PROTEIN KINASE INHIBITORY PROPERTIES OF EXTRACTS DERIVED FROM *BOCCONIA FRUTESCENS* AND *GOMPHOCARPUS PHYSOCARPUS*

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Abstract: *Bocconia frutescens* is an invasive shrub originally introduced to Hawai'i as an ornamental plant. *Gomphocarpus physocarpus* is a shrub that is native to southern Africa, also introduced to Hawai'i. These two plants were selected for study due to the possibility of alkaloid contents and biological activities. The objective of this study was to examine the protein kinase inhibitory activity (PKI) of crude methanol extracts derived from *Bocconia frutescens* and *Gomphocarpus physocarpus* using hyphae formation inhibition assay against *Streptomyces 85E* at concentrations of 20 µg/disk. Extracts that showed protein kinase inhibitory activity at this concentration were tested at lower concentrations. Results found that all *B. frutescens* extracts showed PKI activity, whereas none of the *G. physocarpus* showed biological activity. In addition, the presence of chelerythrine and berberine alkaloids in *B. frutescens* extracts were assessed during this study using thin layer chromatography (TLC) and liquid chromatography-massspectrometry (LC-MS) analysis. The results showed that both alkaloids were present in all *B. frutescens* extracts.

Keywords: Protein kinase inhibition, hyphae formation inhibition assay, *Bocconia frutescens*, *Gomphocarpus physocarpus*

INTRODUCTION

Bocconia frutescens, or plume poppy in English, is native to Mexico, Central America, and South America. Practitioners of traditional Mexican medicine referred to *B. frutescens* as "gordolobo" or "llorasangre." The indigenous people of Mexico used various parts of this plant to treat dermatitis, skin ulcers, tuberculosis, and some respiratory tract infections (Sánchez-Arreola *et al.*, 2006). The sap of this plant has also been used to make anesthetics and treat bronchitis (Quattrocchi, 2012).

Bocconia frutescens is an invasive shrub that was introduced to Hawaiʻi as an ornamental plant (Sánchez-Arreola *et al.*, 2006). Subsequently the State of Hawai'i classified *B. frutescens* as a noxious weed. Infestations exist on the islands of Maui and Hawai'i. The Maui infestation covers the southern and western slopes of Haleakalā. The Hawai'i island infestations are located in the Ka'ū and South Kona districts. Dense *B. frutescens* populations have been found on *Eucalyptus* plantations. Native forests and sugar cane lands have also harbored *B. frutescens* populations (Benitez *et al*., 2007).

Extracts from *B. frutescens* displayed antimicrobial activity against a number of pathogenic bacterial species including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* (Sánchez-Arreola *et al.*, 2006). The leaves, stems, and roots contain phytochemicals that exhibited antibacterial properties against these pathogens (Yu *et al.*, 2014). The compounds with antimicrobial activity include alkaloids such as berberine, chelerythrine, columbamine, and protopine (Tavares *et al.*, 2014; Stermitz *et al.*, 2000; Liang *et al.*, 2014; Su *et al.*, 2011).

Gomphocarpus physocarpus, or balloon plant, is a shrub that is native to southern Africa that has spread worldwide. *G. physocarpus* has been introduced to Australia, China, Hawai'i

India, and Mexico among other places. This plant invades pastures and kills livestock in Hawai'i and Australia (Rojas-Sandoval & Acevedo-Rodríguez, 2016).

Africans used all parts of this plant in traditional medicine to treat warts (Munsamy & Naidoo, 2015). They also used the roots to treat stomach aches and the leaves to treat headaches. *G. physocarpus* has a variety of African names including balbossie, balmelkbossie, umsingalwesalukazi, uphuphuma, and usingalwesalukazi (Rojas-Sandoval & Acevedo-Rodríguez, 2016).

The plant produces a white latex which is toxic when ingested. The laticifers contain alkaloids and phenols. Carbohydrates, cardiac glycosides, terpenoids, steroids, phenols, and alkaloids have all been found in crude ethanolic extracts of *G. physocarpus*. These compounds could contribute to the toxic properties of the latex (Munsamy & Naidoo, 2015). The methanol and *n*-hexane extracts from the fruits of the closely related G. *fruticosus* have shown activity against *P. aeruginosa* with a minimum inhibition concentration (MIC) of 31 µg/mL (Madureira *et al.*, 2011).

