Chapter 4
Hyphenated Technique of LC-PDA-MS/MS for Phytochemical Profiling of *Ficus deltoidea*

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Abstract *Ficus deltoidea* is one of the widely used traditional herbal medicines in Malaysia. However, scientific data for this herb are very limited, particularly its phytochemical profile. The complexity of the phytochemical profile has hindered the development of this herb to wider application. Recent advancement of hybrid technology in analytical tools has eased the work of phytochemical profiling by simultaneous separation and identification using liquid chromatography integrated with photo diode array and tandem mass spectrometer (LC-PDA-MS/MS). This hyphenated technique was successfully used in the present study. A wide range of phytochemicals including amino acids, organic acids, alkaloids, terpenoids, polyphenols, and their derivatives have been identified. The marker compound, isovitexin (apigenin) was identified at the retention time 17.5 min in the negative ion mode of mass spectrometry. Besides flavone (apigenin), this non-targeted mass screening detected isoflavones (genistein), flavanone (naringenin), flavonol (kaempferol), and flavanol (catechin) in the leaves extract of the plant. Several organic acids and phenolic acids which are very important for plant physiology and defense mechanism were also found in this study. The identification was carried out by matching the fragmentation pattern of the compound to the literature data. The presence of product ions which are the characteristic fragments for a particular compound was used for phytochemical identification. In other words, the fragmentation pattern of phytochemical is similar to the fingerprint of human beings.

Keywords LC-PDA-MS/MS · *Ficus deltoidea* · Phytochemical · Secondary metabolites · Isovitexin

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P. Ramasami et al. (eds.), *Crystallizing Ideas – The Role of Chemistry*,
DOI 10.1007/978-3-319-31759-5_4

4.1 Introduction

The genus *Ficus* belongs to the Moraceae family which comprises about 750 species [1]. Most of the *Ficus* species can be found in the tropical countries as an evergreen shrub tree. According to Harrison [2], the rainforests of Borneo contain more than 160 *Ficus* species. The common species in Malaysia are *Ficus deltoidea* var. *kunstleri*, var. *trengganuensis,* and var. *angustifolia* [3]. The leaves of *F. deltoidea* have traditionally been used as herbal drink by people from South East Asia to treat numerous ailments such as diabetes, diarrhea, high blood pressure, skin infection, as well as to strengthen uterus of women after childbirth since ancient time [4, 5].

However, literature scientific data for this plant is very limited. Because of the wide application and usefulness of this herbal plant, many researchers have started their efforts to generate technical data for this herb, in terms of the pharmacological activities and its phytochemical profile recently. It was found that most researchers used methanolic extract of the plant in their studies. This is because the solvent system containing methanol would enhance the extraction of secondary metabolites from plant samples. Secondary metabolites are produced by plants through a number of defense mechanisms against both biotic and abiotic stresses for survival. In particular, flavonoids and terpenoids are considered as the major secondary metabolites attributed to the reported pharmacological properties.

Till date, few studies have been conducted on this herb, particularly on the pharmacological activities such as anti-nociceptivity [5], anti-inflammatory [6], and anti-diabetic [7, 8] by Malaysian researchers. The acute toxicity at the lethal dose of 50 % on Sprague Dawley rats was found to be greater than 5 g/kg [9]. There were also no significant adverse effects on the body weight, mortality, hematology, and histopathology of the rats from the subchronic toxicity. Furthermore, the toxicity effect was also evaluated based on the content of the characteristic phytochemicals in *F. deltoidea*, namely vitexin and isovitexin (C-glycosyl flavonoid). The result showed that there was no significant toxicity effect on normoglycemic mice and induced diabetic rats treated with the highest dose of 2 g vitexin and isovitexin per kilogram of body weight [10]. Another research team from France also investigated the volatile compounds, mostly from the chemical group of terpenoids and other oxygenated hydrocarbon compounds for 13–20 *Ficus* species [1, 11]. To the best of our knowledge, limited data are available for the phytochemicals in *F. deltoidea*. Therefore, the present study was carried out to identify the phytochemicals from the plant using a high sensitivity and mass accuracy hyphenated technique, LC-PDA-MS/MS.

