Current Topics in Phytochemistry
Vol. 15, 2019

Original Article

Polyphenols from the sagebrush *Artemisia tridentata* ssp. *tridentata* affect the redox state of cultured hepatocytes by direct and indirect mechanisms

Carolyn Y. Dadabay[1,*], Paula Barrientos Spaulding[1], Erick Valenzuela[1], Matthew Turner[3], Kaitlyn E. Eckert[1], Riitta Julkunen-Tiitto[4], Nedra Noblit[1] and Donald H. Mansfield[2]

[1]Chemistry and [2]Biology Departments, The College of Idaho, Caldwell, Idaho, USA;
[3]Biomolecular Research Center, Boise State University, Boise, Idaho, USA.
[4]Department of Environmental and Biological Sciences, University of Eastern Finland, Joensuu, Finland.

ABSTRACT

Basin big sagebrush (*Artemisia tridentata* Nutt. ssp. *tridentata* (Asteraceae)), is a widespread North American shrub which produces a variety of polyphenolic compounds. Although sagebrush has been used as a traditional remedy by natives and settlers to the region, the polyphenols in *Artemisia tridentata* ssp. *tridentata* have not been highly investigated for their bioactive properties. To determine whether these polyphenols affect the intracellular redox state, we measured their ability to neutralize radicals *in vitro* and in a human liver carcinoma cell line (HepG2), and their effects on intracellular glutathione levels. Extracts from *Artemisia tridentata* ssp. *tridentata* decreased the oxidation of 2'7'-dichlorofluorescin *in vitro* and in cultured cells. Cells treated with polyphenolic extracts showed increased levels of glutathione in a time and dose-dependent manner. Approximately 48 polyphenolic compounds were distinguishable in extracts, by HPLC/UV absorbance detection. Mass spectroscopy was used to identify thirteen compounds as aesculin, aesculetin, apigenin, apigenin-7-O-glucoside, axillarin, casticin, chlorogenic acid, isoscopoletin, kaempferol, luteolin, methyl-axillarin, quercetin, and scopoletin. These results indicate that polyphenols produced in *Artemisia tridentata* ssp. *tridentata* affect the redox state of living cells by multiple mechanisms.

*Corresponding author: cdadabay@collegeofidaho.edu

KEYWORDS: *Artemisia tridentata*, sagebrush, polyphenol, antioxidant, glutathione.

INTRODUCTION

Desert, or big sagebrush (*Artemisia tridentata*), is estimated to be one of the most common shrubs in North America, comprising up to 70% of the relative vegetative cover and 90% of the phytomass in the arid Great Basin region of the western United States [1]. In addition to being an important source of forage and shelter for wildlife, sagebrush has been used by Native Americans and settlers to the region for its antifungal, antiparasitic, antimicrobial and analgesic effects [2-4]. However, despite the wide occurrence of sagebrush and its historic usage, modern research into its medicinal properties is only recently gaining momentum [1, 5, 6]. Like other plants of the genus *Artemisia*, *Artemisia tridentata* produces several classes of compounds that are likely to be bioactive [5]: polyphenols [6-10], monoterpenes [6, 10-14], and sesquiterpenes [6, 10, 15].

The focus of this work is the biological activity of polyphenolic compounds produced in basin big sagebrush, *Artemisia tridentata* Nutt. ssp. *tridentata* (Asteraceae) (abbreviation *A.t.t.*), the most widespread sagebrush subspecies of the American Great Basin. This subspecies has been reported to produce at least 25 different polyphenolic compounds, including coumarins and flavonoids [5-8] and in a related

subspecies, *Artemisia tridentata* ssp. *wyomingensis*, the production of these polyphenolic compounds correlates with *in vitro* antioxidant capacity [16]. However, polyphenolics from these plant sources have not been investigated for their antioxidant properties in eukaryotic cell systems. In this study, we demonstrate that polyphenols extracted from *A.t.t* not only act as antioxidants *in vitro*, but also affect the redox state of cultured HepG2 human hepatocellular carcinoma cells by the dual mechanism of reducing the level of intracellular reactive oxygen species (ROS) and increasing the level of endogenous glutathione (GSH). Furthermore, while early publications identified at least 25 different polyphenolic compounds, including coumarins and flavonoids, in *A.t.t.*, [5, 7, 8], there has been no recent confirmation of individual compounds. We confirm the presence of nine previously reported phenolic compounds (aesculin, aesculetin, apigenin. apigenin-7-O-glucoside, isoscopoletin, kaempferol, luteolin, quercetin, scopoletin; [5]) and present evidence for 4 previously unreported compounds (chlorogenic acid, axillarin, methyl axillarin, and casticin) in *A.t.t.* The demonstration of the bioactivity of polyphenols from *Artemisia tridentata* suggests a new pharmacological utility for this vast plant resource of North America.

MATERIALS AND METHODS

Cell lines, chemicals and biochemicals

HepG2 (human hepatocellular carcinoma) and Saos-2 (human osteosarcoma) cell lines, fetal bovine serum (FBS), Eagle's minimum essential medium (EMEM), Dulbecco's modified Eagle's medium (DMEM), and penicillin-streptomycin solution (P/S) were obtained from ATCC. HPLC grade methanol was obtained from Fisher Scientific. DC Protein Assay Kit and Quick Start Bradford 1X Dye Reagent were purchased from Bio-Rad. 2,2'azobis(amidinopropane) (ABAP) was purchased from Wako. Glutathione reductase was obtained from Calbiochem. Aesculin, eupafolin, isoscopoletin, and luteolin, naringenin, quercetagetin, scopoletin, and umbelliferne were from Indofine Chemical Company. All other reagents and reference compounds were obtained from Sigma-Aldrich.