It is well known that eukaryotic kinases play a vital role in signal transduction and signaling pathways by phosphorylating their substrates. Recently, protein phosphorylation of tyrosine has been reported in bacterial species, which might be associated with developmental processes (Bakal, 2000). Further studies by Bakal (2001) showed that *Streptomyces* have Signal Transducer and Activator of Transcription (Stat3)-like proteins similar in amino acid sequence surrounding a phosphorylated tyrosine which plays a role in cellular regulation in *Streptomycetes*. Abnormal phosphorylations by these kinases can lead to diseases (Bakal, 2002; Shanbhag *et al.*, 2015). Recently, protein phosphorylation kinase inhibitors have been identified using an assay based on the inhibition of hyphae formation against *Streptomyces* 85E (Waters *et*

al., 2002; Shanbhag *et al.*, 2015). Protein kinases play a role in the formation of aerial hyphae, spores, and pigmentation in *Streptomyces.* Additionally, protein kinases have a role in antibiotic production in some strains. Certain compounds, including eukaryotic kinase inhibitors, exhibit protein kinase inhibition properties because they can inhibit these kinases (Shanbhag *et al.*, 2015; Waters *et al.*, 2002). Kinase inhibitors are of significant pharmaceutical interest as potential breakthroughs for treating diseases including cancers, in part because abnormal phosphorylations by protein kinases have caused these diseases (Shanbhag *et al.*, 2015). Chelerythrine has shown cytotoxic activity against human cancer cells because of its ability to inhibit protein kinases (Chmura *et al.*, 2000).

In our continuing search for plant-derived protein kinase inhibitors, a methanol extract of *B. frutescens* and *G. physocarpus* were prepared and selected for further study.

MATERIALS AND METHODS

Plant material

The plant specimens, *B. frutescens* and *G. physocarpus*, were collected from Miloli'i, Hawai'i, USA on July 5, 2016. The plant materials were identified by Jimmy Parker (Early Detection Program Coordinator, Big Island Invasive Species Committee). The voucher specimens (No. 0001 and 0002) were deposited at the Natural Products Chemistry Laboratory at the Daniel K. Inouye College of Pharmacy, University of Hawai'i at Hilo. The plant materials were air dried for 7 days at the laboratory of DKICP, UH Hilo. The air dried plant materials were separated and cut into smaller pieces. Most of these pieces were between one and five centimeters long. The plant materials that were used are listed in Table 1.

Extractions

Samples (50 grams each) of the plant material were individually extracted in 1800 mL of methanol for 24 h three times. The supernatants were filtered with (VWR Whatman No. 417) filter paper. The combined filtrates were then concentrated using a rotary evaporator under a vacuum at 45° C. Table 1 lists the weight of the dried crude extracts.

Inhibition of hyphae formation assay in Streptomyces

The inhibition of hyphae formation assay in *Streptomyces* 85E was performed on extracts through an agar well diffusion method described previously (Waters *et al.*, 2002). The mycelia fragments of *Streptomyces* were spread on minimal medium ISP4 agar plates to produce a bacterial lawn. Extracts and partitions of known concentration dissolved in DMSO were dispensed onto disks in 20 µL aliquots. The air-dried disks were applied directly onto the plates and incubated at 30° C. After 48 hours of incubation, zones of inhibition were evaluated, and the results were identified by a clear zone of inhibition and/or bald phenotype around the disk. An inhibition zone greater than 8 mm is considered active. Samples were tested at 80 µg/disk on 7 mm filter disks. Active extracts and compounds were tested at lower concentrations of 40, 20, 10, and 5 µg/disk (Waters *et al*., 2002). DMSO solvent (Sigma-Aldrich Co., St. Louis, MO, USA) was used as a negative control. Berberine chloride and chelerythrine chloride standards (Sigma-Aldrich Co., St. Louis, MO, USA) were used as positive controls.