4.2 Methodology

4.2.1 Plant Material and Chemicals

The plant materials of *F. deltoidea* Jack var. *trengganuensis* Corner used in this study were harvested from the garden of Institute of Bioproduct Development,

Universiti Teknologi Malaysia (Johor bahru, Malaysia). The species was authenticated and deposited in the Universiti Kebangsaan Malaysia Herbarium (Bangi, Selangor, Malaysia) with voucher specimen 40213. HPLC grade of methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Nanopure water (18.2 MΩ cm) was produced from Barnstead NANOpure Diamond water purification system (Thermo, Waltham, MA).

4.2.2 Herbal Extraction

Fifty gram of dried and ground leaves were extracted with 650 ml of 50 % methanol in a reflux extraction system for 60 min. The crude extract solution was filtered and dried in vacuo for the determination of extraction yield (9.13 %w/w). Consequently, 1 g/ml of the crude extract was prepared for LC-PDA-MS/MS analysis.

4.2.3 LC-PDA-MS/MS

A capillary liquid chromatography (Dionex Corporation Ultimate 3000; Sunnyvale, CA) system was integrated with a diode array detector (Dionex Ultimate 3000) and a quadrupole and time-of-flight, QTOF mass spectrometer (AB SCIEX QSTAR Elite; Foster City, CA). A C18 reversed phase XSelect HSS T3 column (2.1 × 100 mm, 2.5 µm) with a flow rate of 150 µl/min was used for separation. Samples were scanned for a wide range of wavelengths from 200 to 500 nm. The binary gradient system consists of solvent A (water with 0.1 % formic acid) and solvent B (acetonitrile). The LC gradient was: 0–30 min, 2–30 % B; 30–40 min, 30–98 % B; 98 % B hold for 0.1 min and 40.1–60 min, 98–8 % B. The injection volume was 5 µl. All samples were filtered with 0.2-µm nylon membrane filter prior to injection.

The QTOF mass spectrometer was used for the small metabolite screening from m/z 100–1000. It was calibrated using 1 pmol of reserpine (m/z 609.2807) before use to ensure mass accuracy. A single Information Dependent Acquisition (IDA) method was created to acquire both TOF MS and three dependent runs of product ion scan with rolling collision energy. Nitrogen gas was used for nebulizing (40 psi) and as curtain gas (25 psi). Collision gas was set at 3, the accumulation time was 1 s for TOF MS and 2 s for each product ion scan. The voltage of ion spray was 5500 V for positive ion mode and −4500 V for negative ion mode. The declustering potential was 40 V and the focusing potential was set at 200 V.

4.3 Results and Discussion

The plant crude extract was separated by decreasing the polarity of mobile phases through a C18 column for compound elution. The most polar phytochemicals would be eluted first from the non-polar column, followed by less polar phytochemicals. Therefore, the phytochemicals would be eluted accordingly based on their polarity and scanned at a wide range of wavelengths by the photo diode array detector. The chromatograms at the four wavelengths with the most intense peaks such as 254, 282, 310, and 340 nm are presented in Fig. 4.1. Mostly, plant secondary metabolites could be detected at these wavelengths. The compounds which were eluted from the column consequently ionized at the ion source before fragmentation and detection by mass spectrometer.

Significantly, the number of peaks detected in the chromatograms (Fig. 4.1) was less than compounds ionized in mass analyzer as presented in the mass spectra (Fig. 4.2).

The lower number of compounds detected by the chromatographic method compared to the mass spectrometric technique can be explained by the weak absorbance of many compounds under UV and visible range of light. Therefore, this hybrid system appears to be more effective for phytochemical profiling compared to single technique of chromatographic and/or mass spectrometric approach. Moreover, the combination of quadrupole and time-of-flight technology in a mass analyzer is known to be a powerful analytical tool for unknown compound elucidation because of its high resolution and mass accuracy.

Based on the mass screening, a number of secondary metabolites were identified from both positive and negative ion modes. The identification was carried out by matching with the literature data. The compounds included amino acids, organic acids, alkaloids, terpenoids, flavonoids, and their glycosides (Table 4.1). The table shows that the majority of the phytochemicals, particularly organic acids, phenolic acids, flavonoids, and other hydrocarbons were detected at the negative ion mode. Several organic acids were detected in this study and they are intermediates in the plant carbon metabolism. Previous experimental evidence has proven organic acids as key components in various plant mechanisms to cope with nutrient deficiencies, metal tolerance and plant–microbe interactions [12]. The activation of the plant defense mechanism also produces phenolic acids and flavonoids as by-products from the phenylpropanoid pathway. These secondary metabolites are important to regulate plant growth hormones and to protect plants from ultraviolet radiation.

