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Original Article

An on-line LC-MS/ DPPH approach towards the quality control of antioxidative ingredients in Sahastara

Nitra Nuengchamnong[1, 2*] and Kornkanok Ingkaninan[3]

[1] *Science Laboratory Centre, Faculty of Science, Naresuan University, Mueang, Phitsanulok, 65000 Thailand*

[2] *Regional Medical Sciences Centre Phitsanulok, Department of Medical Sciences, Ministry of Public Health, Mueang, 65000 Thailand*

[3] *Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, Mueang, Phitsanulok, 65000 Thailand*

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Abstract

The holistic approach of herbal medicines is becoming acceptable in many parts of the world. In order to determine, not only chemical but also biological activity profiles of traditional medicines, on-line liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) coupled to antioxidant assay was introduced. Here, we demonstrate the performance of this on-line monitor for quantitatively and qualitatively analyses of antioxidants in Sahasthara, a Thai traditional medicine. HPLC real-time coupling to ESI-MS and DPPH assay was applied to separate and identify the constituent compounds responsible for antioxidant activity. The antioxidant fingerprint was obtained together with the LC-MS chromatogram. The full scan MS and the multiple reaction monitoring (MRM) were used for structural confirmation and quantitation. Peak identification was performed by comparing retention times and mass spectra with reference compounds and published data. Based on their mass spectra, 13 antioxidant compounds were identified as gallic acid, chebulic acid, digalloyl glucoside, caffeoylferuloylquinic lactone, galloylshikimic acid, trigalloyl glucoside, corilagin, chebulanin, chebulagic acid, dicaffeoylquinic lactone, dicaffeoyl quinic acid, ellagic acid rhamnoside and ellagic acid. Gallic acid and ellagic acid were further quantitated and comprised 126.8±6.0 mg/g and 253.7±17.6 mg/g of Sahasthara, respectively. This on-line monitor is shown to be an effective tool for fast and simultaneous analyses of antioxidant compounds in complex formulations characteristic of many traditional medicines.

Keywords: DPPH assay, holistic, LC-MS, on-line chromatography, herbal medicine

1. Introduction

Traditional medicines commonly contain several herbal ingredients which make their quality control difficult. The quantitative analyses of individual chemical markers might not fully reflect the quality of these complex samples. HPLC and TLC fingerprints are, therefore, often used to express holistic chemical constituents of these medicines. However, the combination of bioassay with chemical analyses should be more suitable for the quality evaluation of these samples.

LC-MS is very useful technique for chemical profiling of plant metabolites (Pellati *et al.*, 2013; Pfundstein *et al.*, 2010; Zywicki *et al.*, 2002). Several groups have successfully coupled this technique online to bioassays for the analyses of biologically active compounds in natural products

* Corresponding author.
Email address: nitran@nu.ac.th

(Nuengchamnong *et al.*, 2011; Su *et al.*, 2010; Yao *et al.*, 2012). The online technique has proved to be a beneficial method to evaluate antioxidant activity while simultaneously identifying the compounds responsible for such activity in complex mixtures. Moreover, the quantitative analyses and the confirmation of structure can be conducted by MRM mode using selected molecular ion and fragmentation of the compounds of interest.

"Sahasthara" is described in the national list of essential medicines in Thailand as an antipyretic, anti-inflammatory and analgesic. This medicine is composed of 21 medicinal plants (Thai FDA, 2012). The names of the constituent plants, the parts used, their proportions and chemical constituents cited from references are shown in Table 1. The alcoholic extracts of this medicine showed anti-inflammatory and antioxidant activities (Kakatum *et al.*, 2012). This is in agreement with the observation from Middleton (Middleton *et al.*, 2000) that the healing properties of medicinal plants sometimes relate to phenolic contents and their roles in the prevention of diseases might be associated with relieving oxidative stress. However, to the best of our knowledge, neither the profile of chemical constituents in these preparations nor the compounds responsible for antioxidative activity have been investigated so far.

In this study, the chemical compositions related to the antioxidant activity of this preparation were determined by LC-ESI-MS/MS coupled to an antioxidant assay. The fingerprint of antioxidative activity as well as the biomarkers found in the study can be used for quality control of Sahasthara herbal medicine in the future.

