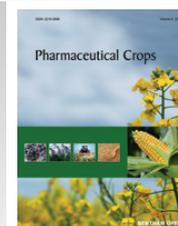




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RESEARCH ARTICLE

Assessment of Chemopreventive Contents of Native American Juneberries (*Amelanchier alnifolia* (Nutt.) Nutt. ex M. Roem.)

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Abstract: Juneberry (*Amelanchier alnifolia*) is a shrub with edible berry-like fruit commonly found across the Northern Great Plains of North America and widely used by Native Americans as a medicinal plant. This study was an effort to assess the chemopreventive contents of Juneberry extracts in ethanol by measuring the total phenolic content, orthophenolic content, DPPH radical scavenging activity, ABTS radical cation decolorization assay, and cytoprotective effects on HepG2 cells. The total phenolic content upon treatment with Folin-Ciocalteu's reagent ranged from 0.2261 ± 0.0031 to 19.311 ± 1.651 mg.g⁻¹ tannic acid equivalents. Orthophenolic content quantified by monitoring the bathochromic shifts upon reacting with sodium molybdate ranged from 0.12 ± 0.03 to 2.38 ± 0.15 mg.g⁻¹ of FW. Antioxidant activity using DPPH assay ranged from 50.08 ± 0.77 to $98.60 \pm 1.12\%$, in contrast to quercetin dihydrate with an activity of $86.20 \pm 0.05\%$. ABTS radical scavenging assay produced inhibition from 1.90 ± 0.11 to $60.25 \pm 1.65\%$ as opposed to 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) with an inhibition of $94.03 \pm 0.21\%$. HepG2 cells treated with 100 µg/µL of sample WC2B showed 56% cell-viability against tert-Butyl hydroperoxide in comparison to quercetin dihydrate which showed 90% cytoprotection. Total phenolic content and orthophenolic content in Juneberries from 2012 were higher than in Juneberries obtained from the 2013 and 2014. Similarly fruit grown in the wild showed significantly higher antioxidant activity and phenolic content than those grown in domestic cultivars.

Keywords: Juneberry, *Amelanchier alnifolia*, Chemopreventive, Antioxidant activity, Native American, Cytoprotection, phenolics, DPPH, ABTS, Orthophenolic, TBHP.

INTRODUCTION

Human cells are repeatedly exposed to free radicals, species with unpaired electrons some of which are vital for physiological function. However, an uncontrolled exposure of cells to oxidants gives rise to oxidative stress which could be harmful to biomolecules. By-products of aerobic respiration, phagocytic cells, peroxisomes, and cytochrome P450 enzymes are found to be responsible for degenerative diseases such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, cataract, and overall aging [1]. Dietary antioxidants quench reactive oxygen species (ROS) and reactive nitrogen species (RNS) to either halt radical chain reactions or prevent the formation of further ROS/RNS. Antioxidants consumed by food are mainly radical chain reaction inhibitors, metal chelators, oxidative enzymes inhibitors and antioxidant enzyme cofactors [2]. Many studies have shown an inverse relationship between dietary antioxidant flavonoid intake such as black tea, onions, apples, and risk of coronary heart disease and prostate cancer [3, 4]. The majority of the renowned natural compounds having chemotherapeutic effects against cancer are phytochemicals, and these are further categorized into phenolic compounds, alkaloids, lectins, terpenoids, isoprenoids, and quinones [5]. These components could individually or collectively display chemotherapeutic effects by a mechanism known as antioxidant activity [6].

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Amelanchier alnifolia (Nutt.) Nutt. ex M. Roem. (Rosaceae) is a shrub native to the Northern Great Plains of North America. Although commonly referred to as juneberry, it has a veritable book of aliases such as serviceberry, saskatoon berry, and shadbush. Native Americans are known for the extensive use of juneberries as a source of food and medicine. Historically, fruits were consumed both fresh, dried and mixed with meat, while native tribes also used tea made of juneberry branches to recover from colds, bark to relieve stomach problems, and twigs to gain strength following childbirth [7].