Flavonoids could be detected at both positive and negative ion modes depending upon the capability of the compound to be protonated or deprotonated under high voltage condition of ion source. Interestingly, isoflavones were detected at the positive ion mode, whereas the other classes of flavonoids such as flavone (apigenin), flavanone (naringenin), and flavonol (kaempferol) were detected at the negative ion mode. Isovitexin (apigenin-6-C-glycoside), which is the characteristic phytochemical in *F. deltoidea,* was detected in the negative ion modes of mass screening. According to Sanchez-Rabaneda et al. [13], isovitexin could be differentiated from its isomer,

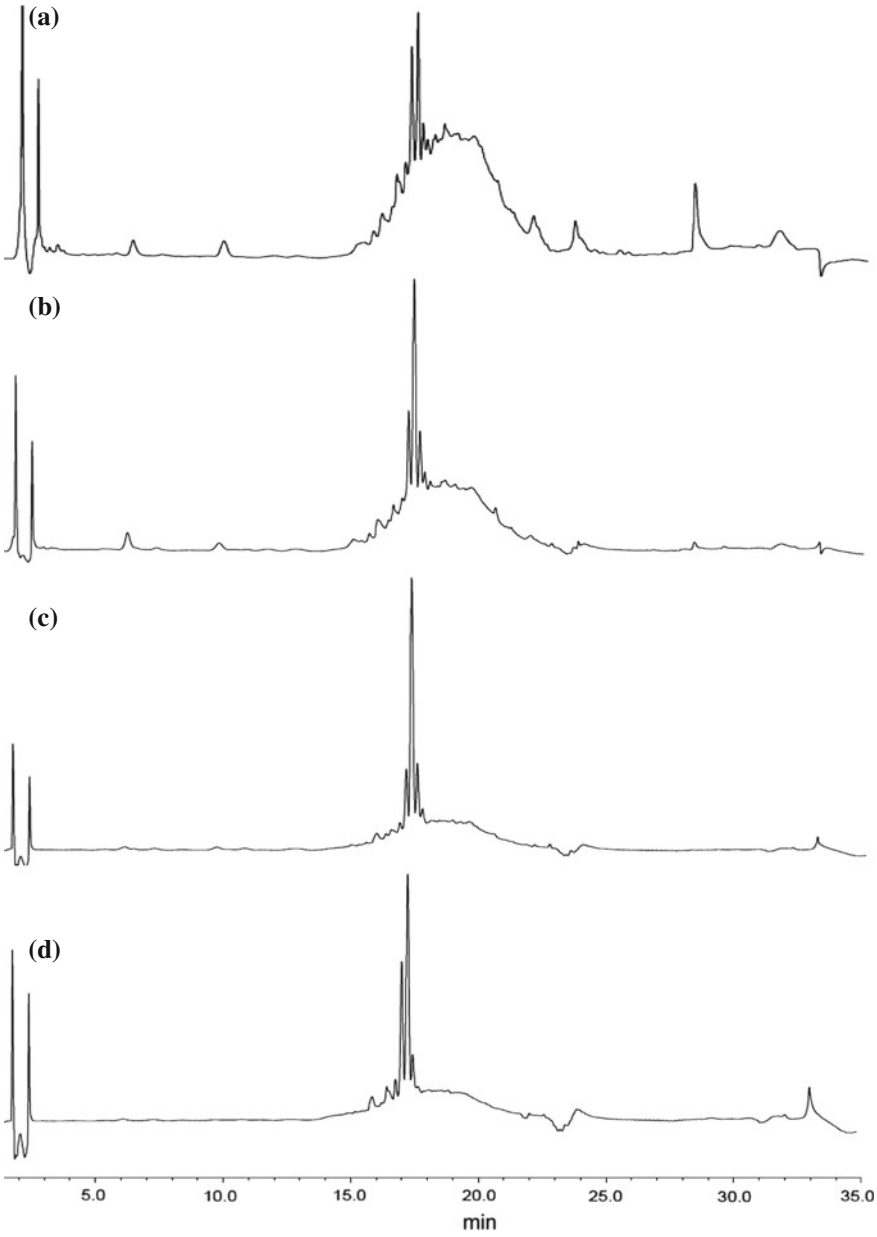

Fig. 4.1 Chromatogram of leaves extract of *Ficus deltoidea* at **a** 254 nm, **b** 282 nm, **c** 310 nm and **d** 340 nm

vitexin based on the presence of the only diagnostic ion, m/z 353 as the product ion in the negative ion. Kaempferol C-glycoside was also detected in the plant extract with its aglycone at m/z 285. Kaempferol could easily be differentiated from its