Plant material

Leaves of *Artemisia tridentata* Nuttall ssp. *tridentata* (Asteraceae) were collected May-July of 2008 through 2011 from plants growing on site at The College of Idaho campus, Caldwell, Idaho, USA. The plants were transplanted from a site near Succor Creek, Oregon, USA (N 43.4975^{o} W-117.1344^{o}) in 2007, and identified by Dr. Donald Mansfield, Biology Department, The College of Idaho. A voucher specimen (DiNicola 2011-41) was deposited at the Harold M. Tucker Herbarium (CIC), The College of Idaho, Caldwell, Idaho, USA.

Extraction of polyphenols

Fresh leaves (7-10 g) were combined with 10 mL methanol/g foliage and homogenized in a Waring blender for 10 min, then gravity filtered through coarse filter paper. Sagebrush extracts (SBE) were stored at -20 ºC. Relative concentrations of SBE were estimated by comparing the absorbance at 340 nm to a standard curve of quercetin in methanol.

Peroxyl radical scavenging capacity (PSC) assay

Antioxidant activity of SBE was measured *in vitro* as the decrease in oxidation of 2'7'-dichlorofluorescin (DCFH) to fluorescent dichlorofluorescin (DCF) by peroxyl radicals generated during the thermal degradation of ABAP [17]. SBE or reconstituted SBE HPLC fractions (see below) were diluted 1:40 in methanol, reference compounds were dissolved in methanol, and control measurements were made on methanol alone. Fluorescence values for DCF (Ex/Em 485/528nm) were recorded every 2 minutes for one hour at 37 ºC, then integrated to obtain areas under the curve (AUC) for each sample. AUC values were averaged for 4 replicates of sample or control and the antioxidant activity of the SBE calculated according to the equation [17]:

$$\text{PSC units} = 1-(SA/CA)$$

where PSC is the peroxyl radical scavenging capacity units, SA is the AUC for the SBE or reference sample, and CA is the AUC for control reaction which contained methanol solvent but no plant extract.

ROS assay on HepG2 and Saos-2 cells

HepG2 cells were plated in 96 well plates at a density of 2.0 x 10^4 cells/well in EMEM supplemented with 10% FBS and 1% P/S. The following day SBE, quercetin dissolved in methanol, or an equal volume of methanol (6.25% final concentration) was added to quadruplicate wells for pre-incubation times ranging from 0.5 to 4 hours

at 37 ºC. Following pre-incubation, the media was aspirated and wells washed twice with phosphate buffered saline solution (PBS) (0.010 M sodium phosphate, 0.154 M NaCl, pH 7.4). The cells were then incubated with 200 μM DCFH-DA dissolved in PBS with 0.3% BSA for 0.5 hours at 37 ºC. DCFH-DA is de-acetylated to the DCFH form and oxidized by endogenous ROS to form the fluorescent DCF (Ex/Em 485/525 nm) measured directly in wells [18]. Following fluorescence readings, the media was aspirated, wells washed twice with PBS, and the protein content determined using the Bio-Rad DC protein assay. Fluorescence measurements were normalized for total cellular protein. An identical procedure was used to determine ROS in Saos-2 cells treated with SBE except that the cells were cultured in DMEM supplemented with 10% FBS and 1% P/S.

HPLC-DAD analysis

Reverse phase HPLC was carried out using a Varian ProStar HPLC system equipped with a ProStar 335 photodiode array detector. For analytical HPLC, a Varian Microsorb-MV 100 C18 150 x 4.6 mm column was used at 25 ºC. SBE was passed through a 0.45 μ PTFE filter, then 5 μL was applied to the column and eluted at a flow rate of 0.75 mL/min as follows: 0-15 min at 100% solution A (10% methanol, 2.5% acetic acid); 15-30 min at 75% solution A and 25% solution B (97.5% methanol, 2.5% acetic acid); 30-40 min at 50% solution A and 50% solution B; 40-50 minutes consisted of a linear change to 100% solution B; and 50-55 min at 100% solution B. For preparative scale HPLC a Varian Pursuit C18 5 μ 250 x 10.0 mm column was used at room temperature. Samples (500 μL of SBE or 0.20 μmol of reference compounds) were filtered as above, then were applied to the column and eluted over 60 min. The elution consisted of a linear gradient starting with 100% solution C (30% methanol, 2.5% acetic acid) and ending at 100% solution B at a flow rate of 1.0 ml/min. All elutions were monitored for absorbance at 280 nm. Data was analyzed using Galaxie Chromatography Data System software version 1.9.302.530. For experiments using HPLC-fractionated SBE in further assays, HPLC eluate fractions were dried down under N_2 then re-suspended in 250 μL of methanol. In order to rule out the possibility that solvent residue remaining from the HPLC gradient in the reconstituted pools was responsible for the differences seen, the gradient was run without SBE, solvent fractions were collected and processed identically to SBE-containing fractions, and tested for PSC. These solvent pools alone did not contain significant levels of PSC or GSH-inducing activity (data not shown).

