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Original Research

Untargeted profiling of field cultivated bush tea (*Athrixia phylicoides* DC.) based on metabolite analysis

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Abstract: Bush tea (*Athrixia phylicoides* DC.) is an aromatic South African indigenous plant used for many decades as a health beverage and medicine. Several studies have extensively investigated wild bush tea's secondary metabolites, but the entire profiling of cultivated bush tea's metabolites is limited in the literature. Thus, the objective of this study was to profile cultivated bush tea metabolites using liquid chromatography-quadrupole time of flight-tandem mass spectrometry (LC-QTOF-MS). The 31 metabolites profiled included; benjaminamide, chlorogenate, chrysosplenetin, coumarin, 6*Z*-docosenamide, naringenin 7-*O*-β-D-glucoside, 5-*p*-coumaroylquinic acid, integrastatin A, luteolin 7-*O*-(6-*O*-malonyl-β-D-glucoside), 1,3-dicaffeoylquinic acid, magnoshinin, okanin, (2*S*)-5-hydroxy-7-methoxy-6,8-dimethylflavanone, (9*Z*,12*Z*,15*Z*)-octadecatrienoic acid, 2″-deamino-2″-hydroxy-6″-dehydroparomamine, *O*-butanoylcarnitine, myricitrin, gorlic acid, tetracenomycin X, sakuranin, D-tryptophan, linoleamide, laricitrin 7-monoglucoside, L-β-phenylalanine, L-proline, pheophytin A, pheophorbide A, PI(18:0/20:4(8*Z*,11*Z*,14*Z*,17*Z*), stearidonic acid, and gibberellin A14 aldehyde. These annotated metabolites included phenolics, flavonoids, and quinic acids, indicating that bush tea is rich in metabolites, which have a potential wide range of health benefits.

Key words: *Athrixia phylicoides*; Metabolites; Bush tea; LC-QTOF-MS; Phytochemistry.

Introduction

The effects of consumption of herbal teas, which contain thousands of phytochemicals, on general health and relief of symptoms in severe diseases have been reported (1-4). The quality of tea is inevitably associated with the presence and concentration of phytochemicals (5-8), thus, the discovery of novel tea compounds has increased globally since about 80% of the world's population depends on traditional medicines and herbal teas for primary health care (9).

Bush tea (*Athrixia phylicoides* DC.), an aromatic leafy shrub native to South Africa (10), holds an impressive history treating various ailments (11) and contributed to the primary health care of ancient people (12). Several studies have been conducted to assess bush tea's potential as an herbal tea, and demonstrated that bush plant has phytochemicals and secondary metabolites, which have healing effects and pharmacological properties (13-16). However, these studies have only identified and characterized bush tea sampled from the wild. Metabolomics approaches such as untargeted ultraperformance liquid chromatography–mass spectrometry (UPLC-MS), targeted UPLC-MS, and untargeted ^{1}H NMR have been recommended for tea phytochemical composition profiling to evaluate the tea quality (17). In bush tea, the whole profiling of cultivated bush tea's metabolites using metabolomic analysis is still not investigated. In this study, cultivated bush tea metabolites were profiled using liquid chromatography-quadrupole

time of flight-tandem mass spectrometry (LC-QTOF-MS/MS).

Materials and Methods

Experimental site

The trial was carried out at the Agricultural Research Council Vegetable and Ornamental Plant Institute situated about 25 km north of central Pretoria on the Moloto/KwaMhlanga Road (R573), GPS coordinates 25º 59″ S; 28º 35″ E. The farm covers approximately 4000 ha, of which only 650 ha is under irrigation. The bush tea was grown under 40% shade net and drip irrigation at frequency of 2.5 mm/h. Agronomic practices such as fertilization was applied as per Tshivhandekano et al. (18) who recommended that 150 N kg/ha.

Sample preparation

Two-year mature leaf and twig samples were harvested from bush tea plants. Harvested leaves were air-dried at room temperature in the shade. Samples were then ground to a powder using a benchtop grinder and stored in glass vials below -50°C until extraction.

Extraction of bush tea samples for LC-MS

Ten milligrams of each sample were weighed into 2-mL Eppendorf tubes. One mL solvent (0.1% formic acid in methanol) was added to dissolve the samples and vortexed for 1 min then placed in a sonicator waterbath for 30 min. Samples were then centrifuged for 5 min at 10 000 rpm on a benchtop centrifuge. Approximately 700 µL of the supernatant were pipetted into HPLC vials and fitted with metal caps with rubber septa and secured with a crimper.