Chemicals and Analysis

The analytical thin layer chromatography (TLC) was performed on Merck precoated silica gel 60 F_{254} plates. The crude organic extracts from *B. frutescens* and *G. physocarpus* and compounds

berberine chloride and chelerythrine chloride were chromatographed on the TLC plate. Plates were developed in the solvent system chloroform-methanol (10:1). Dragendorff's reagent was used to detect the alkaloid contents (Nayeem *et al*., 2011). High-performance liquid chromatography (HPLC) grade acetonitrile (Sigma-Aldrich Co, St. Louis, MO, USA) and HPLC grade water were used for the LC-MS analysis.

Performing LC-MS and MS/MS on extracts that displayed antibacterial activity

In this study, liquid chromatography mass spectrometry (LC-MS) analysis was performed on an Agilent 6530 Q-TOF mass spectrometer with an Agilent HPLC 1260 binary pump. A Waters Sunfire C18 column (3.5 μm, 2.1 × 150 mm) was used as the stationary phase. The mobile phase was run at a gradient with 1% formic acid in water and acetonitrile at a flow rate of 0.2 mL/min. The mass spectrometer was equipped with an Agilent Jet Stream electrospray ionization (ESI) source. LC-MS was performed in positive ionization on a mass range of *m/z* 100 – 1700 at a scan rate of 3 spectra/second. The capillary voltage set at 3500 V. Nebulizer pressure was set at 35 psi. Drying gas temperature was set at 300° C with a flow rate of 8 L/min, and the sheath gas temperature was 350° C with a flow rate of 11 L/min. The MS/MS conditions were set to automatically collect at a relative threshold of 0.010%, and the collision energy was ramped with a slope of 5 and an offset of 2.5.

RESULTS AND DISCUSSION

All crude extracts were evaluated against protein kinase inhibition activity using hyphae formation inhibition assay against *Streptomyces* 85E (Table 1). The negative control, DMSO solvent, did not inhibit hyphae formation while both of the positive controls, berberine chloride and chelerythrine chloride, inhibit hyphae formation inhibition. Chelerythrine chloride showed more potent protein kinase inhibition activity against *Streptomyces* 85E than berberine chloride. None of the *G. physocarpus* extracts showed protein kinase inhibition activity at the highest concentration that was used during this study (80 µg/disk). Therefore, the *G. physocarpus* parts that were tested have very limited, if any, protein kinase inhibition properties. Extracts from the stems and roots of *B. frutescens* showed biological activity at 80 µg/disk and 40 µg/disk, but the roots showed stronger activity against *Streptomyces* 85E than the stems. The leaves of *B. frutescens* did not display any biological activity after 48 h, but they did show some activity after 24 h. The activity was only measured quantitatively after 48 h.

A preliminary TLC analysis indicated the presence of alkaloids in all crude extracts of *B. frutescens* using Dragendorff's reagent. During TLC analysis spraying with Dragendorff's reagent, compounds found in the crude extracted formed spots in similar positions to those found in the standards providing evidence for the presence of both compounds in the crude extracts. Other red and orange marks, beside the standards, were present for the crude extracts providing evidence for the existence of other alkaloids in *B. frutescens*.

In addition, the compounds of the organic extract of *B. frutescens* (leaves, roots and stems) were analyzed using HPLC-ESI-QTOF-MS/MS against two standards (chelerythrine and berberine). LC-MS mass spectrometry analysis also supported the presence of both alkaloids in the *B. frutescens* crude extracts. Compounds with (*m/*z) values of the chelerythrine standard,

348.1236, and the berberine standard, 336.1236, were found in all three *B. frutescens* extracts. Of note, the same retention time of these respective alkaloids eluted with the same solvent systems matched with the signals observed with the *B. frutescens* crude extracts. MS/MS fragmentation data provided further evidence for the presence of chelerythrine and berberine in the extracts derived from *B. frutescens*. The fragmentation profile of the chelerythrine and berberine standards were also observed in all three extracts. In conclusion, by matching retention time, mass to charge ratio (*m/z*) and fragmentation pattern, it was evident that *B. frutescens* extracts contain chelerythrine and berberine.