HPLC/tandem mass spectrometry, University of Eastern Finland

SBE was dried down, then resuspended in 5X volume of 1:1 water:methanol. A volume of 2 uL was analyzed by high performance liquid chromatography mass spectrometry (HPLC-MS) using an Agilent 6540 Ultra High Definition Accurate-Mass Q-TOF instrument. The AJI electrospray ionization (ESI) source was operated under the following conditions: positive ion mode; nebulizer pressure: 35psig; flow rate of drying gas (N_2): 12 L/min; drying gas temperature: 350 °C; MS abs/ threshold 200 (0.010%). Acquisition mode 100 m/z min to 3000 m/z max, scan rate of 1.00 spectra/second. HPLC separation was achieved using an Agilent Extend C18 column, 1.8 μm, 2.1 x 50 mm. The flow rate was 400 μL/min. The mobile phases were: water with 1.5% tetrehydrofuran and 0.25% acetic acid in MilliQ water (buffer A) and 100% methanol (buffer B). A step gradient method was used to separate analytes, starting at 100% buffer A for 1.5 min, linearly increasing buffer B to 15% by 3 min, to 30% by 6 min, to 50% by 12 minutes, then holding at 50% buffer B until 20 min. Data was analyzed using the Agilent MassHunter Workstation Acquisition 2003-2011; Data Acquisition TOF/Q-TOF B.04.00 (B4033.1).

HPLC/tandem mass spectrometry, Boise State University Biomolecular Research Center

SBE was analyzed by HPLC-MS using an ultra-high resolution Quadrupole Time of Flight (QTOF) instrument (Bruker maXis). The electrospray ionization (ESI) source was operated under the following conditions: positive ion mode; nebulizer pressure: 1.2 Bar; flow rate of drying gas (N_2): 8 L/min; drying gas temperature: 200 °C; voltage between HV capillary and HV end-plate offset: 3000 V to -500 V; mass range was set from 80 to 800 m/z; and the quadrupole ion energy was 6.0 eV. Sodium formate was used to calibrate the system in the mass range. HPLC separation was achieved using a XTerra MS C18 column, 3.5 μm, 2.1 x 150 mm (Waters, Milford, MA). The flow rate was

250 µL/min. The mobile phases were 5% acetonitrile and 0.1% formic acid in water (buffer A) and acetonitrile and 0.1% formic acid (buffer B). The linear gradient method was used to separate analytes starting at 5% Buffer B, increasing Buffer B to 40% from 3 to 31 minutes, then increasing to 80% at 46 min and holding for 5 minutes. A 10 µL sample injection was used. MS/MS fragmentation was achieved using multiple reaction monitoring (MRM) with collision energies optimized for each target molecule. The optimized collision energies for each molecule are listed in Table 1. Collision energies were optimized using chemical standards and by direct injection to the MS with a syringe pump at a flow rate of 240 µL/hr. For direct injection samples, the MS was operated under the conditions listed above, except the drying gas flow rate was 4 L/min and the nebulizer pressure was set to 0.4 Bar. Data was analyzed using the Compass Data Analysis software package (Bruker Corporation, Billerica, Massachusetts).

Statistical analysis for in vitro and cell-based assays

Results are expressed as the mean ± s.d. Test values were statistically compared to controls using paired t-tests. Probability values of $p < 0.05$ were considered statistically significant and are indicated by *.

RESULTS AND DISCUSSION

In vitro peroxyl radical scavenging capacity

Polyphenol-containing methanolic extracts of *A.t.t.* (sagebrush extract or SBE) were found to decrease the *in vitro* oxidation of 2'7'-dichlorofluorescin by peroxyl radicals generated through the thermal degradation of 2,2'azobis(amidinopropane). SBE with polyphenol concentrations ranging between 0.10 and 4.0 mM were found to exhibit peroxyl radical scavenging capacity (PSC) [17] as shown in Figure 1. As a demonstration of relative potency, SBE antioxidant capacity was compared to the antioxidant capacity of quercetin, a well-documented antioxidant flavonol, and was found to have significantly higher PSC at each concentration over the same range (Figure 1).

Scavenging of reactive oxygen species (ROS) in cultured hepatocytes

Cultured HepG2 cells were exposed to SBE containing a polyphenol concentration of 100 µM for times up to 4 hours then the levels of intracellular ROS measured. SBE treatment caused a 28% decrease in the level of intracellular ROS within 0.5 hours, with a decrease of 64% reached by 4 hours (Figure 2). SBE was 2.6-fold more effective at reducing ROS than 100 µM quercetin at 4 hours. Treatment of cells with SBE for longer periods did not result in greater decreases in ROS levels (data not shown).

Induction of intracellular glutathione (GSH) in cultured hepatocytes

To determine whether SBE affected the intracellular redox state through a mechanism involving the endogenous antioxidant GSH, the level of total GSH was determined following exposure of HepG2 cells

Table 1. Optimized collision energy for molecules of interest.