A Finnish study profiling the phytochemicals in *A. alnifolia* plant, leaves, stems, and berries using HPLC-DAD and LPLC-ESI/MS reported that the berry portion was majorly comprised of cyanidin-based anthocyanins, hydroxycinnamic acid, and quercetin-derived flavonol glycosides, while the stem portion was comprised of flavanone, flavonol glycosides, catechins, and hydroxybenzoic acid [8]. Rop *et al.*, measured flavonoid content among nine different cultivars and reported “smoky” to have highest level (550.5 ± 20.5 mg of rutin/kg FW) of flavonoid [9]. Further, a study by Wang and Mazza found that strong crude extract of juneberries has the ability to prevent the production of nitric oxide in LPS/IFN-gamma-activated RAW 264.7 macrophages [10]. Another study comparing two cultivars of juneberries discovered that “Thiessen” and “Smoky” has the highest radical scavenging activity due to the high concentration of anthocyanin [11]. As many studies have shown *A. alnifolia* to have antioxidant and chemopreventive effects, this study hypothesizes that the main chemical agents responsible for chemopreventive effect are phenolic compounds and investigates the total phenolic content, orthophenolic content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging antioxidant activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity, and cell based antioxidant activity of juneberry extracts for the purpose of discovering novel therapeutics.

MATERIALS AND METHODS

Plant Material

The frozen berries of *Amelanchier alnifolia* grown commercially and in the wild were provided from cultivars near Fort Berthold Community College, North Dakota. All samples were chosen by Prof. K Hartman, Chair Science Department, Fort-Berthold Community College, North Dakota. The frozen berries were stored at -20 °C prior to investigation.

Preparation of Plant Extracts

Frozen material (1 g) was weighed and 10 mL of 95% ethanol was used to homogenize using IKA (Wilmington, NC) homogenizer at 14,000 rpm for 5 min. The sample test tube was rinsed with 4 mL of ethanol and centrifuged for 20 min at 4,000 rpm. Supernatant was diluted to 20 mL with 95% ethanol, sealed and stored in the refrigerator until use. This method is thought to extract free phenolics, bound phenolics and soluble-conjugated phenolics.

Cell-free Antioxidant Chemical Assays

Total Phenolic content Assay: A 35% sodium carbonate solution was prepared. 50 μ L of Folin Ciocalteu’s phenol reagent, 50 μ L of the sample extract, 300 μ L of the sodium carbonate solution, and 3.5 mL of deionized water was added to each cuvette. The reaction mixture in the cuvette was left to sit for 15 min before analysis. Samples were analysis at 730 nm on SpectroVis Plus (Vernier) spectrophotometer, using five extracts from each sample set. A blank sample was used for the calibration of the instrument. The samples were measured against a standard curve using tannic acid (Alfa Aesar, 93%) as the sample extract, in concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL (Fig. S1A).

Antioxidant Activity Assay: A DPPH solution of 100 μ M was prepared by dissolving 32 mg of DPPH into 800 mL of 95% ethanol. A 500 μ L volume of the sample extract was mixed with 3.0 mL of the DPPH solution in a cuvette. The reaction mixture was allowed to sit for 10 min. The samples were analyzed on a Shimadzu spectrophotometer at 517 nm in triplicates. A control solution was prepared by adding 500 μ L of 95% ethanol to a cuvette with 3.0 mL of the DPPH solution, and 95% ethanol was used as a blank for calibration of the instrumentation. Antioxidant activity is measured using the following equation:

$$\text{Antioxidant activity (\%)} = \frac{[A(\text{control}) - A(\text{sample})]}{A(\text{control})} \times 100$$

For a comparison, a 1.4 mg/mL quercetin dihydrate (Sigma Aldrich, 98%) was used.

Orthophenolic Content Assay: 1 mL aliquots of the sample extract were dried under force, re-dissolved into 1 mL of pure water and vortexed for ten seconds to facilitate the compound going into the solution. To this 1 mL solution, 1 mL of 0.1 M, pH 6.5 phosphate buffer and 2 mL of a 5% (v/v) sodium molybdate solution were sequentially added, and the reaction mixture was left to sit for 15 min prior to analysis. Samples were analyzed using Molecular Devices SpectraMax spectrophotometer at 350 nm. The sample extract was compared against a standard curve of Caffeic acid (Indofine, 97%) in the range of 0-10 µg/mL (Fig. S1B).

ABTS Radical Cation Decolorization Assay: ABTS was dissolved in water to form a 7 mM solution. ABTS radical cations were then produced by reacting ABTS stock solution with 2.45 mM solution of potassium persulfate (final concentration). The mixture was allowed to stand in the dark at room temperature for 12–16 h. The ABTS radical solution was adjusted with methanol to an absorbance of 0.700 ± 0.020 at 734 nm. To 280 µL of this solution of ABTS was added to 20 µL of sample in a 96-well plate. The mixture was incubated for 5 min at 30°C and the absorbance at 734 nm was measured using a synergy H1 Biotek microplate reader and the inhibition percentage of the radical scavenging activity was then calculated. For a comparison, a 0.13 mg/mL Trolox (ACROS Organics, 97%) was used.