Table 1. Plant Materials and Extracts

Samples	**Part**	**Extract (g)**
Bocconia frutsecens	Stems	1.8
	Leaves	7.5
	Roots	5.1
Gomphocarpus physocarpus	Stems	2.2
	Leaves	7.9
	Roots	1.7
	Seed Capsules*	5.3

*Seeds were removed from their seed capsules. The antibacterial activity of the seeds was not evaluated during this study due to paucity of materials

Table 2. Biological Activity of Tested Extracts and Compounds on Growth and Sporulation of *Streptomyces 85E*

	Zone of Inhibition at Concentrations of Samples (mm) (mean ± SD)				
Sample	80 µg/disk	40 µg/disk	20 µg/disk	10 µg/disk	5 µg/disk
B. frutescens Stem Extract	B: 17.7 ± 1.5	B: 9.7 ± 1.2	0	NT	NT
B. frutescens Root Extract	B&C: 20.0 ± 1.0 C: 13.7 ± 2.1	B: 13.3 ± 0.6	0	NT	NT
B. frutescens Leaf Extract	0	NT	NT	NT	NT
G. physocarpus Stem Extract	0	NT	NT	NT	NT
G. physocarpus Root Extract	0	NT	NT	NT	NT
G. physocarpus Leaf Extract	0	NT	NT	NT	NT
G. physocarpus Seed Pod Extract	0	NT	NT	NT	NT
Berberine chloride standard	B: 8.6 ± 0.3	0	NT	NT	NT
Chelerythrine chloride standard	C: 26.6 ± 0.6	B&C: 26.0 ± 1.0 C: 18.7 ± 0.6	B: 20.7 ± 0.6	B: 15.0 ± 1.0	B:14.7 ± 0.6

Stock solutions were prepared in DMSO. No zone of inhibition was observed with DMSO as a negative control. All extracts and compounds were tested at 80 µg/disk. Active extracts were tested at lower concentrations (20, 10, 5 µg/disk). B indicates bald phenotype. C indicates clear phenotype. B and C indicates that a clear phenotype was within a balding phenotype. NT indicates no test necessary, because substance showed no activity at higher concentrations.

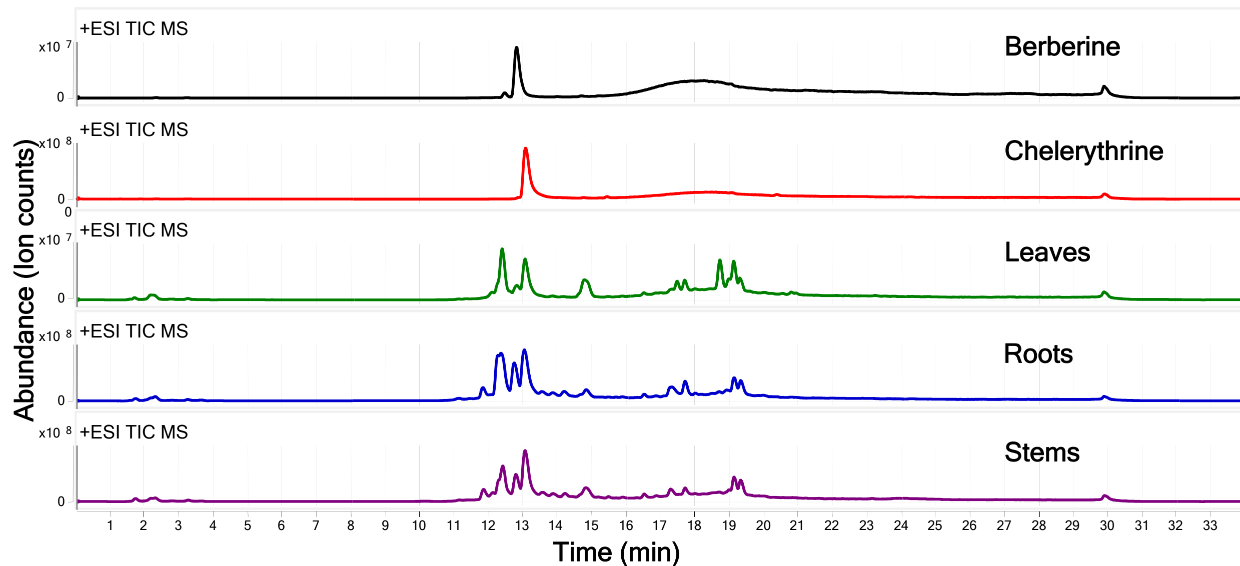

Figure 1. Total ion chromatogram of berberine, chelerythrine, and extracts derived from *B. frutescens* (leaves, roots and stems).