Compound	Molecular formula	[M+H]	Isolation width	Collision energy (eV)
Aesculetin	$C_9H_6O_4$	179.0415	4	3
Aesculin	$C_{15}H_{16}O_9$	341.1005	6	15
Apigenin-7-glucoside	$C_{21}H_{20}O_{10}$	433.1191	6	18
Apigenin	$C_{15}H_{10}O_5$	271.0805	6	45
Casticin	$C_{19}H_{18}O_8$	375.1174	6	33
Scopoletin	$C_{10}H_8O_4$	193.0608	6	25
Chlorogenic Acid	$C_{16}H_{18}O_9$	355.1097	6	10
Luteolin	$C_{15}H_{10}O_6$	287.0627	6	45
Kaempferol	$C_{15}H_{10}O_6$	287.0627	6	45
Quercetin	$C_{15}H_{10}O_7$	303.0572	6	40
Umbelliferone	$C_9H_6O_3$	163.0435	6	30

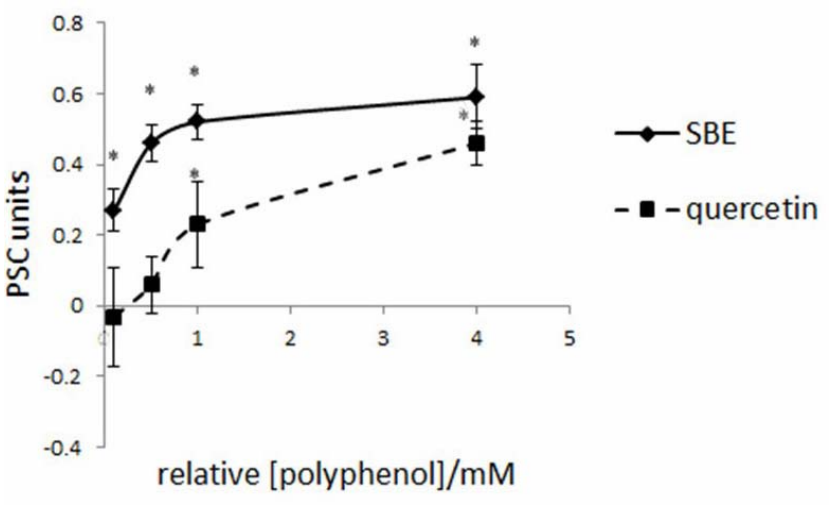

Figure 1. Peroxyl radical scavenging capacity of methanol extracts of sagebrush. Higher PSC units indicate stronger antioxidant activity. PSC values for commercially obtained quercetin were measured under the same conditions. Results indicate the mean + s.d. of two experiments. *indicates PSC differs from methanol control at $p < 0.05$.

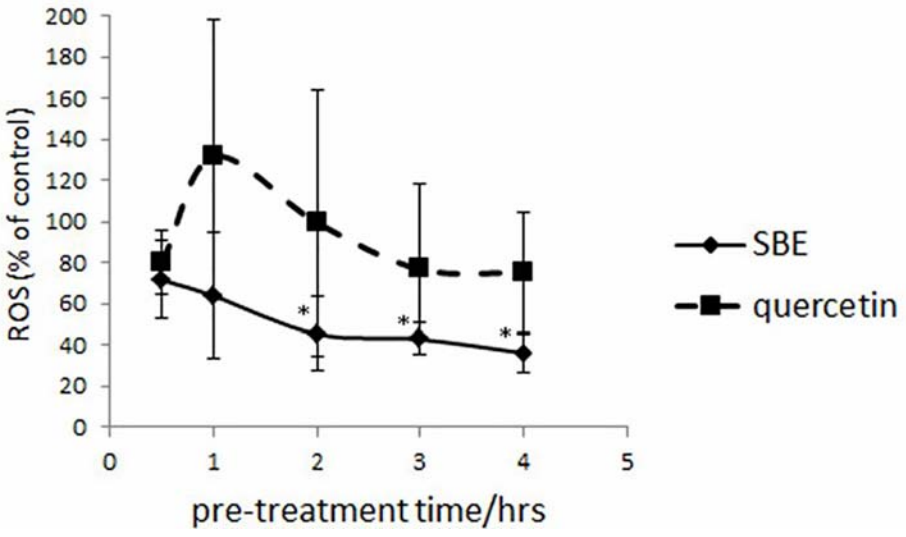

Figure 2. Effect of sagebrush extract on levels of reactive oxygen species in HepG2 cells. Cells were pretreated with either SBE or commercially obtained quercetin for the indicated times then the levels of intracellular ROS determined. Values represent the percent of intracellular ROS as compared to control cells treated with methanol alone. Results indicate the mean + s.d. of three experiments. *indicates ROS differs from control at $p < 0.05$.

to SBE. Following 24 hours of exposure to 10, 50, or 100 μM concentrations of SBE, significant increases in the level of total GSH were observed (Figure 3(a)). The maximum increase of 2.9-fold was seen at 100 μM treatment. A time course of GSH induction in response to 100 μM SBE indicated that induction of GSH was not significant following 12 hours of exposure (Figure 3(b)) but became significant after 24 hours. Treatment of cells with 10 nM SBE increased the levels of total GSH at only the 12 hour time point but was not sustained through the 24 hour time point (Figure 3(b)). Interestingly, the induction of GSH extends over a longer period (24 hours, Figure 3(b)) than the reduction in ROS in HepG2 cells (4 hours, Figure 2). These data indicate that in addition to direct

