายกิตติวุฒิ ไตย์ยน	1. รศ.ดร.กรกนก อิงคนินันท์ 2. รศ.ดร.เนติ วรธนุช 3. นพ.พีระพงศ์ เอียราวัฒน์ 4. อ.วิธรา แก้วมหานิล 5. ผศ.ดร.จันทร์จิรา วสุนธรา วัฒน์ 6. ผศ.ดร.ธรระวี คงธนกิติ 7. นายณฐกร คำแก้ว 8. พญ.ดวงนภา รุ่งพิบูลโสภิษฐ์ 9. รศ.ดร.จินตนาภรณ์ วัฒนธร 10. น.ส.นิพรา เนื่องจำนงค์ 11. น.ส.ศุทธินี วิสุทธธรรม 12. น.ส.ยุพผา จัตุรงค์ 13. น.ส.รามรยุ รามดอกไม้ 14. น.ส.ธัญจิรีย์ ยินจันทร์ 15. นส.ชิดาภรณ์ ปิยะศ 16. นายกิตติวุฒิ ไตย์ยน	เนื่องจาก plasma ของอาสาสมัครที่ Aliquot เก็บไว้แล้วใน 80°C นำมาวิเคราะห์ต่อ ดังนี้ 1. วิเคราะห์เชิงปริมาณของสาร metabolites หรือสารสำคัญของน้ำสกัดพรมมีเข้มข้นใน plasma ทำโดย Mrs. Genet Minale Yeshanew 2. วิเคราะห์เชิงปริมาณของ endothelial markers ใน plasma ทำโดย น.ส.วิรดา รักเสนาะ #Protocol synopsis หน้า 5

APPENDIX B TIC CHROMATOGRAMS OF BRAHMI ESSENCE (A) AND METABOLITES IN PLASMA SAMPLE OF VOLUNTEERS IN GROUP A AT BASE LINE (B), AFTER TAKING OF A PLACEBO FOR 2 WEEKS (C) AND AFTER TAKING OF BRAHMI ESSENCE FOR 12 WEEKS (D)

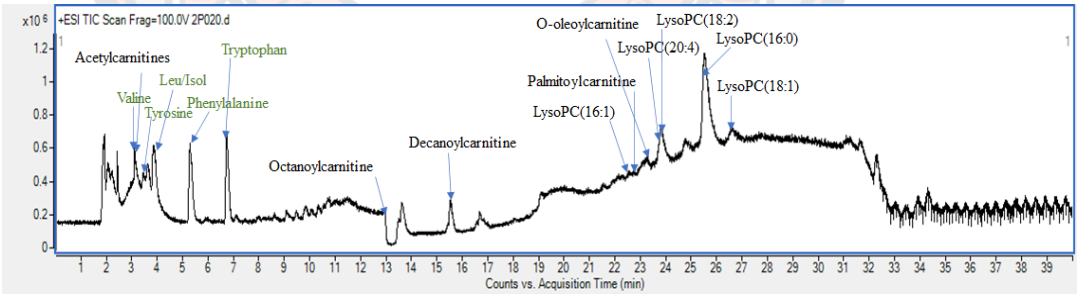
A)



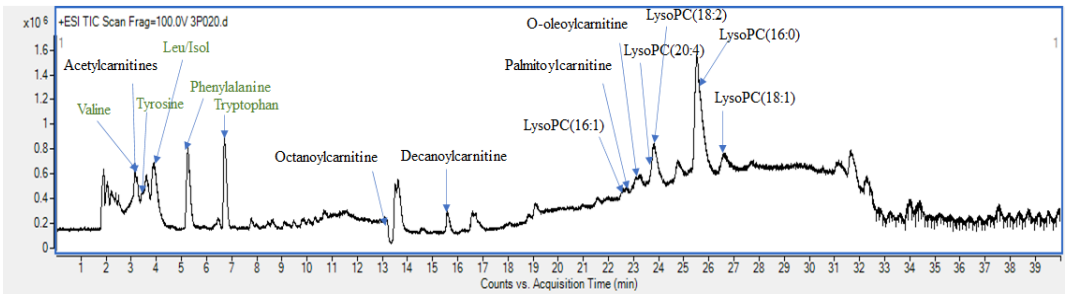
B)