QTOF LC-MS analysis

The supernatants were subsequently injected into the QTOF LC-MS (Thermo Scientific, Dionex UltiMate 3000Dionex Softron GmbH, Dornierstr. 4, Germany). Metabolites were separated using a gradient of H_2O with 0.1% formic acid (solvent A) and acetonitrile (solvent B), using a Dionex UltiMate 3000 UPLC at a flow rate 0.3 mL/min on a Waters BEH C18, 2.1 × 100 mm column. Mass spectrometry data was obtained on a QTOF Bruker Impact II (Bruker Daltonics GmbH Fahrensheitstr. 4, Bremen, Germany) using electron spray ionization (ESI) running in positive mode, that scans between 50-1600 m/z, with nebulizer at 1.8 bar and dry gas as 8 L/min.

Liquid chromatography-MS data were analyzed using the Compass data analysis tool version 4.3.110 and converted into buckets after peak integration and Pareto scaling. Peaks were identified according to actual mass, MS/MS and retention time (RT). Accurate mass and MS/MS spectral data were compared to the Kyoto Encyclopedia of Genes and Genomes (KEGG), Chemical Entities of Biological Interest (ChEBI), Chemspider and MetFrag databases.

Results and Discussion

There is lack of distinct literature on compounds in cultivated bush tea. The previous studies reported compounds from bush tea that were harvested from the wild, which is considered unsustainable since the domestication of the wild plant is critical to avoid extirpation of this plant. The results from this study in Tables 1 and 2 demonstrate the 31 compounds detected from cultivated bush tea using LC-QTOF-MS. The compounds annotated were validated through fragment ions denoted in Table 1 and 2, and chromatogram in Figure 1. The selected extracted ion chromatograms of the cultivated bush tea extract included *O*-butanoylcarnitine, L-β-phenylalanine, D-tryptophan, chlorogenate, coumarin, naringenin-7-*O*-β-D-glucoside, chrysosplenetin, stearidonic acid, gibberellin A14 aldehyde, (9*Z*,12*Z*,15*Z*)-octadecatrienoic acid, 6*Z*-docosenamide (Figure 1). About nine compounds were previously reported to be present in bush tea while 23 compounds are newly detected bush tea compounds (Table 3).

The secondary metabolites profiled included benjaminamide (mass 681.627; RT 8.85 min; $C_{42}H_{83}NO_5$) which was isolated from *Ficus mucuso* Welw. ex Ficalho and possess antimicrobial activities (19). Another secondary metabolite annotated chlorogenate or chlorogenic acid (mass 354.095; RT 4.61 min; $C_{16}H_{18}O_9$) was also profiled as one of the compounds in bush tea (Figure 2). This finding is consistent to with results reported by de Beer et al. (20) who reported the presence of chlorogenic acid in bush tea. Chlorogenate was also reported as a compound that lowers the risk of blood pressure (21). Chrysosplenetin (mass 374.107; RT 8.96 min; $C_{19}H_{18}O_8$), a secondary metabolite that was repor-

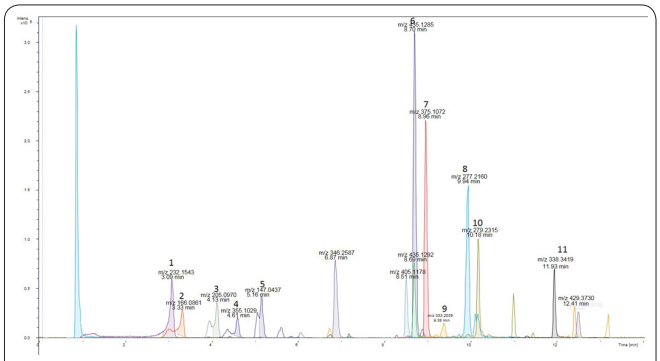

Figure 1. The selected extracted ion chromatograms of the cultivated bush tea extract analysis by LC/Q-TOF-MS. 1) *O*-butanoylcarnitine; 2) L-β-phenylalanine; 3) D-tryptophan; 4) chlorogenate; 5) coumarin; 6) naringenin 7-*O*-β-D-glucoside; 7) chrysosplenetin; 8) stearidonic acid; 9) gibberellin A14 aldehyde; 10) (9*Z*,12*Z*,15*Z*)-octadecatrienoic acid; 11) 6*Z*-docosenamide.