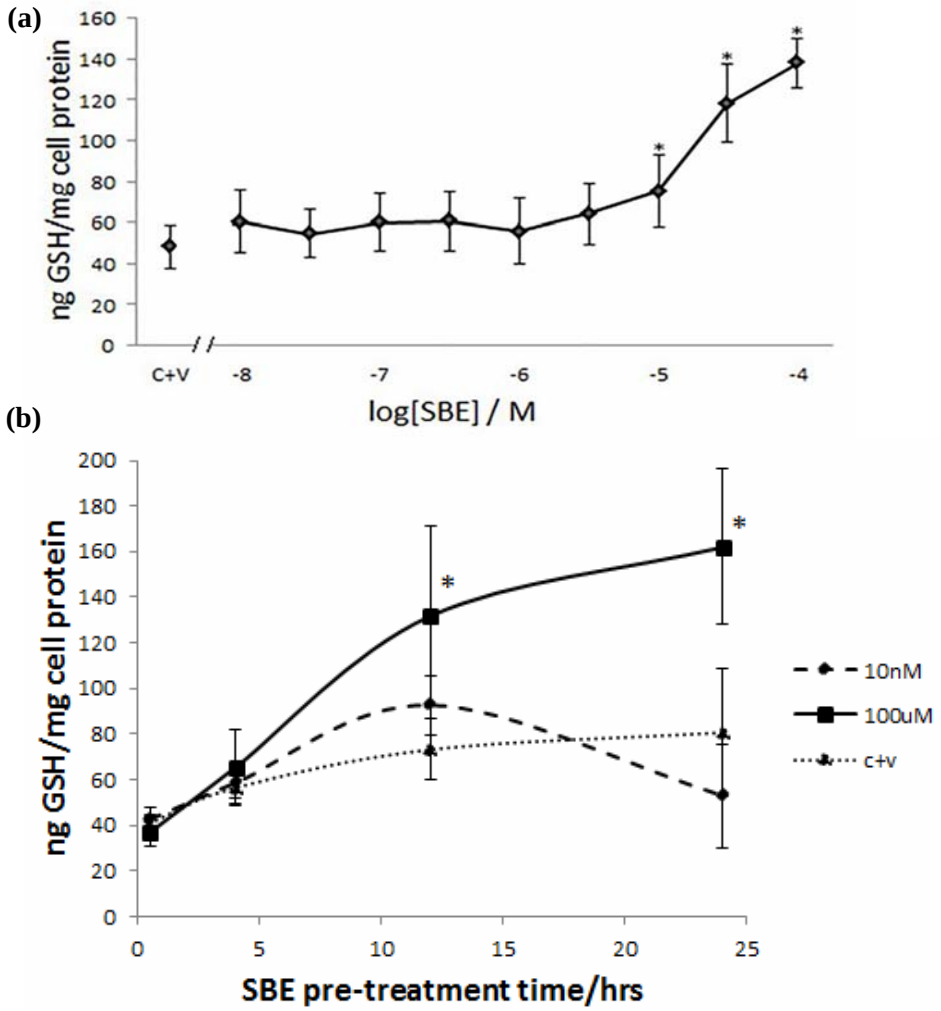

Figure 3. Effect of sagebrush extract on total cellular levels of glutathione in HepG2 cells. **(a)** Increasing doses of SBE were applied to cells for 24 hours. C + V indicates cells treated with methanol alone. Results indicate the mean + s.d. of six experiments. *indicates value differs from C + V at $p < 0.05$. **(b)** Cells were treated with 10 nM SBE, 100 μM SBE, or methanol alone (C + V) for the times indicated. Results indicate the mean + s.d. of four experiments. *indicates value differs from C + V at $p < 0.05$.

chemical neutralization of free radicals, SBE polyphenols influence the intracellular redox state by increasing the levels of GSH. An antioxidant mechanism involving both direct chemical neutralization and GSH induction was supported by our finding of different time courses for these two effects: the neutralization of intracellular ROS occurred in the first few hours following exposure to SBE, while the induction of GSH occurred over a longer 24 hour period (Figure 2 vs Figure 3(b)). Our finding that SBE-induced GSH is consistent with others who have shown increased GSH and GSH biosynthetic enzymes in response to exposure to flavonoids [19, 20].

HPLC fractionation polyphenolic compounds in SBE

Previous analyses of *A.t.t.* for polyphenolic compounds by HPLC suggested up to 25 polyphenolic compounds in the foliage, including coumarins, coumarin glycosides, flavonoids, and flavonoid glycosides [5, 7, 8, 21, 22]. Using current C-18 reverse phase HPLC technology with diode array detection to confirm those early studies, we detected at least 48 individual compound peaks in SBE (see Figure 4).

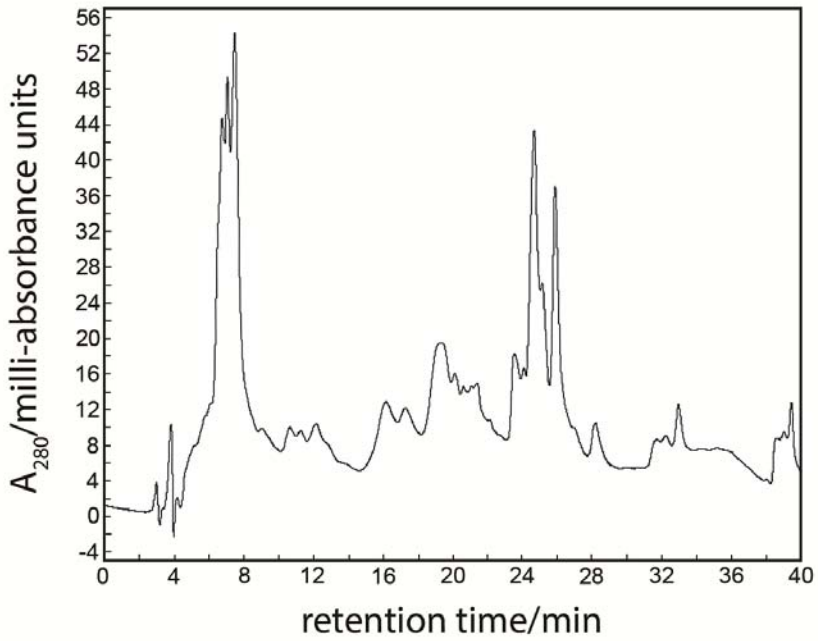

Figure 4. Separation of compounds in SBE by analytical reverse phase HPLC. Detection of eluting compounds was by absorbance at 280 nm.