Table 1. The list of ingredients in Sahasthara herbal medicine

Scientific name	Family	Part used	Weight (%)	Chemical constituents
Piper nigrum L.	Piperaceae	fruit	24.0	volatile oils, alkaloid (Nakatami *et al.*, 1986)
Plumbago indica L.	Plumbaginaceae	root	22.4	napthaquinones (Dinda and Chel,1992)
Terminalia chebula Retz.	Combretaceae	fruit	10.4	hydrolysable tannins (Surveswaran *et al.*, 2007)
Piper longum L.	Piperaceae	fruit	9.6	volatile oils, alkaloid (Nakatami *et al.*, 1986)
Acorus calamus L.	Acoraceae	rhizome	8.8	sesquiterpenes (Dong *et al.*, 2010)
Baliospermum montanum Muell. Arg.	Euphorbiaceae	root	8.0	phorbol esters (Ogura *et al.*, 1978)
Holarrhena curtisii King & Gamble	Apocynaceae	stem and root	4.8	alkaloids (Cannon *et al.*, 1980)
Cinnamomum camphora Sieb	Lauraceae	bark	1.4	volatile oils (Frizzo *et al.*, 1999)
Myristica fragrans. Houtt	Myristicaceae	aril	1.3	volatile oils (Surveswaran *et al.*, 2007)
Myristica fragrans. Houtt	Myristicaceae	seed	1.2	volatile oils (Surveswaran *et al.*, 2007)
Lepidium sativum L.	Cruciferae	seed	1.1	imidazole alkaloids (Maier *et al.*, 1998)
Ferula asafoetida L.	Umbelliferae	oleogumresin	1.0	sesquiterpenes, coumarins, sulfur containing compounds (Iranshahy *et al.*, 2011)
Anethum graveolens L.	Umbelliferae	fruit	1.0	volatile oils (Rádulescu *et al.*, 2010)
Pimpinella anisum L.	Umbelliferae	fruit	0.9	phenolic compounds (Marques and Farah, 2009)
Merremia vitifolia (Burm. f.) Hallier f.	Convolvulaceae	root	0.8	alkaloids (Jenett-Siems *et al.*, 2005)
Cuminium cyminum L.	Umbelliferae	fruit	0.8	volatile oils, phenolic acids, flavonoids, coumarins (Surveswaran *et al.*, 2007)
Nigella sativa L.	Ranunculaceae	seed	0.7	volatile oils (Nickavar *et al.*, 2003)
Anacyclus pyrethrum(L.) DC.	Compositae	root	0.6	terpenoids, phenolic acids (Surveswaran *et al.*, 2007)
Atractylodes lancea(Thunb) DC.	Compositae	rhizome	0.5	sesquiterpenes (Wang *et al.*, 2008)
Picrorhiza kurroa Benth.	Scrophulariaceae	root	0.4	flavonoids, phenolic acids (Surveswaran *et al.*, 2007)
Terminalia chebula Retz.	Combretaceae	gall	0.3	-

2. Materials and Methods

2.1 Materials and reagents

Sahasthara, was obtained from Bangkratum Hospital, Phitsanulok, Thailand. The production and quality control process were conducted under the regulation of the Thai Ministry of Public Health. The reference compounds, gallic acid and ellagic acid were purchased from Sigma (St. Louis, MO, USA). L-Ascorbic acid and 1,1 diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was purchased from Fluka Chemie AG (Buchs, Switzerland). Methanol (LC/MS reagent) was purchased from JT Baker (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA). Formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Water was purified using an Elga USF system (Bucks, England).

2.2 Sample preparation

Sahasthara in powder form (0.5 g) was extracted 3 times with 70% (v/v) methanol by sonication for 10 min, and then filtered. The filtrate was collected, adjusted to 50.0 mL and its antioxidant activity determined using both the standard batch DPPH assay and an online LC-ESI-MS/MS coupled to DPPH analyses. For LC-ESI-MS/MS determination, the filtrate was filtered through a 0.2 μm nylon syringe filter (Chrom Tech, Inc. MN, USA) prior to the analysis.

2.3 Off line DPPH assay for determination of antioxidant activity

The antioxidant activity of Sahasthara in methanolic extract was determined using the DPPH assay on 96 well plates (Nuengchamnong *et al.*, 2009).

The IC_{50}, which was defined as the concentration of extract required to scavenge 50% of the DPPH radical was determined by extrapolation from the regression line using Excel program. Measurements were performed in triplicate and L-ascorbic acid and Trolox were used as positive controls.