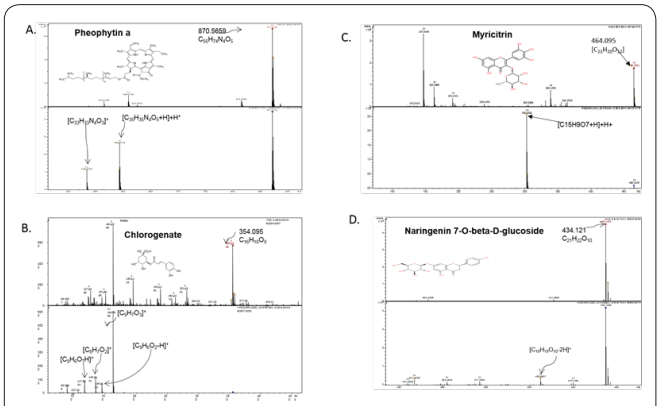

Figure 2. Selected MS-product ion spectra of four compounds: A) pheophytin a; B) chlorogenate; C) myritrin; D) naringenin-7-*O*-D-glucoside.

Table 1. LC/Q-TOF-MS exact masses for cultivated bush tea secondary metabolites and their main fragment ions.

Compound	Retention time (min)	Elemental composition	[M+H]+	Frag ion 1	Frag ion 2	Frag ion 3	Frag ion 4
Benjaminamide	8.85	$C_{42}H_{83}NO_5$	681.627	298.2740			
Chlorogenate	4.61	$C_{16}H_{18}O_9$	354.0951	117.0335	135.0441	145.0284	163.0390
Chrysosplenetin	8.96	$C_{19}H_{18}O_8$	374.1072	135.0441	299.0550	302.0421	317.0655
Coumarin	5.16	$C_9H_6O_2$	146.037	91.0542	119.0491		
6*Z*-Docosenamide	11.93	$C_{22}H_{43}NO$	337.334	71.0855	85.1012	86.0600	97.0648
Naringenin 7-*O*-β-D-glucoside	8.70	$C_{21}H_{22}O_{10}$	434.121	362.0632	377.0867	405.0816	
5-*p*-Coumaroylquinic acid	4.21	$C_{16}H_{18}O_8$	338.108	91.0542	119.0491	147.0441	
Integrastatin A	7.25	$C_{20}H_{20}O_9$	332.0526	347.0761	375.0710	390.0945	
Luteolin 7-*O*-(6-*O*-malonyl-β-D-glucoside)	6.29	$C_{24}H_{22}O_{14}$	534.101	135.0441	145.0284	163.0389	
1,3-Dicaffeoylquinic acid	5.18	$C_{25}H_{24}O_{12}$	516.127	135.04405	145.0284	163.0390	499.1234
Magnoshinin	8.85	$C_{24}H_{30}O_6$	414.204	119.0855	135.0804	135.0804	
Okanin	6.55	$C_{15}H_{12}O_6$	288.063	153.01822	163.03896		
(2*S*)-5-Hydroxy-7-methoxy-6,8-dimethylflavanone	9.00	$C_{18}H_{18}O_4$	298.121	107.0491	119.0491	135.0804	147.0441
(9*Z*,12*Z*,15*Z*)-Octadecatrienoic acid	10.18	$C_{18}H_{30}O_2$	278.225	81.0699	95.0855	109.1012	123.1168
2″-Deamino-2″-hydroxy-6″-dehydroparomamine	6.48	$C_{12}H_{22}N_2O_8$	322.138				
O-Butanoylcarnitine	3.09	$C_{11}H_{21}NO_4$	231.147	214.1438			
Myricitrin	6.90	$C_{21}H_{20}O_{12}$	464.095	303.0499			
Gorlic acid	10.18	$C_{18}H_{30}O_2$	278.225	67.0542	81.0699	83.0855	95.0855
Tetracenomycin X	8.18	$C_{24}H_{20}O_{11}$	484.101	119.0491	119.0491	147.0441	147.0441
Sakuranin	6.36	$C_{22}H_{24}O_{10}$	448.137	419.0972			
N-(2-Hydroxyheptadecanoyl)-1-*O*-β-D-glucosyl-15-methylhexadecasphing-4-enine	5.52	$C_{40}H_{77}NO_9$	301.0490	536.5037	554.5143		

Table 2. LC/Q-TOF-MS exact masses for the cultivated bush tea' metabolites and their main fragment ions.