HPLC-tandem mass spectrometry and 2D ms/ms to identify constituents of SBE

Identity of chemical components of SBE was established using tandem liquid chromatography/ mass spectrometry retention times for M+H parent ion masses (University of Eastern Finland), and with extracted ion chromatographs (EIC), and ms/ms fragmentation patterns (Boise State University Biomolecular Research Center), (see Table 2, panel A). SBE data were compared to reference standards and also compared to entries in the MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu).

The identity of five polyphenolic compounds were strongly confirmed by M+H parent ion mass values, 2D ms/ms fragmentation patterns, and retention times comparable to authentic standards (see Table 2, panel A): apigenin-7-O-glucoside, casticin, chlorogenic acid, isoscopoletin, and scopoletin. Seven additional polyphenolic compounds were detected through comparison of M+H parent ion mass values and retention times with authentic standards: aesculin, aesculetin, apigenin, axillarin, kaempferol, luteolin, and quercetin (see Table 2, panel B). For these compounds, 2D ms/ms fragmentation patterns could not be obtained, most likely due to low abundance in SBE. Of the fourteen phenolic compounds detected in our SBE, four were compounds which have not previously been reported in *A.t.t.*: axillarin, casticin, chlorogenic acid, and methyl axillarin.

HPLC fractionation of peroxyl radical scavenging activity and GSH-inducing activity

In order to further characterize the bioactivity of compounds in SBE, preparative scale reverse phase HPLC was used to prepare enriched fractions (Pools I-V) for further testing (Figure 5). Two of these pools contained significant PSC relative to controls: Pool II containing relatively more polar compounds and Pool IV containing relatively less polar compounds (Figure 6(a)). To characterize GSH-inducing activity, HepG2 cells were exposed to equal dilutions of the pools I-V for 12 hours then the levels of GSH determined. The pattern of GSH induction shown by the SBE eluent pools in living cells was markedly different than the pattern seen for the *in vitro* PSC assay for direct chemical reactivity (Figure 6(b) vs. (a)). The eluent pool which caused the greatest induction of GSH was pool IV (Figure 6(b)) with lesser but still statistically significant effects seen with treatment using pools II, III, and V. Pool IV contained high

Table 2. Polyphenolic compounds detected in SBE using HPLC-tandem mass spectrometry and 2D ms/ms. SBE compounds listed exhibited HPLC retention times (not shown) which matched authentic reference samples, except in the cases of axillarin and methyl axillarin (no reference samples available). Panels A and B: SBE compounds exhibited parent ion positive M+H masses shown which matched those of the indicated compound, and of authentic reference standards except in the case of axillarin and methyl axillarin. Values determined at *the Department of Environmental and Biological Sciences, University of Eastern Finland; and/or ^the Biomolecular Research Center, Boise State University. Panel A. SBE compounds exhibited 2D ms/ms fragementation patterns which matched those of authentic reference standards (see 'Materials and Methods' section) as well as patterns described in MassBank of North America (MoNA).

Panel A.		
Component	**Parent ion pos(M+H) mass**	**ms/ms pos fragment mass^**
Apigenin -7-O-glucoside	433.11^	433.11, 271.06
Casticin	375.11^	375.11
Chlorogenic acid	355.0971*, 355.1^	355.10, 163.04
Isoscopoletin	193.05^, 193.05^	193.05, 178.03, 133.03
Scopoletin	193.05^, 193.05^	193.05, 178.03, 133.03
Panel B.		
Component	**Primary ion M+H mass**	
Aesculin	341.09^	
Aesculetin	179.03^	
Apigenin	271.06^	
Axillarin	347.0688*, 347.08^	
Kaempferol	287.06^	
Luteolin	287.06^	
Methylaxillarin	361.0926*, 361.09^	

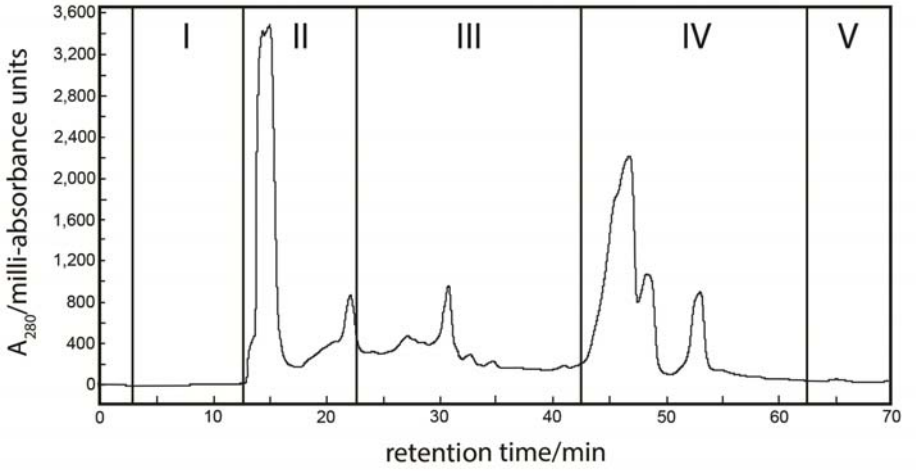

Figure 5. Fractionation of SBE by preparative-scale HPLC. Eluting compounds were detected by absorbance at 280 nm. Eluate was collected in pools I (2.5-12.5min), II (12.5-22.5min), III (22.5-42.5 min), IV (42.5-62.5 min), and V (62.5-67.5min).