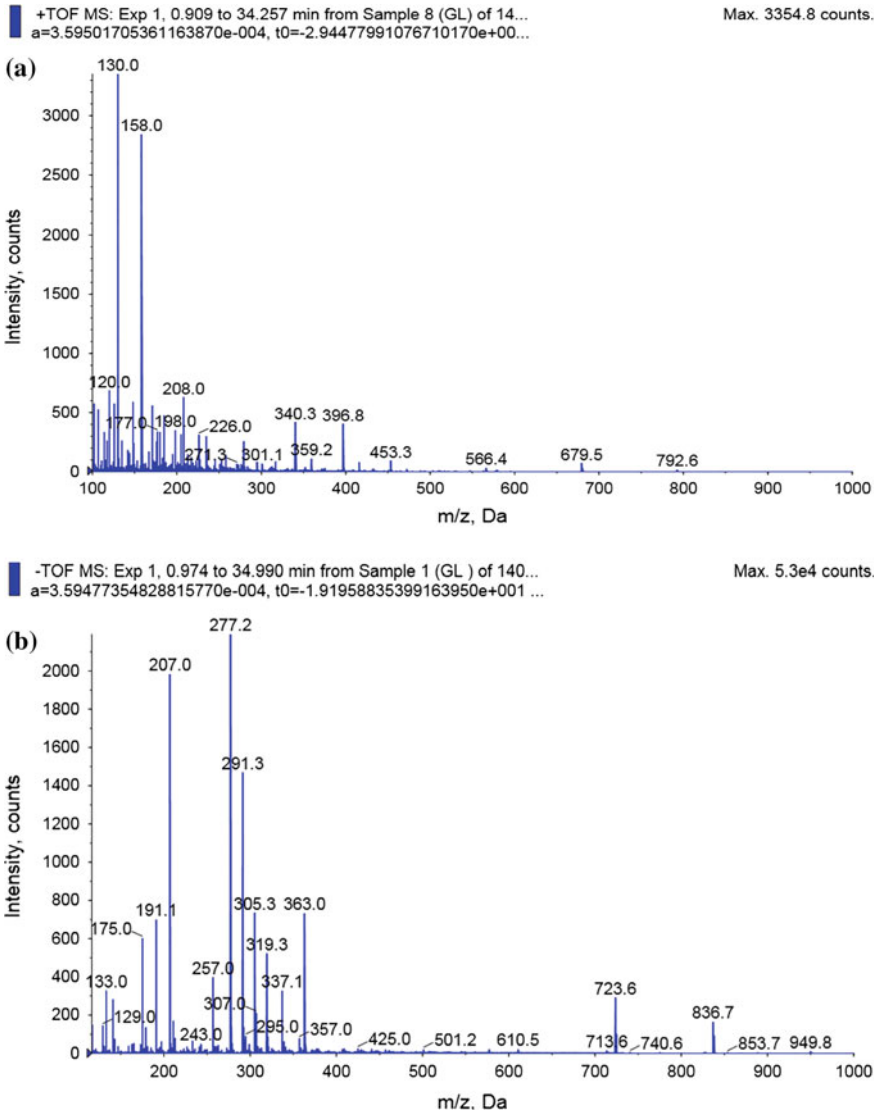

Fig. 4.2 Mass spectra of *Ficus deltoidea* leaves extract at the positive (**a**) and negative (**b**) ion modes

counterpart, luteolin (flavone) which has similar aglycone ion at m/z 285, by the absence of neutral loss 42 in the flavonol [14].