C)

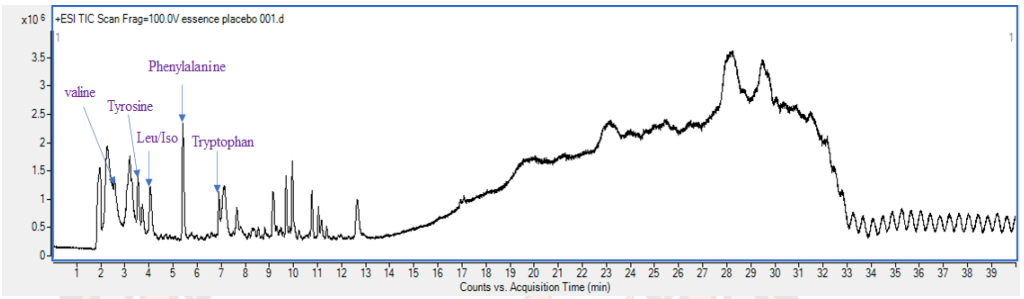


D)

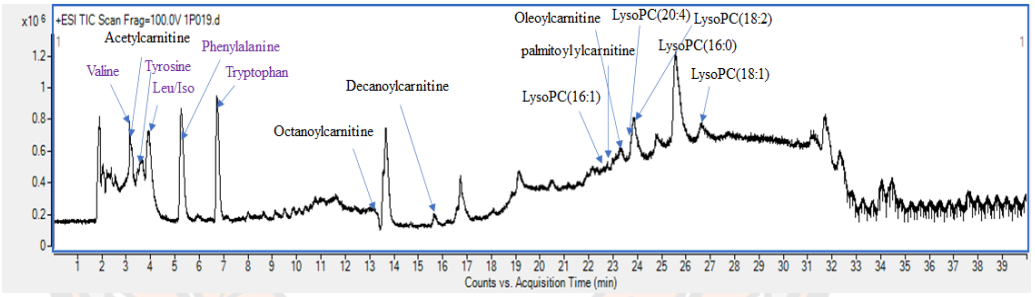


APPENDIX C TIC CHROMATOGRAMS OF PLACEBO (A) AND METABOLITES IN PLASMA SAMPLE OF VOLUNTEERS GROUP B AT BASE LINE (B), AFTER TAKING OF A PLACEBO FOR 2 WEEKS (C) AND TAKING OF A PLACEBO FOR 12 WEEKS (D)

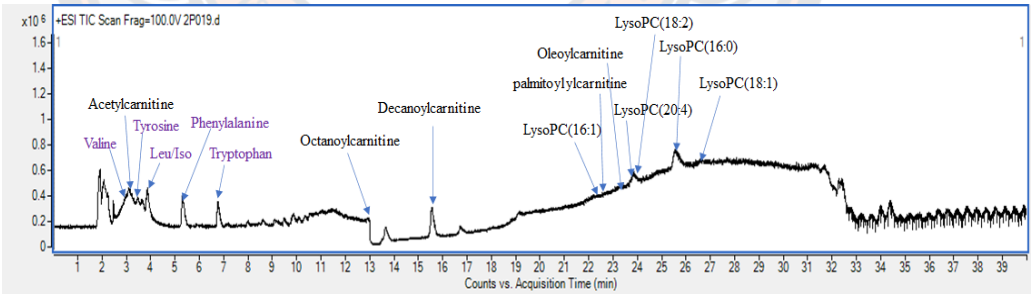
A)



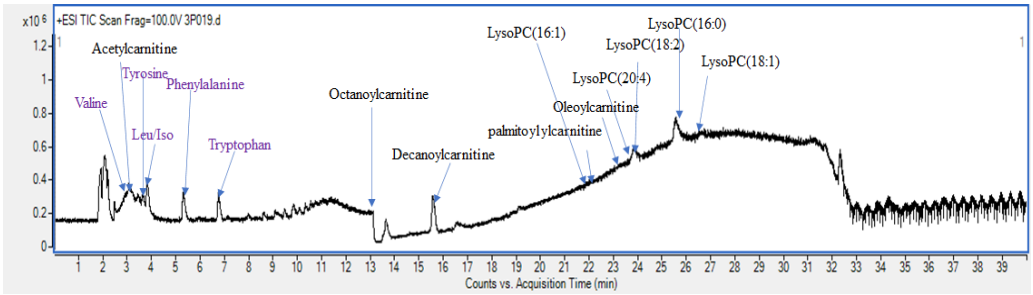
B)