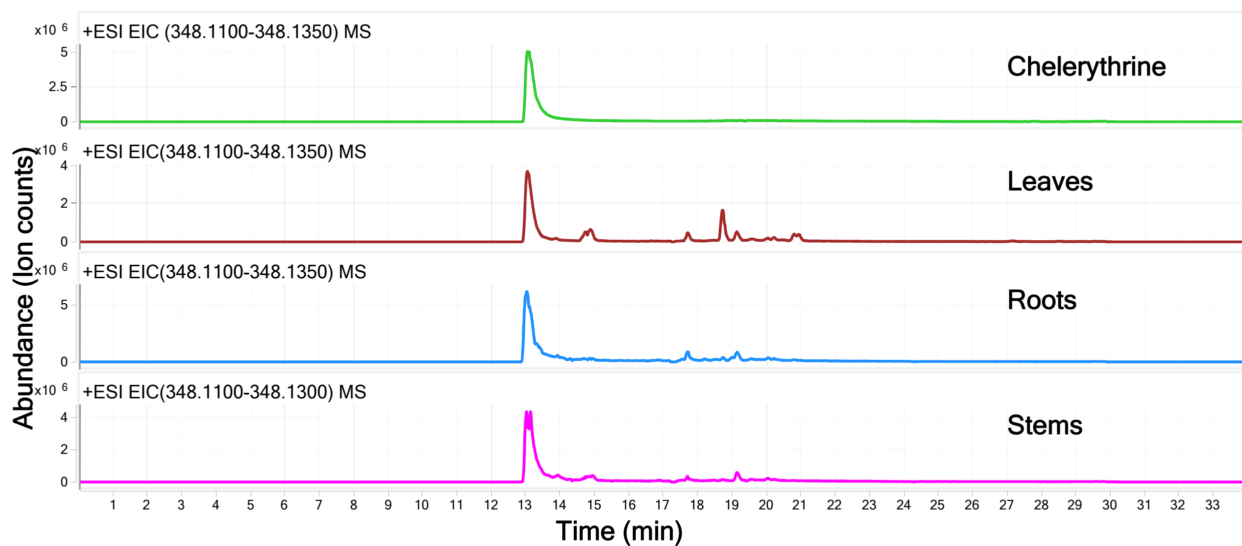

Figure 2. Extracted ion chromatogram of chelerythrine, and extracts derived from *B. frutescens* (leaves, roots and stems).

Table 3. Accurate Mass (*m/z*) of Chelerythrine

Sample	Observed *m/z*	Theoretical *m/z*	Difference (ppm)
Chelerythrine	348.1237	348.1236	-0.34
Roots	348.1224	348.1236	3.40
Stems	348.1223	348.1236	3.69
Leaves	348.1242	348.1236	-1.77

Table 3 indicates retention time, observed *m/z* and calculated *m/z*, relative errors of both the standards and samples. Standard chelerythrine yielded an observed $[M + H]^+$ peak at a (*m/*z) of 348.1237 (Table 3). By comparison, crude organic extract of *B. frutescens* leaves ($[M + H]^+$ peak at a (*m/*z) of 348.1242], stems ($[M + H]^+$ peak at a (*m/*z) of 348.1223], and roots ($[M + H]^+$ peak at a (*m/*z) of 348.1224] produced peaks around that mass. A difference of 10 ppm was the cut off for homology. These (*m/*z) values were obtained from the graph in Figure 2. The peak corresponding to chelerythrine exhibited mass-to-charge (*m/z*) ratios corresponding to the molecular ions $(M + H)^+$ of standard chelerythrine, confirming the presence of this alkaloid in all three parts of plant materials.