2.4 LC-ESI-MS coupled to DPPH assay

The HPLC was coupled on-line to MS and a continuous flow DPPH assay as mentioned before (Nuengchamnong *et al.*, 2011). An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) was connected to a PE SCIEX API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem, Foster city, CA) equipped with an electrospray ionization interface. The chromatographic separation was achieved with a Phenomenex Gemini C18 column (5 μm, 250 x 4.6 mm i.d.) (Phenomenex, Torrance, CA) protected with an ODS C18 guard column, and operated at 25°C. The mobile phase consisted of solvent A (0.1% v/v formic acid in H_2O) and solvent B (methanol). The elution program started from 90:10 solvent A:B for 4 min, then changed to 80:20 in 6 min, linearly increased to 10:90 over 30 min. After that, the ratio of solvent A:B was constant at 10:90 for 5 min, then changed to 90:10 for 5 min and kept constant at 90:10 for 5 min to recondition the column.

Mass spectra were recorded within 55 min. The injection volume was 5 μL. The flow rate was set to 600 μL/min. Analyst 1.5.1 software (Applied Biosystem, Foster city, CA) was used for data acquisition and processing. The full scan mass spectra from *m/z* 100-1000 amu were acquired in negative ion modes. The optimum conditions of the interface were as follows: ESI-negative; ion spray voltage of -4500 V, curtain gas (N_2) at 69 Kpa (10 psi), ion source gas 1 (air, for nebulizing) at 450 Kpa (65 psi), ion source gas 2 (air, for drying solvent) at 380 Kpa (55 psi). The interface temperature was set to 400°C. The entrance potential (EP) and declustering potential (DP) were -10V and -80V, respectively. The eluate from the LC separation was split into two parts at the ratio of 8:2. The minor part flowed into the continuous flow system for antioxidant activity detection. The system consisted of an HPLC pump, LC20AD prominence (Shimadzu, Kyoto, Japan), home-made knitted reaction coil PEEK tubing with an inner diameter of 180 μm and a total reaction coil volume of 100 μL. The flow of 0.1mM DPPH was set to 200 μL/min and the resultant bleaching was detected at 515 nm using the UV-VIS detector, all at 25°C. (SPD 20AV, Shimadzu, Kyoto, Japan). The LC solution software was used for data acquisition and processing.

To characterize antioxidant activity peaks, the fragment ions from their corresponding parent ions in negative mode were induced with collision gas (CAD) of 41 Kpa (6 psi), collision energy (CE) between -5 to -50V and collision cell exit potential (CXP) of -6 V, DP in the range of -20 to -110 V. MS/MS in MRM mode was used for structural confirmation and quantitation of antioxidant compounds.

The extract mass in MRM mode was employed to confirm the structure identification and quantification by using the precursor ion and their product ions. The DP, CE and CXP were set at-(70-100), -(20-60) and -(6-20)V to obtain high intensity of signal.

Peak identification was performed by comparing the retention time, mass spectra and fragmentation patterns with reference compounds and published data (Table 2). The identification of these compounds was confirmed using MRM scan mode.

2.5 Quantitative analyses

The major antioxidant compounds in Sahasthara were quantitated using the same HPLC condition in previous section. The MS parameters were operated in negative MRM mode using the precursor ion $[M-H]^-$ and their product ions. Gallic acid and ellagic acid at various concentrations were used to prepare calibration curves that were generated by linear regression based on peak area**.** The limits of detection

Table 2. Identification of antioxidant compounds in the methanolic extract of Sahasthara using LC-ESI-MS/MS-DPPH assay. Data was recorded in negative ionization mode. Retention time (t_R) was from the activity trace.