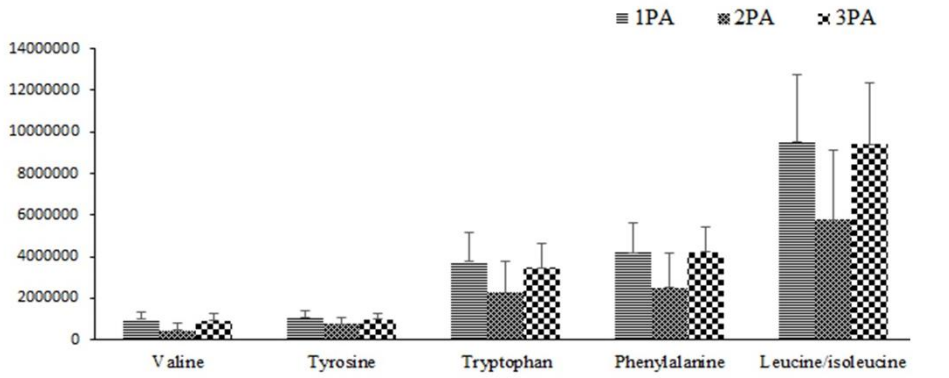
C)

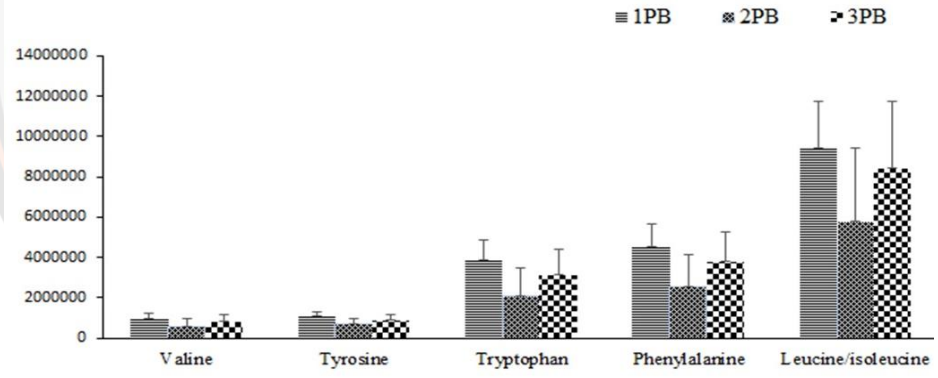


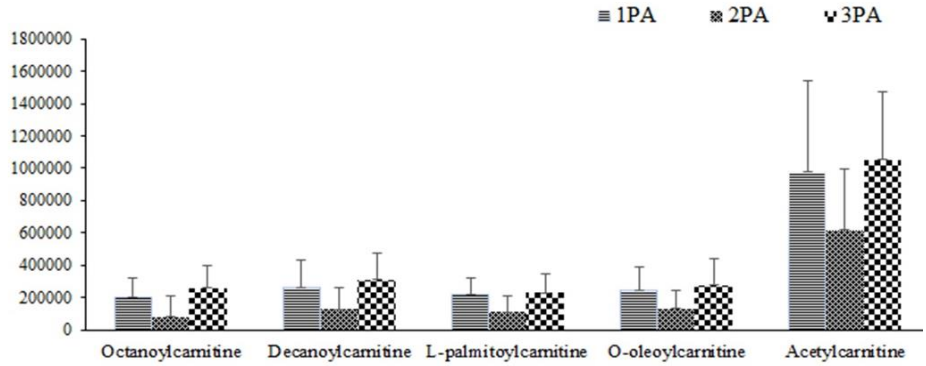
D)