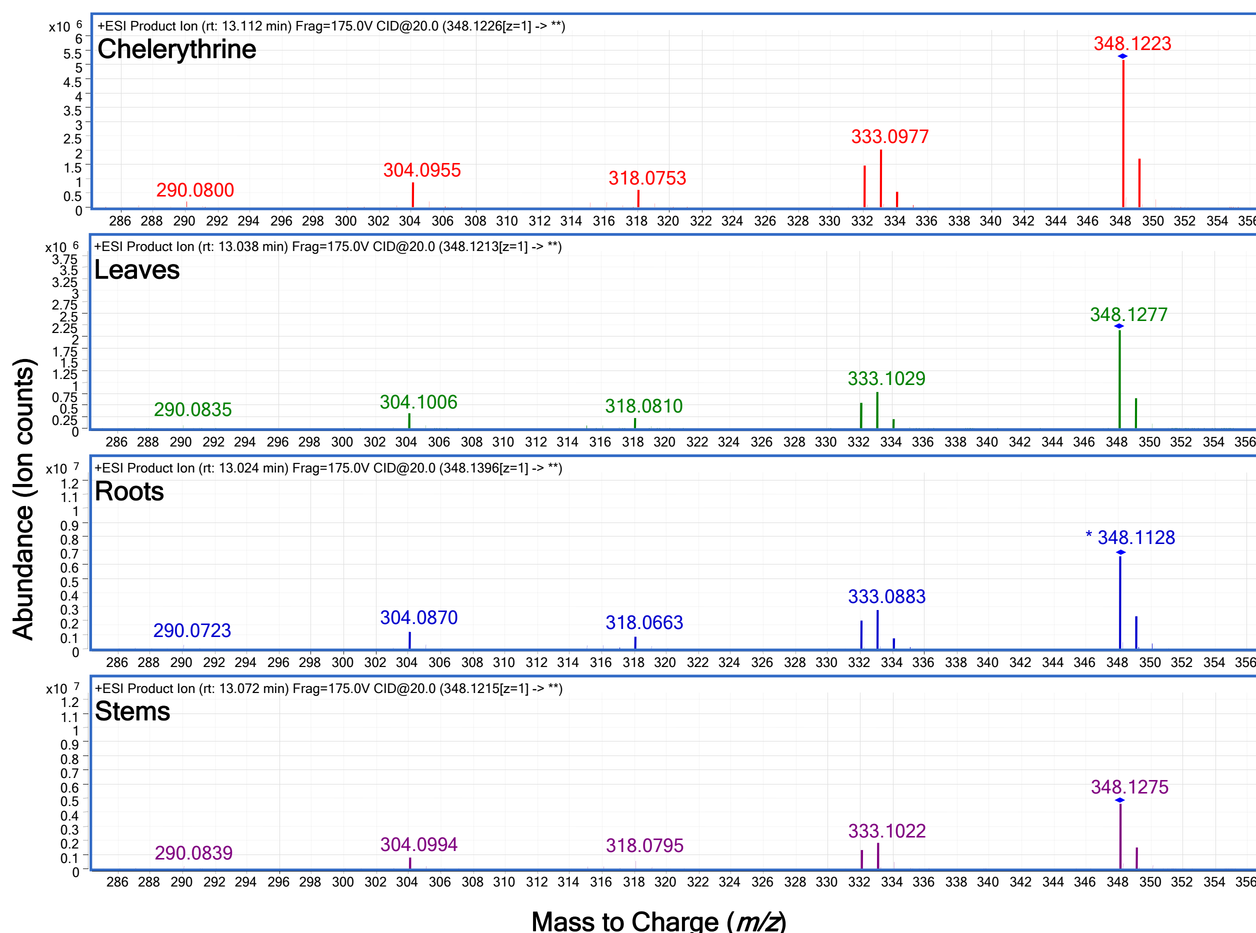

Figure 3. MS/MS of of Chelerythrine

Standard chelerythrine chloride yielded an $[M + H]^+$ peak at a (*m/z*) of 348.1223 (Figure 3). The fragmentation pattern MS/MS spectrum of standard chelerythrine analyzed by ESI-MS/MS yielded 318.0753 $[M - OCH_2]^+$, 304.0995 $[M - CO\text{-}CH_3\text{-}H]^+$, and 290.0800 $[M - CH_2O\text{-}CO]^+$, respectively. The fragmentation patterns of the samples (leaves, roots, and stems) were similar to chelerythrine's fragmentation pattern. The ESI-MS/MS results confirm the presence of the alkaloid chelerythrine (Son *et al*., 2014) in leaves, stems and roots of *B. frutescens*.