In the positive ion mode, the C-glycosyl of isoflavone, namely genistein-8-C-glucoside (m/z 433) and its diglycoside (m/z 579) were detected in the plant extract. The fragmentation of this compound matched with the data reported by Rauter et al. [15] for 5,7,4′-trihydroxyisoflavone-8-C-glucoside

Table 4.1 Secondary metabolites identified from both positive and negative ion modes

Putative compounds	m/z (+)	Fragment ions	Ref
Amino acid			
Hydroxyproline	132	132/114/86	[27]
Ornithine	133	133/116/87	[27]
Tryptophan	205	205/188/170/146/118	[28]
4-Amino-S-phenylthiopene-2-enedionic acid	210	210/192/164/147/146/118	[29]
Phenolic acid			
Caffeic acid	181	181/163/145/135/121/107/93	[30]
Flavonoid			
Epicatechin	291	291/273/255/207/165/147/139/123	[31]
Genistein-8-C-glucoside (5,7,4′-trihydroxyisoflavone-8-C-glucoside)	433	433/415/397/379/355/337/313/283/271/147/121	[15]
Genistein-C–O-diglycoside	579	579/433/415/397/379/355/337/313/283/271/129	[15]
Terpenoid			
Cleavage of the seven membered unsaturated ring C for duboscic acid	263	263/245/235/217/199/191/173	[24]
Ligustilide isopentenaldehyde	275	275/257/233/191/149/139/107	[32, 33]
β-Lapachone	277	277/259/241/235/221/199/159/149/135/121/107/93	[34]
Ebracteolatanolide B or Yuexiandujisu E or D	351	351/333/315/287/241/213/177/163	[25, 26]
Hydrocarbon			
Decatriendioic acid	197	197/179/161/133/105/93	na
Putative compounds	m/z (−)	Fragment ions	Ref
Amino acid			
Tryptophan	203	203/185/175/157/142/116/89	[35]
Carboxyl phenylalanine	208	208/191/164/147/135/120/103	[35, 36]

(continued)

Table 4.1 (continued)

Putative compounds	m/z (+)	Fragment ions	Ref
Organic acid			
Succinic acid	117	117/99	[37]
Malic acid	133	133/115/89	[35]
Oxoadipic acid	159	159/131/115/113/103	[35]
Shikimic acid	173	173/155/111/85	[38]
Citric acid	191	191/173/155/147/131/129/117/111/103/87	[35]
Gluconic acid	195	195/177/159/129/105/99/87	[39]
Galactaric acid	209	209/191/173/147/133/129/115/113/103/89/87/85	[40]
Xylosyl muconic acid	273	273/181/159/131/115/103/97/87	[35]
Glycosyl citric acid	337	337/191/173/155/129/111/85	[39]
Quinyl rutinoside	501	501//455/416/398/371/191/173/155/129/111	[41]
Phenolic acid			
Hydroxybenzoic acid	137	137/93	[35]
Coumaric acid	163	163/119	[42]
Hydroxyl cinnamic acid	164	164/147/119/103/91	[36]
Ethoxybenzoic acid	165	165/137/121/97	[35]
Dihydrocamalexic acid	245	245/203/130/116/98	[43]
Phenol dihydrocaffeic acid	273	273/227/205/183/181/137/97	[44]
Glycosyl coumaric acid	325	325/229/211/185/183/163/151/119	[42]
Dodecadienyl coumaric acid	327	327/309/283/265/237/163/143/119/93	na
Chlorogenic acid	353	353/191/173/155/135/129/111/85	[35, 45]
Phenyl benzoyl isopropylmalic acid	371	371/249/175/157/121/113/99/85	[35]
Galloyl glucuronyl hydroxyl shikimic acid	519	519/349/191/173/155/129/111/85	na
Rhamnosyl glycosyl tauryl coumaric acid	578	578/458/414/413/294/293/282/175/163/119/103	[46]

(continued)

Table 4.1 (continued)

Putative compounds	m/z (+)	Fragment ions	Ref
Terpenoid			
Rhamnosyl glycosyl trihydroxyl genipin	565	519/387/225/207/191/163/149/119/113/99/89	[35]
Flavonoid			
Kaempferol	285	285/152/108	[35]
Catechin	289	289/245/203/187/179/161/151/137/125/123/121/109/97	[14, 23, 47, 48]
Epicatechin	289	289/245/203/187/179/161/151/137/125/123/121/109/97	[14, 23, 47, 48]
Apigenin-6-C-glycoside (isovitexin)	431	431/353/341/311/283/269/191/161/117	[48]
Kaempferol-C-glycoside	447	447/357/327/297/285	[14, 23]
Dimer of phloretin	545	545/499/448/419/401/312/273/164/151/137/125/97	[35]
Vitexin rhamnoside	577	577/457/413/293/173	[23]
Hydrocarbon			
Hept-di-enol	111	111/93/84	na
Ketovaline	115	115/98/97	[35]
Hydroxyl benzofuran	135	135/117/89	[49–51]
Methyl benzoate	135	135/121/107/91	[35]
Dihydroxyl methyl acetophenone	165	165/147/129/105/87	[52]
Hydroxyl methyl coumarin	175	175/157/147/131/119/115/113/103	[35]