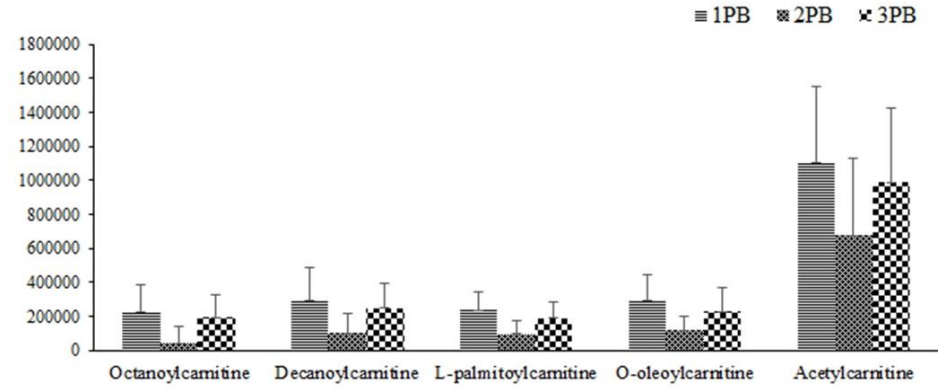


APPENDIX D THE INTENSITIES OF TENTATIVELY IDENTIFIED METABOLITES IN PLASMA SAMPLES OF VOLUNTEERS FROM GROUP A AND GROUP B AT BASELINE (1PA, 1PB), AFTER TAKING OF A PLACEBO FOR 2 WEEKS (2PA, 2PB) AND AFTER TAKING OF A PLACEBO OR BRAHMI ESSENCE FOR 12 WEEKS (3PA, 3PB)





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3PB
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1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
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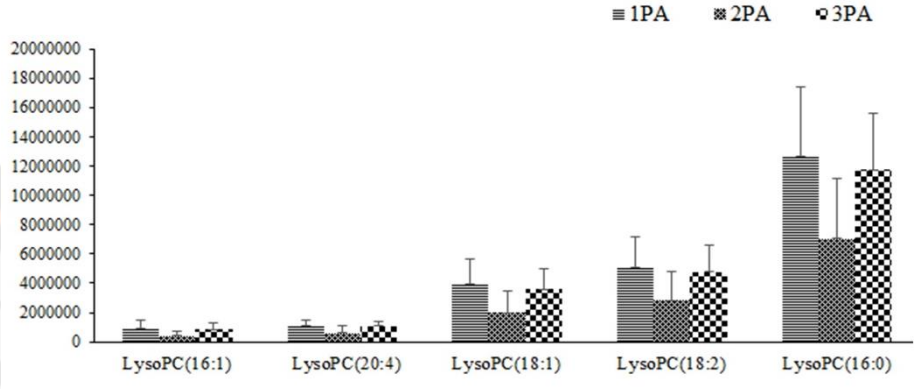
Decanoylcarnitine
L-palmitoylcarnitine
O-oleoylcarnitine
Acetylcarnitine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3PA
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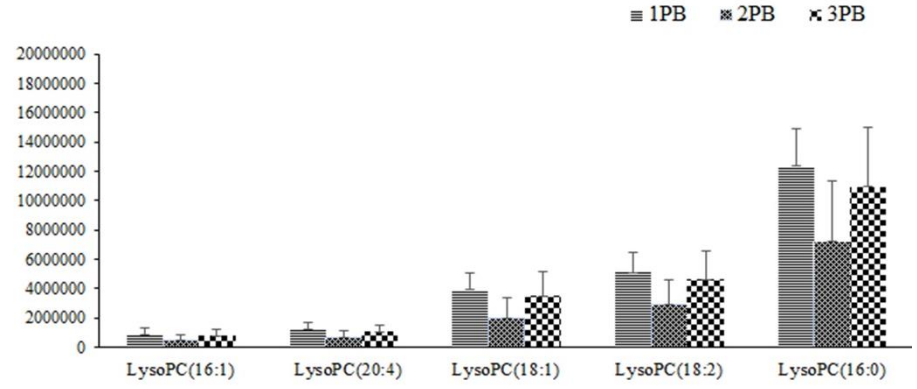
LysoPC(16:1)
LysoPC(20:4)
LysoPC(18:1)
LysoPC(18:2)
LysoPC(16:0)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3PB
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LysoPC(20:4)
LysoPC(18:1)
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