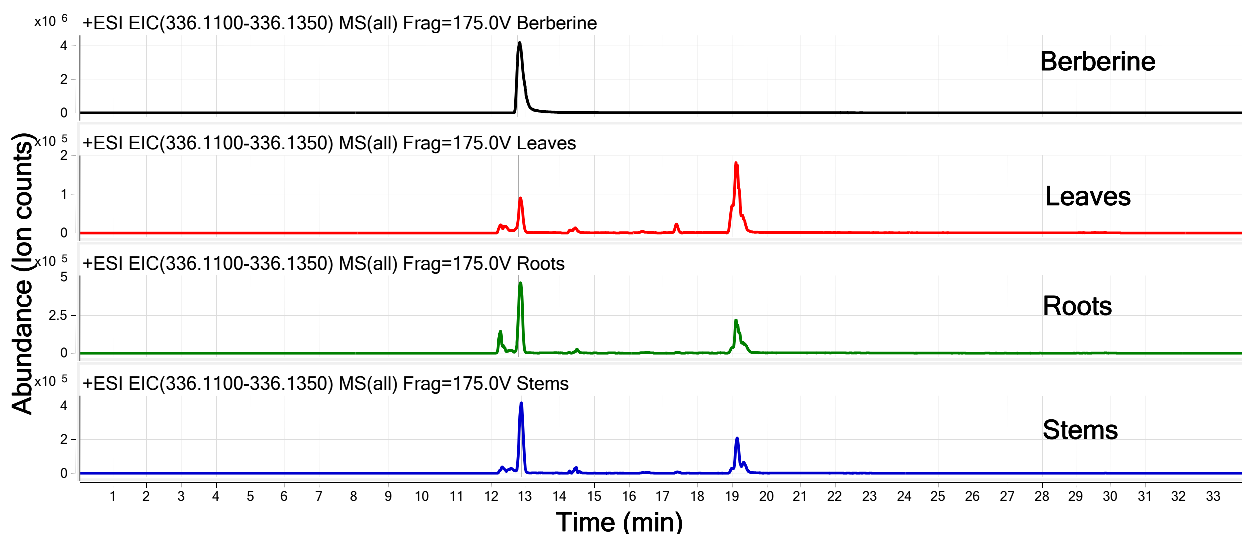

Figure 4. Extracted ion chromatogram of berberine

Table 4. Accurate Mass of berberine (*m/z*)

Sample	**Observed Mass**	**Theoretical Mass**	**Difference (ppm)**
Berberine	336.1239	336.1236	-0.94
Roots	336.1243	336.1236	-2.13
Stems	336.1228	336.1236	2.33
Leaves	336.1219	336.1236	5.00

Standard berberine yielded an observed mass $[M + H]^+$ peak at a (*m/*z) of 336.1239. By comparison, crude organic extract of *B. frutescens* leaves, stems, and roots produced peaks around that mass. The peak corresponding to berberine exhibited mass-to-charge (*m/z*) ratios corresponding to the molecular ions $(M + H)^+$ of standard berberine, confirming the presence of the alkaloid in all three plant materials. A difference of 10 ppm was the cut off for homology. These (*m/*z) values were obtained from the graph in Figure 4.