No	t_R (min)	ESI-MS (*m/z*)		Tentative identification	Ref
		MS[M-H]$^-$	MS/MS		
1	15.3	169.2	**125.0**[M-H-CO_2]$^-$, 339.3[2M-H]$^-$	Gallic acid*	
2	15.8	354.9	**205.0**, 248.9, 173.2, 193.0	Chebulic acid	1
3	18.0	483.4	**313.1**[M-galloyl-H_2O]$^-$, 331.4[M-galloyl]$^-$, 270.6, 169.1[Gallic acid-H]$^-$	Digalloyl glucoside	2,3
4	19.2	511.5	**337.3**[M-H-quinic lactone]$^-$, 255.4[M-2H]$^{2-}$, 178.99 [caffeic acid-H]$^-$	Caffeoylferuloyl-quinic lactone	4
5	20.6	325.2	**169.0**[gallic acid-H]$^-$, 154.9[shikimic acid; 174-H_2O-H]$^-$	Galloylshikimic acid	5
6	25.6	635.4	**465.1**[M-gallic acid]$^-$, 317.3[M-2H]$^{2-}$, 483.1[M-galloyl]$^-$	Trigalloyl- glucoside	1,6
7	25.7	633.4	463.2[M-gallic acid]$^-$, 300.9[Hexahydroxy diphenoyl-H]$^-$	Corilagin	1,6
8	25.8	651.3	481.1[M-H-gallic acid]$^-$ 169.0[gallic acid-H]$^-$, 325.2[M-2H]$^{2-}$	Chebulanin	1
9	27.5	953.2	300.7[Hexahydroxy diphenoyl-H]$^-$, 275.2[Chebulic-2H_2O-CO_2-H]$^-$, 318.6[Chebulic-2H_2O-H]$^-$, 476.2[M-2H]$^{2-}$	Chebulagic acid	1
10	29.8	497.5	**451.5**, 335.2[M-caffeoyl-H]$^-$, 160.7[caffeic-H-H_2O]$^-$	Dicaffeoylquinic lactone	7
11	30.6	514.9	**353.1**[M-H-caffeoyl]$^-$, 335.1[M -H-caffeoyl-H_2O]$^-$, 299.2[M-H-caffeoyl-3H_2O]$^-$	Dicaffeoylquinic acid	8
12	33.6	447.3	**300.1**[M-H-147]$^-$, 301.2[M-H-146]$^-$	Ellagic acid rhamnoside	2
13	34.6	301.3	**229.0**[M-H-72]$^-$, 169.1[gallic acid-H]$^-$, 134.6[169-2OH-H]$^-$	Ellagic acid*	

* compared with reference compound; bold *m/z* was daughter ion used in MRM
Reference (1) Pfundstein *et al.*, 2010, (2) Mämmelä *et al.*, 2000, (3) Zywicki *et al.*, 2002, (4) Marques and Farah, 2009, (5) Powlowska *et al.*, 2006, (6) Pellati *et al.*, 2013, (7) Marques and Farah, 2009, (8) Han *et al.*, 2009

(LOD) with a signal-to-noise ratio of three were determined. The analyses were performed in triplicate. The ion spray (IS) was set to -4500V and dwell time set to 100 m sec for all compounds.

3. Results and Discussion

The antioxidant activity of Sahasthara in methanolic extracts was assessed on the basis of the scavenging activity of the stable DPPH free radical (Brand-Williams *et al.,* 1995). The IC_{50} of Sahasthara was 219.2±5.8 µg/mL while the reference compound; L-ascorbic acid and Trolox were 6.71±0.17 and 7.96±1.34 µg/mL. The extract was further analysed by the LC-ESI-MS/MS coupled to DPPH assay to obtain the antioxidant fingerprint of its chemical constituents separated by LC (Figure 1).

3.1 Identification of antioxidant compounds in Sahasthara

The separation of chemical constituents in Sahasthara was achieved using a reversed phase C_{18} column with gradient elution of 0.1% v/v formic acid: methanol as a mobile phase. The eluate was split to the continuous flow system for antioxidant activity detection and to the mass spectrometer