Compound	Retention time (min)	Element composition	[M+H]+	Frag ion 1	Frag ion 2	Frag ion 3	Frag ion 4
D-Tryptophan	4.13	$C_{11}H_{12}N_2O_2$	204.093	91.0542	118.0651	132.0808	144.0808
Linoleamide	3.83	$C_{18}H_{33}NO$	279.256	83.0491	83.0855	86.0600	
Laricitrin 7-monoglucoside	8.26	$C_{22}H_{22}O_{13}$	494.106	333.0605	318.037	85.0284	
L-β-Phenylalanine	3.33	$C_9H_{11}NO_2$	165.079	120.0808			
L-Proline	9.22	$C_5H_9NO_2$	115.063	70.0651			
Pheophytin A	11.25	$C_{55}H_{74}N_4O_5$	870.5659	533.2547	533.25469	593.2758	
Pheophorbide A	11.57	$C_{35}H_{36}N_4O_5$	592.2686	461.2336	461.23357		
PI(18:0/20:4(8*Z*,11*Z*,14*Z*,17*Z*)	12.46	$C_{47}H_{83}O_{13}P$	886.5571	549.2459	591.2564	609.2670	
Stearidonic acid	9.94	$C_{18}H_{28}O_2$	276.209	93.0699	109.1012	135.1168	149.1325
Gibberellin A14 aldehyde	9.38	$C_{20}H_{28}O_4$	332.199	127.03896	287.2006		

Table 3. Compounds previously found in bush tea and newly annotated.

Novel compounds found in this study	Compounds found previously in wild bush tea	References
Benjaminamide		
	Chlorogenate	(16, 20)
	Chrysosplenetin	(25)
	Coumarin	(23)
6*Z*-Docosenamide		
	Naringenin 7-*O*-beta-D-glucoside	(25)
	5-*p*-Coumaroylquinic acid	(16)
Integrastatin A		
Luteolin 7-*O*-(6-*O*-malonyl-β-D-glucoside)		
	1,3-Dicaffeoylquinic acid	(16, 20)
Magnoshinin		
Okanin		
PI(18:0/20:4(8*Z*,11*Z*,14*Z*,17*Z*)		
(2*S*)-5-Hydroxy-7-methoxy-6,8-dimethylflavanone		
(9*Z*,12*Z*,15*Z*)-Octadecatrienoic acid		
	Myricitrin	(25)
Gorlic acid		
Sakuranin		
Tetracenomycin X		
D-Tryptophan		
2″-Deamino-2″-hydroxy-6″-dehydroparomamine		
O-Butanoylcarnitine		
Linoleamide		
N-(2-Hydroxyheptadecanoyl)-1-*O*-β-D-glucosyl-15-methylhexadecasphing-4-enine		
Laricitrin 7-monoglucoside		
	L-β-Phenylalanine	(25)
	L-Proline	(25)
Pheophytin A		(25)
Pheophorbide A		
Stearidonic acid		
Gibberellin A14 aldehyde		

ted to have been isolated from the root of *Berneuxia thibetica* Decne. and possesses an anti-viral activity (22), was also annotated from bush tea extracts in this study. Consistent with Padayachee (23), a phenylpropanoidcoumarin (mass 146.037; RT 5.16 min; $C_9H_6O_2$) was present in bush tea and this secondary metabolite was reported to play a significant role in the sweet and aromatic odor quality of green tea (24) and possesses anti-microbial properties. Bush tea extract from this study had a flavonone glycoside naringenin-7-*O*-β-D-glucoside (mass 434.121; RT 8.70 min; $C_{21}H_{22}O_{10}$) also known as prunin (Figure 2), this metabolite was detected in wild bush tea (25). A polyphenolic acid, 5-*p*-coumaroylquinic acid (mass 338.108; RT 6.48 min; $C_{16}H_{18}O_8$), a compound commonly found in teas and fruits (26) was detected.

Bush tea extracts in this study also contained an antiviral compound, integrastatin A (mass 332.0526; RT 7.25 min; $C_{20}H_{20}O_9$) also found in bush tea. This study also reported the presence of luteolin 7-*O*-β-D-glucoside (mass 534.101; RT 6.29 min; $C_{24}H_{22}O_{14}$), a plant flavonoid that possesses antioxidant, antiviral, and antibacterial properties (27). A caffeoylquinic acid (CQA), the main constituent of chlorogenic acid, 1,3-dicaffeoylquinic acid (mass 516.127; RT 5.18 min; $C_{25}H_{24}O_{12}$), was also found in bush tea extract.