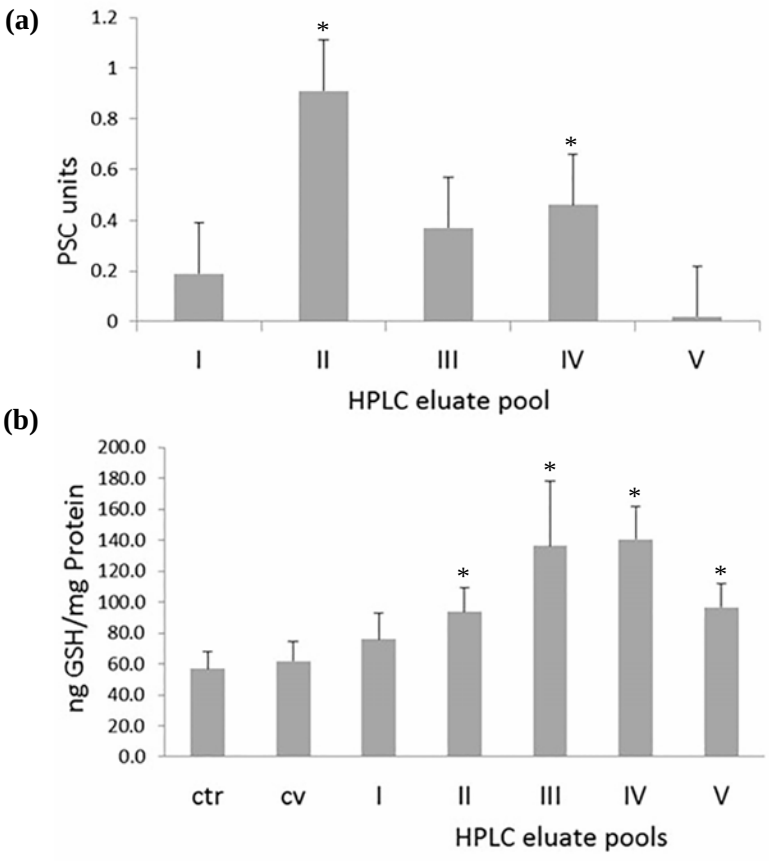

Figure 6. Antioxidant and bioactive effects of SBE fractionated by HPLC. (a) Peroxyl radical scavenging capacity (PSC) of reconstituted HPLC eluate pools I-V. Equal volumes of reconstituted pools were used in the assay. Results indicate the mean + s.d. of two experiments. *indicates value is different than methanol control at $p < 0.05$. (b) Effect of reconstituted HPLC eluate pools I-V on glutathione levels in HepG2 cells. Cells were treated with reconstituted HPLC fractions at a dilution which was equivalent to that used to give 100 μM of the original SBE, an equal volume of methanol (cv), or left untreated (ctr) for 12 hours. Results indicate the mean ± s.d. of five experiments. *indicates value different from cv at $p < 0.05$.

levels of both PSC and GSH-inducing bioactivity indicating that it contained compounds capable of acting both as direct chemical and GSH-inducing antioxidants. In contrast, pool II, which showed the highest PSC, caused induction of GSH to a much lesser extent than pool IV. Furthermore, pool III had significant activity in inducing GSH, yet its PSC was not statistically significant. SBE Pools with higher GSH-inducing ability eluted later in the HPLC gradient, and therefore contained compounds which were relatively less polar. It is possible that these more lipophilic compounds pass more readily across the cell membrane than the more polar, earlier-eluting compounds, resulting in more capacity to influence intracellular events.

Comparison of compounds in HPLC pools I-V to reference standards

In order to investigate the chemical composition of preparative HPLC pools I-IV, the retention times of authentic standards were obtained separately but under the same elution conditions (Table 3). Of the standards used, relatively more hydrophilic coumarins emerged with lower retention times corresponding to SBE eluent Pools II and III. More lipophilic flavonol standards were found to have retention times corresponding to SBE pools III and IV. Flavone and flavone glycoside standards also had retention times corresponding to SBE pools III and IV. Direct identification of compounds in SBE pools I-V by

Table 3. Comparison of HPLC retention times for reference compounds versus SBE pools I-V using the same preparative scale HPLC protocol in each case. Eluting compounds were detected by absorbance at 280 nm.

Reference cpd	Observed ret. time (min)	SBE pool	Collection time (min)
		Pool I	2.50-12.50
Aesculin	21.84	Pool II	12.50-22.50
Aesculetin	30.85	Pool III	22.40-42.50
Isoscopoletin	34.15		
Scopoletin	34.79		
Umbelliferone	35.95		
Quercetagetin	40.31		
Apigenin-7-O-glucoside	40.76		
Quercetin	49.28	Pool IV	42.50-62.50
Naringenin	51.13		
Luteolin	51.57		
Eupafolin	52.2		
Kaempferol	55.41		
		Pool V	62.50-67.5

further LCMS processing was not possible due to limited quantities and residual solvent interference with LCMS signals.