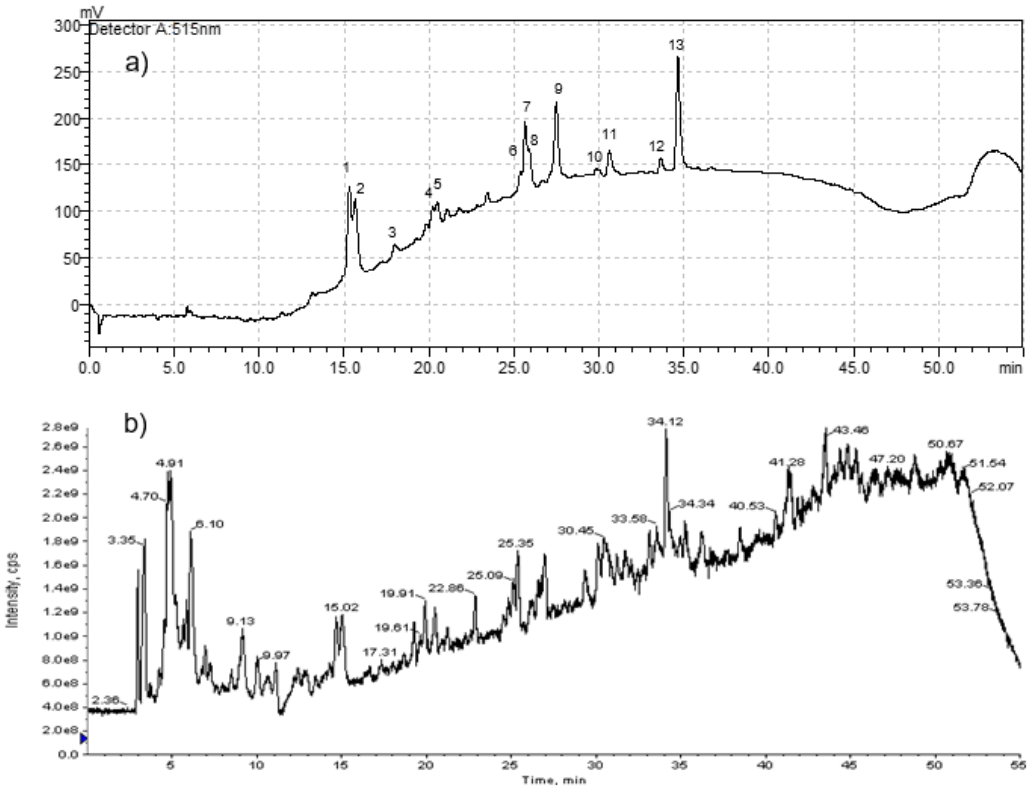

Figure 1. Time synchronized analyses of Sahasthara methanolic extract using LC-ESI-MS/MS coupled to DPPH assay: a) the trace of the DPPH based antioxidant activity detected at 515 nm, b) the total ion current (TIC) output from the ESI-MS in negative mode. For peak assignments, see Table 2. Conditions are described in the text.

with an electrospray interface generated in full scan MS mode. The sample was analyzed in negative and positive ionization modes. In this experiment, the negative mode gave a high intensity of signals than the positive mode, therefore it was selected for the analyses. Figure 1 shows the trace of the DPPH based antioxidant activity monitored at 515 nm and the corresponding chromatogram of the total ion current (TIC) output from the ESI-MS. The antioxidant trace suggested that at least thirteen compounds had antioxidant activity (Figure 1a). The delay between the MS read-out and the corresponding peak from the antioxidant activity assay was calculated as ca. 0.63 min based on retention times for gallic acid and ellagic acid as reference standards. The negative ions of the major antioxidants are listed in Table 2 and the identification of these compounds is proposed. The retention times depicted in Table 2 were from the DPPH antioxidant activity trace. It is noted that the upward shifting of the trace of the DPPH based antioxidant activity (Figure 1a) was observed due to the gradient elution. However, it affected neither the synchronized analyses of the antioxidant peaks to MS/MS, nor the repeatability of the assay.

Based on the DPPH antioxidant activity trace from the online system (Figure 1a), the major antioxidative activity of Sahasthara came from gallic acid (**1**), chebulic acid (**2**), corilagin (**7**), chebulagic acid (**9**), and ellagic acid (**13**). These compounds were commonly found in the fruit of *Terminalia chebula* Retz. (Pfundstein *et al.*, 2010) which is one of the main ingredients of Sahasthara. *T. chebula* fruit was related to various pharmacological activities (Bag *et al.*, 2013). Reddy and his co-worker proved that the antioxidant, chebulagic acid and gallic acid, can inhibit cyclooxygenase-2 (COX-2) (Reddy *et al.*, 2009a, 2009b, 2012). This finding suggests that its antioxidant activity might correlate with its anti-inflammatory activity.

3.2 Quantitative analysis of antioxidant compounds in Sahasthara using LC-MS/MS

Two major antioxidant compounds in Sahasthara, gallic acid and ellagic acid, were quantitatively analysed using LC-MS/MS in MRM mode. The regression equation with $1/(x^2)$ weighting for gallic acid and ellagic acid with working range at concentrations of 0.1-50 μg/mL was $y = 1.46x10^5x + 631$; with a correlation coefficient value (r) of 0.9994. For ellagic acid, the regression equation was $y = 1.75 x10^4x+1.99x10^4$; with $r = 0.9994$. The LODs of gallic acid and ellagic acid were 0.05 ng and 0.5 ng on-column, respectively. The results showed that 126.8±6.0 mg/g of gallic acid and 253.7±17.6 mg/g ellagic acid were found in this herbal medicine.

4. Conclusions

A Thai traditional medicine, Sahasthara has been investigated using on-line LC-ESI-MS/MS coupled with DPPH assay. Hydrolysable tannins were identified as major anti-oxidative components in this medicine. The study successfully demonstrated the use of the hydrolysable tannins, gallic acid and ellegic acid as markers for quantitative analysis along with the antioxidative activity fingerprint for holistic qualitative analysis for quality assessment of this medicine.

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