Cell-based Antioxidant Assay (Anti-TBHP)

Cell Culture: Human hepatocellular liver carcinoma cell line hepG2 was used to test cytoprotective activity of juneberry extracts. These Cells were maintained as a monolayer culture grown in a 75 cm² flask in an incubator in DMEM supplemented by 10% fetal bovine serum (FBS) and 1% antibiotic.

Bioassay Procedure: Cytoprotection assay was carried out according to Hamed *et al.* 2012. Plant extracts were evaporated by nitrogen gas and dissolved in DMSO for the cell assay. HepG2 cells were seeded into a 96 well plates (10×10^4 cells/mL). Cells were grown until confluent monolayers were obtained. Cells were then pretreated with the *A. alnifolia* extracts (100 µg, 50 µg, 25 µg and 12.5 µg) and 5% DMSO control for 20 hours. The medium was removed, cells were washed with 200 µL of Dulbecco's modified phosphate buffered saline (DPBS) (200 µL/well). Media containing 500 µM tertiary-butyl hydroperoxide (TBHP) (Acros, 70%) with 2% FBS final concentration was added to the cells and incubated for 5 hours. Following TBHP oxidative stress treatment, Cell viability was assessed using MTT assay. Reference compound, quercetin dihydrate (Sigma Aldrich, 98%) was used parallel in this assay (Figs. S2-S8).

RESULTS AND DISCUSSION

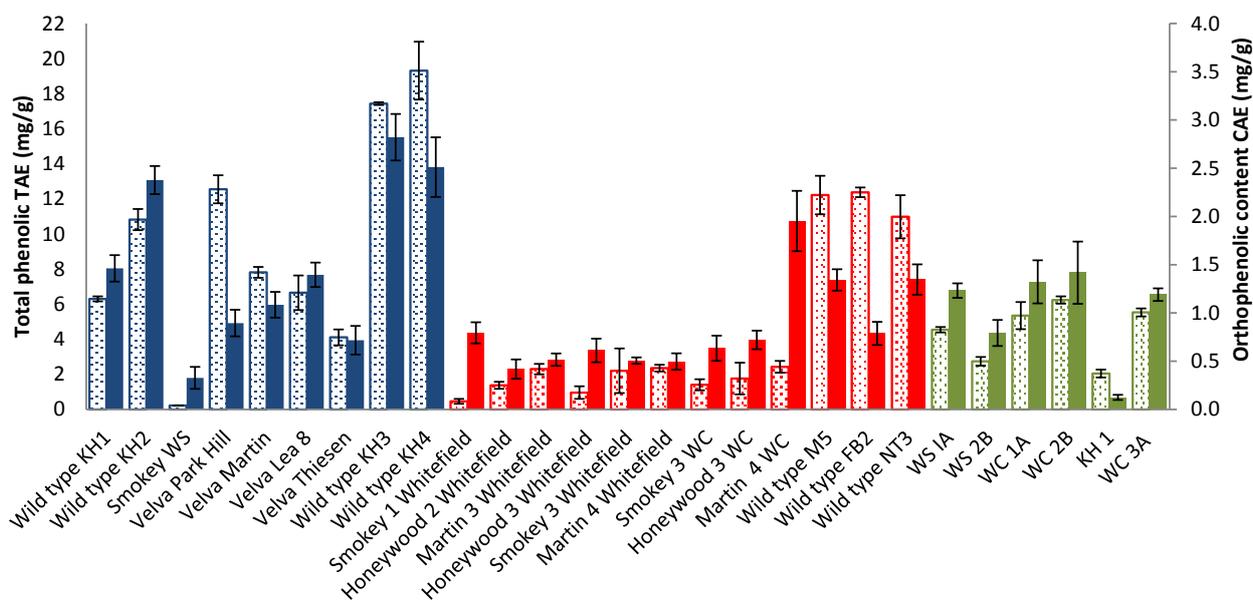


Fig. (1). Representative data showing the total phenolic content (solid) and Orthophenolic Content (pattern) in ethanol extracts of juneberries from cultivars in 2012 (blue), 2013 (red), and 2014 (green). Data shown are means \pm standard deviation (n=5 experiments).

Table 1. Total phenolic content, DPPH antioxidant activity, ABTS radical inhibition and orthophenolic content in juneberries.