This study showed that bush tea also contains a neolignan, an anti-inflammatory compound magnoshinin (mass 414.204; RT 8.85 min; $C_{24}H_{30}O_6$) (28). A chalconoid compound, okanin (mass 288.063; RT 6.55 min; $C_{15}H_{12}O_6$) was annotated and it possess antimicrobial and anti-inflammatory properties (29). Bush tea extract in this study contained a flavanone, (2*S*)-5-hydroxy-7-methoxy-6,8-dimethylflavanone (mass 298.121; RT 9.00 min; $C_{18}H_{18}O_4$). A compound (9*Z*,12*Z*,15*Z*)-octadecatrienoic acid (mass 298.121; RT 10.18 min; $C_{18}H_{30}O_2$) was detected. A flavonoid, myricitrin (mass 464.095; RT 6.90 min; $C_{21}H_{20}O_{12}$), was also found in cultivated bush tea extract (Figure 2). This compound is a nitric oxide and protein kinase C inhibitor, which possesses antipsychotic-like and anxiolytic-like effects in animal models of psychosis and anxiety (30). Gorlic acid (mass 278.225; RT 10.18 min; $C_{18}H_{30}O_2$) was also detected. The study also showed that bush tea extracts contain a

flavanone compound called sakuranin (mass 448.137; RT 6.36 min; $C_{22}H_{24}O_{10}$), which exhibits antioxidant activity (31).

The primary metabolites annotated in this study included a compound D-tryptophan (mass 204.093; RT 4.13 min; $C_{11}H_{12}N_2O_2$), which is an α-amino acid. Linoleamide (mass 279.256; RT 3.83 min; $C_{18}H_{33}NO$), a fatty amide lipid molecule was also detected from bush tea extracts. A lipid, laricitrin 7-monoglucoside (mass 494.106; RT 8.26 min; $C_{22}H_{22}O_{13}$), was also detected. An essential amino acid, L-β-phenylalanine (mass 165.079; RT 3.33 min; $C_9H_{11}NO_2$), was found in bush tea extract. The amino acid L-proline (mass 115.063; RT 9.22 min; $C_5H_9NO_2$) was present in cultivated bush tea extract. A chlorophyll compound, pheophytin a (mass 870.565; RT 11.25 min; $C_{55}H_{74}N_4O_5$), was also profiled (Figure 2) and it has been reported to possess antimicrobial, antioxidant and anti-aging effect (32). Consistent with Malongane and McGaw and Nyoni and Mudau (25) pheophorbide A (mass 592.2686; RT 11.57 min; $C_{35}H_{36}N_4O_5$), a product of chlorophyll breakdown, which was found to possess anticancer activity (33), was also present in bush tea. An acidic phosphatidylinositol known as PI(18:0/20:4(8*Z*,11*Z*,14*Z*,17*Z*) (mass 886.551; RT 12.46 min; $C_{47}H_{83}O_{13}$) was also found in bush tea. Stearidonic acid (mass 276.209; RT 9.94 min; $C_{18}H_{28}O_2$), an essential ω-3 fatty acid, was detected in bush tea extract. A phytohormone, gibberellin A14 aldehyde (mass 332.199; RT 9.38 min; $C_{20}H_{28}O_4$), was also found in cultivated bush tea extract.

The study successfully profiled 31 metabolites of cultivated bush tea using the LC-MS QTOF. Of the 31 metabolites profiled, 22 were newly detected from bush tea extracts. The annotated compounds included benjaminamide, chlorogenate, chrysosplenetin, coumarin, 6*Z*-docosenamide, naringenin 7-*O*-β-D-glucoside, 5-*p*-coumaroylquinic acid, integrastatin A, luteolin 7-*O*-(6-*O*-malonyl-β-D-glucoside), 1,3-dicaffeoylquinic acid, magnoshinin, okanin, (2*S*)-5-hydroxy-7-methoxy-6,8-dimethylflavanone, (9*Z*,12*Z*,15*Z*)-octadecatrienoic acid, 2″-deamino-2″-hydroxy-6″-dehydroparomamine, *O*-butanoylcarnitine, myricitrin, gorlic acid, tetracenomycin X, sakuranin, D-tryptophan, linoleamide, laricitrin 7-monoglucoside, L-β-phenylalanine, L-proline, pheophytin A, pheophorbide A, PI(18:0/20:4(8*Z*,11*Z*,14*Z*,17*Z*), stearidonic acid, and gibberellin A14 aldehyde. Intriguingly, the detected compounds have a wide range of health benefits, including antioxidant, anti-microbial, anti-inflammatory, anti-aging, antiproliferative and ameliorating cardiovascular and diabetes diseases. The study will form basis of understanding the compounds influence by cultivation of the bush tea with various judicious application of agronomic practices in large scale in South Africa. Hence, the study validates the significance of bush tea as herbal and medicinal tea reputed to have health benefits.

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Interest conflict
The authors declare no conflict of interest.

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