CONCLUSIONS

Our results demonstrate a potent antioxidant capacity of the polyphenols produced by *A.t.t.* both *in vitro* and in cultured cells. The decrease in ROS seen in SBE-treated cells may involve both the direct chemical neutralization of free radicals by polyphenols entering the cells and activation of endogenous pathways which modulate the intracellular redox state. The ability of SBE to neutralize higher levels of peroxyl radicals than a pure form of the well-studied flavonol quercetin at comparable concentrations suggests that although quercetin is one component of SBE, there are other compounds in SBE, highly effective at neutralizing free radicals and affecting cellular events. We provided updated evidence for the presence of fourteen phenolic compounds in extracts of *A.t.t.*, including evidence for four previously unreported constituents: axillarin, casticin, chlorogenic acid, and methyl axillarin. The further characterization and identification of the remaining unknowns of these potent compounds from an abundantly occurring plant source may represent a rich source of potent bioactive compounds for future applications in pharmacology.

ACKNOWLEDGEMENTS

This publication was made possible by the Idaho INBRE Program, NIH Grant Nos. P20 RR016454 (National Center for Research Resources) and P20 GM 103408 (National Institute of General Medical Sciences); and the Burroughs Wellcome Foundation Collaborative Research Travel Grant award number 1016252. The authors wish to thank Dr. Xinzhu Pu of the Biomolecular Research Center, Boise State University for assistance with HPLC tandem mass spectrometry and data analysis; Professor Alice Vinson of The College of Idaho Art Department for assistance in preparing figures;. and Corey Westfall and Eli Harbin of The College of Idaho Chemistry Department for assistance with samples, assays, and HPLC.The authors wish especially to thank Dr. Jennifer Forbey of the Department of Biological Sciences, Boise State University, for research mentorship and assistance in procuring support for this project.

CONFLICT OF INTEREST STATEMENT

To the best of our knowledge, there are no conflicts of interest.

REFERENCES

1. Shultz, L. M. 2009, Monograph of Artemisia Subgenus Tridentatae (Asteracea-Anthemideae). USA: The American Society of Plant Taxonimists.
2. Kane, C. W. 2006, Herbal Medicine of the American Southwest. USA: Lincoln Town Press.
3. Moerman, D. E. 2009, Native American Ethnobotany. Portland, OR: Timber Press Inc.
4. Kelley, B. D., Appelt, J. M. and Appelt, G. D. 1992, The International Journal of the Addictions, 27, 347.
5. Turi, C. E., Shipley, P. R. and Murch, S. J. 2014, Phytochemistry, 98, 9.
6. Turi, C. E., Axwik, K. E. and Murch, S. J. 2014, Plant Growth Regulation, 74, 239.
7. Wilt, F. M. and Miller, G. C. 1992, Biochemical Systematics and Ecology, 20, 53.
8. Wilt, F. M., Geddes, J. D., Tamma, R. V., Miller, G. C. and Everett, R. L. 1992, Biochemical Systematics and Ecology, 20, 41.
9. Brown, D., Asplund, R. O. and McMahon, V. A. 1975, Phytochemistry, 14, 1083.
10. Kelsey, R. G., Stevenson, T. T., Scholl, J. P., Watson, T. J. and Shafizadeh, F. 1978, Biochemical Systematics and Ecology, 6, 193.
11. Rosentreter, R. and Kelsey, R. G. 1991, Journal of Range Management, 44, 330.
12. Huynh, M. D., Page, J. T., Richardson, B. A. and Udall, J. A. 2015, PLoS One, 10, e0127593.
13. Kessler, A., Halitschke, R., Diezel, C. and Baldwin, I. T. 2006, Oecologia, 148, 280.
14. Karban, R., Wetzel, W. C., Shiojiri, K., Ishizaki, S., Ramirez, S. R. and Blande, J. D. 2014, New phytologist, 204, 380.
15. Kelsey, R. G., Morris, M. S. and Shafizadeh, F. 1976, Journal of Range Management, 29, 502.
16. Pu, X., Lam, L., Gehlken, K., Ulappa, A. C., Rachlow, J. L. and Forbey, J. S. 2015, Western North American Naturalist, 75, 78.
17. Adom, K. K. and Liu, R. H. 2005, Journal of Agricultural and Food Chemistry, 53, 6572.
18. Smith, S. S., Rodriguez-Reyes, J., Arbon, K. S., Harvey, W. A., Hunt, L. M. and Heggland, S. J. 2009, Toxicology in Vitro, 23, 60.
19. Martin, M. A., Ramos, S., Mateos, R., Serrano, A. B. G., Izquierdo-Pulido, M., Bravo, L. and Goya, L. 2008, Journal of Agricultural and Food Chemistry, 56, 7765.
20. Myhrstad, M. C. W., Carlsen, H., Nordstrom, O., Blomjoff, R. and Moskaug, J. O. 2002, Free Radical Biology & Medicine, 32, 386.
21. Tamma, R. V., Miller, G. C. and Everett, R. 1985, Journal of Chromatography, 322, 236.
22. Shafizadeh, F. and Melnikoff, A. B. 1970, Phytochemistry, 9, 1311.