Year	Sample Name	Total Phenolic (mg/g)	DPPH Antioxidant Activity (%)	ABTS radical Inhibition (%)	Orthophenolic content (mg/g)
	Quercetin dihydrate	-	86.20 ± 0.05	-	-
	Trolox	-	-	94.03 ± 0.21	-
2012	Wild type KH1	6.301 ± 0.144	92.79 ± 1.49	20.08 ± 1.43	1.46 ± 0.14
	Wild type KH2	10.822 ± 0.603	97.19 ± 0.23	29.90 ± 1.54	2.38 ± 0.15
	Smokey WS	0.226 ± 0.003	68.48 ± 1.46	3.33 ± 0.75	0.33 ± 0.11
	Velva Park Hill	12.552 ± 0.800	90.79 ± 2.47	10.09 ± 1.13	0.89 ± 0.14
	Velva Martin	7.813 ± 0.309	87.80 ± 2.42	8.86 ± 0.87	1.08 ± 0.13
	Velva Lea 8	6.652 ± 0.985	96.45 ± 0.54	28.72 ± 0.37	1.40 ± 0.13
	Velva Thiesen	4.107 ± 0.452	93.39 ± 1.38	7.86 ± 0.41	0.72 ± 0.15
	Wild type KH3	17.429 ± 0.076	93.64 ± 0.97	60.25 ± 1.65	2.82 ± 0.24
	Wild type KH4	19.311 ± 1.651	93.09 ± 1.05	58.82 ± 0.48	2.51 ± 0.31
2013	Smokey 1 Whitefield	0.461 ± 0.137	58.78 ± 1.37	1.81 ± 0.51	0.79 ± 0.11
	Honeywood 2 Whitefield	1.374 ± 0.198	85.01 ± 1.94	15.65 ± 0.65	0.42 ± 0.10
	Martin 3 Whitefield	2.300 ± 0.299	81.66 ± 2.33	13.28 ± 1.41	0.52 ± 0.06
	Honeywood 3 Whitefield	0.959 ± 0.358	79.25 ± 2.09	11.05 ± 1.23	0.61 ± 0.12
	Smokey 3 Whitefield	2.205 ± 1.275	69.01 ± 2.81	1.60 ± 0.55	0.51 ± 0.03
	Martin 4 Whitefield	2.356 ± 0.194	74.78 ± 1.89	6.26 ± 0.67	0.50 ± 0.08
	Smokey 3 WC	1.407 ± 0.317	86.88 ± 1.62	8.25 ± 0.62	0.63 ± 0.13
	Honeywood 3 WC	1.762 ± 0.906	82.69 ± 0.30	10.21 ± 0.62	0.72 ± 0.10
	Martin 4 WC	2.428 ± 0.337	91.16 ± 2.84	14.32 ± 0.93	1.95 ± 0.31
	Wild type M5	12.220 ± 1.101	97.81 ± 0.37	42.89 ± 6.39	1.34 ± 0.11
	Wild type FB2	12.379 ± 0.277	97.91 ± 0.40	44.85 ± 3.81	0.79 ± 0.12
	Wild type NT3	10.984 ± 1.231	97.54 ± 0.57	34.63 ± 1.33	1.35 ± 0.16
2014	WS 1A	4.546 ± 0.156	92.95 ± 1.60	20.71 ± 0.33	1.23 ± 0.07
	WS 2B	2.745 ± 0.248	69.34 ± 1.69	6.97 ± 0.58	0.79 ± 0.14
	WC 1A	5.347 ± 0.773	98.60 ± 1.12	20.51 ± 1.86	1.32 ± 0.22
	WC 2B	6.234 ± 0.209	97.28 ± 0.79	19.62 ± 0.76	1.42 ± 0.32
	KH 1	2.046 ± 0.227	50.08 ± 0.77	1.90 ± 0.11	0.12 ± 0.03
	WC 3A	5.530 ± 0.225	96.95 ± 0.98	18.64 ± 0.36	1.19 ± 0.07

Table 2. Integration of samples that showed significant cytoprotection together with the respective TPC, antioxidant activity, ABTS radical inhibition and orthophenolic content.

Name	Cytoprotection At 100 ug juneberry concentration (%)	Total Phenolic content (mg/g)	DPPH Antioxidant Activity (%)	ABTS radical Inhibition (%)	Orthophenolic content (mg/g)
Velva Martin	48	7.813 ± 0.309	87.80 ± 2.42	8.86 ± 0.87	1.08 ± 0.13
Wild type KH4	46	19.311 ± 1.651	93.09 ± 1.05	58.82 ± 0.48	2.51 ± 0.31
Wild type NT3	42	10.984 ± 1.232	97.54 ± 0.57	34.63 ± 1.33	1.35 ± 0.16
WC 3A	55	5.530 ± 0.225	96.95 ± 0.98	18.64 ± 0.36	1.19 ± 0.07
WC 2B	56	6.234 ± 0.209	97.28 ± 0.79	19.62 ± 0.76	1.42 ± 0.32