na: not available

(genistein-8-C-glucoside) which was found in the anti-hyperglycemic flavonoid extract of *Genista tenera* for diabetic treatment. This is because isoflavones could be aldose reductase inhibitors blocking the sorbitol pathway that is linked to many problems associated with diabetes [16]. In addition, genistein (isoflavone) was found to have anticancer activity [17] and estrogen-like activity against estrogen deficiency for women [18, 19]. The product ions of m/z 415, 397, and 379 were due to the loss of one, two and three water molecules, respectively from the parent ion, m/z 433. Besides that, the detection of product ions such as m/z 367, 337, and 295 was contributed by the further fragmentation of the protonated ions $^{2,3}X^{+}$—loss of two water molecules, $^{0,4}X^{+}$—loss of two water molecules and $^{0,2}X^{+}$—loss of one water molecule, respectively from the sugar moiety. The cleavage of carbon bond $^{0,1}X^{+}$ at the sugar moiety also produced m/z 283 in the spectrum. Previously, Li et al. [20] reported that flavonoid-C-glycosides appeared to be cleaved at the cross-ring of sugar moiety and caused loss of water molecules in the fragmentation. On the other hand, flavonoids-*O*-glycosides were likely to be fragmented at the glycosidic bond at low collision energy [21].

The flavanols of catechin and epicatechin are commonly found in tea. Interestingly, these compounds were also detected in the leaves extract of *F. deltoidea*. The detection of the characteristic ions for flavanol such as m/z 137 and 109 confirmed the presence of catechin and epicatechin in the plant extract [14]. The characteristic ions were found to result from the mechanism of ring contraction as previously reported by Fabre et al. [22]. Based on the findings of Dou et al. [23], epicatechin would elute after catechin using a C18 column in LC-MS/MS analysis. Since catechin is more polar than epicatechin stereogeometrically, the two negative ion peaks of m/z 289 with similar fragmentation pattern will be assigned as catechin and epicatechin at 11.59 and 16.2 min, respectively. However, only epicatechin (Rt 16.2 min) was detected at the positive ion mode. This might be due to the low concentration of catechin for protonation, which was approximately 10 times lower than epicatechin in the plant extract (data not shown).

This study also detected metabolites resulting from the cleavage of seven-membered unsaturated ring C of a triterpenoid of duboscic acid (m/z 501) such as m/z 263, 245, 217, and 199 by the loss of formic acid and water molecule. Based on the observation of Musharraf et al. [24], the fragmentation at the seven-membered ring C became more prominent at high collision energy (35 eV) which was similar to the collision energy applied in this study. Another terpenoid detected in the positive ion mode was yuexiandujisu E or D which was also found in the roots of *Euphorbia ebracteolata* [25, 26]. On the other hand, genipinoside was detected at the negative ion mode.

4.4 Conclusions

LC-PDA-MS/MS is a fast and reliable hyphenated analytical technique for phytochemical profiling. Approximately, 50 phytochemicals have been identified from the plant extract of *F. deltoidea* in this study. The identified phytochemicals are from a broad range chemical classification ranging from amino acid, organic acid, polyphenol, alkaloid, and terpenoid. This high throughput identification is unlikely to be carried out using single chromatographic and/or spectrometric technique. The combination between good separation of chromatographic technique and excellent identification power of mass spectrometric technique appears to be suitable for highly complex samples including herbal plant materials.

Acknowledgments The author would like to acknowledge the financial support of Exploratory Research Grant Scheme (ERGS 4L114) from the Ministry of Education, Malaysia. I also appreciate the help of Dr. Manjunatha Devagondanahalli Hadagali who is a post-doctoral researcher in the laboratory preparing the plant crude extract for analysis.

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