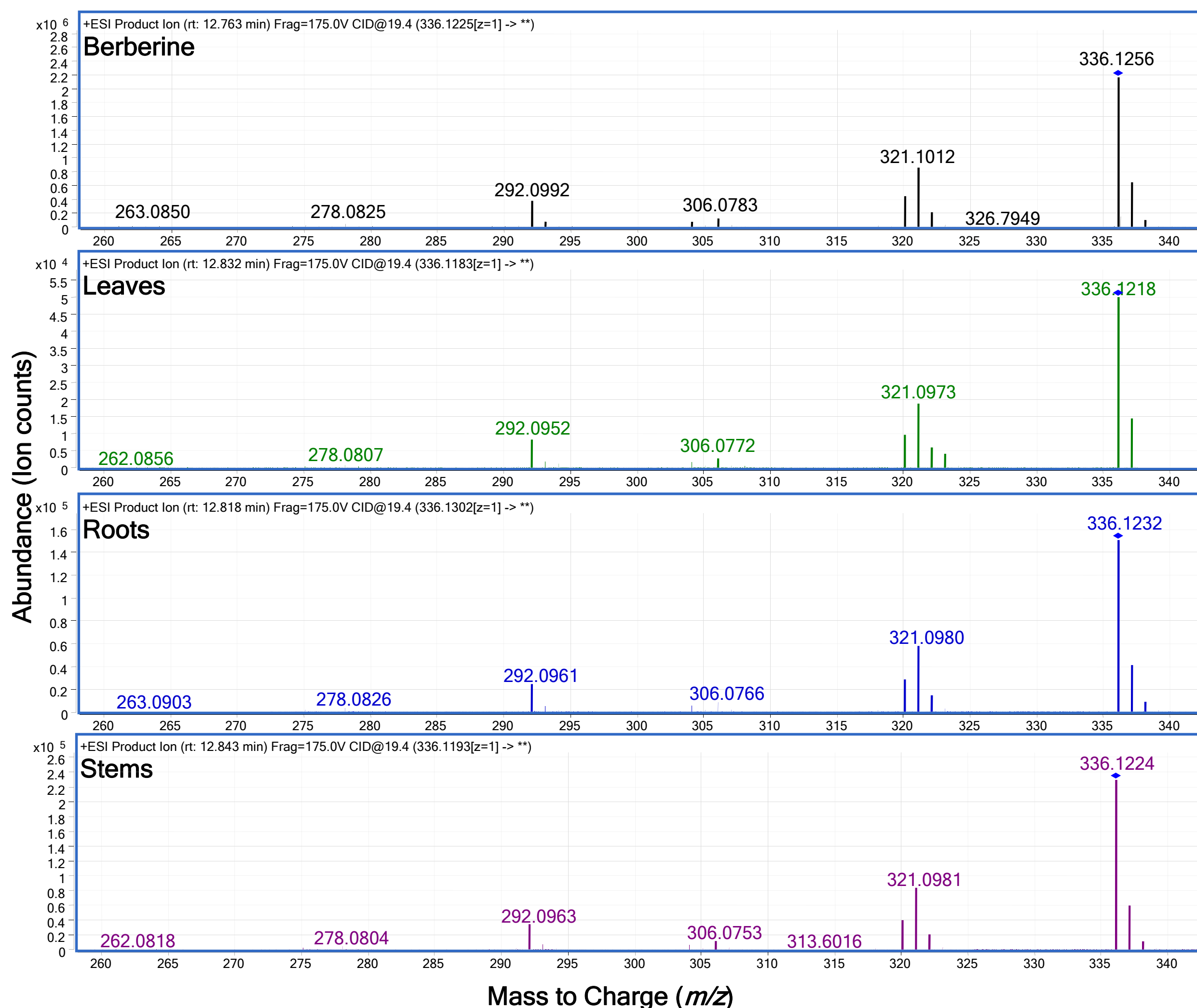

Figure 5. MS/MS of berberine

Standard berberine chloride yielded an $[M + H]^+$ peak at a (*m/z*) value of 336.1256. The MS/MS spectrum of standard berberine analyzed by ESI-MS/MS yielded 306.0783 $[M–OCH_2]^+$, 292.0992 $[M–CO-CH_3-H]^+$, and 278.0825 $[M–CH_2O-CO]^+$ respectively. This result indicates that the ESI-MS/MS were adequate for the confirmation of the presence of alkaloid berberine (Son *et al*., 2014) in leaves, stems and roots of *B. frutescens*.

CONCLUSION

Although *G. physocarpus* possesses medicinal properties, natural products derived from this plant lack potential for protein kinase inhibition activity as evidenced in our study. Further studies on the protein kinase inhibition activity of *B. frutescens* and its alkaloids are currently underway. *B. frutescens* could exhibit these properties by inhibiting protein kinases or via other mechanisms. TLC, LC-MS, and MS/MS all confirmed the presence of berberine and chelerythrine, in crude extracts derived from *B. frutescens*.

In future studies, the percentage that each alkaloid contributes to the protein kinase inhibition properties of *B. frutescens* will be quantified. The other alkaloids and non-alkaloids that were shown by TLC analysis can be investigated. The seeds of *B. frutescens* could not be assessed during this study because of a paucity of materials. Even though crude extracts derived from *G. physocarpus* did not show protein kinase inhibition activity against *Streptomyces 85E*, compounds derived from this plant could display biological activity against other organisms. The medicinal properties of phytochemicals derived from *G. physocarpus* will be investigated.

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