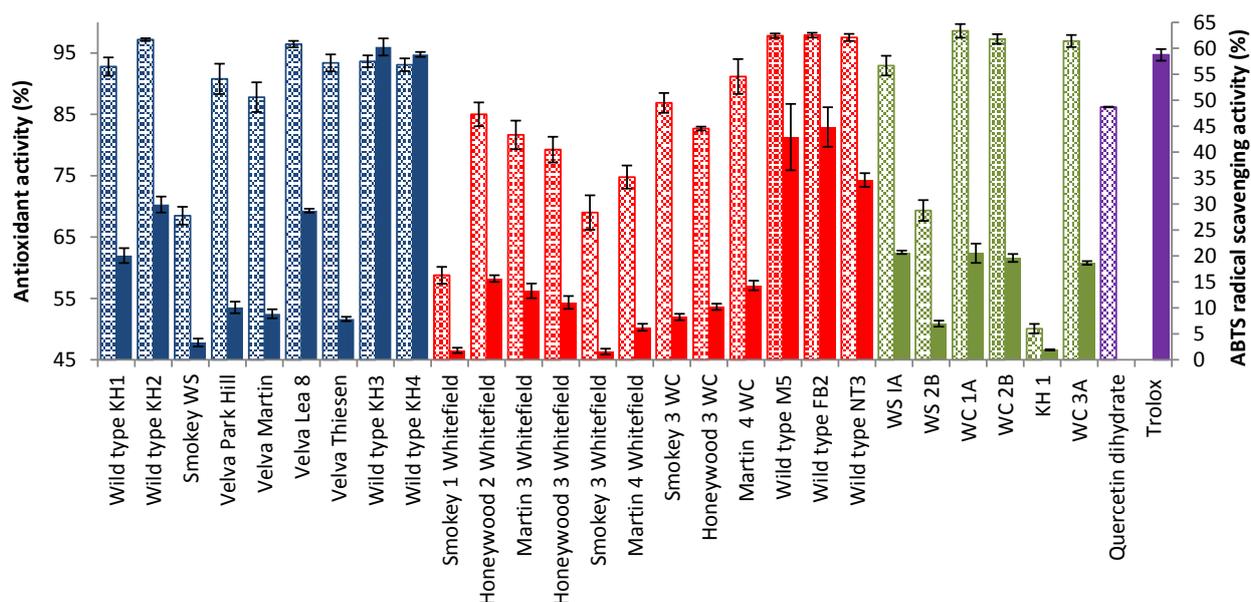


Fig. (2). Representative data showing DPPH radical scavenging antioxidant activity (pattern) and ABTS radical inhibition percentage (solid) by ethanol extracts of juneberries in 2012(blue), 2013(red) and 2014(green). Data shown are means \pm standard deviation ($n=5$ experiments). For comparison, positive controls (purple) quercetin dihydrate (pattern) for the antioxidant activity assay and Trolox (solid) for the ABTS radical inhibition assay are displayed in this plot.

Total Phenolic Contents

Total phenolic content (tannic acid equivalents, TAE) in juneberries (Fig. 1) ranged from 0.2261 ± 0.0031 to 19.311 ± 1.651 mg/g of frozen weight (FW). Data are in accordance with previous studies of Saskatoon berries where TPC was found to be 2.52 to 3.82 g gallic acid Eq·kg⁻¹ of FW [12]. Total phenolic content determined in this study is higher than the reported values in Rop *et al.* However, it must be noted that we used tannic acid as our standard for the assay. Using a similar expression, Fredes, Carolina, *et al.* (2014) observed a TPC, 14.6 g GE kg⁻¹ FW of maqui berry, 3.9 g GE kg⁻¹ FW of pomegranate, and 3.0 g GE kg⁻¹ FW of raspberry [13]. It is clear that TPC determined in juneberries are higher than in most other fruits and therefore is a great source of dietary antioxidants. Significant difference can be seen in TPC between juneberries of the same year and different years' crops. Phenolic content in fruits has been shown to be influenced by cultivar, abiotic stresses such as harvest year, water availability in soil and temperatures [14, 15]. Wild type (juneberries grown in the wild) such as KH2, KH3, KH4 of 2012 and M5, NT3 and FB2 of 2013 showed higher total phenolic content than domestic cultivars, with Velva Park Hill and Velva martin being the exceptions. Which implies that the natural growth conditions are better suited for encouraging production of secondary metabolites beneficial for human health. Overall, juneberries of 2012 showed much higher TPC than other years, environmental factors are however unknown (Figs. S9-S11).

Antioxidant Activity

DPPH assay is the most renowned and widely used assay as there is no other simpler or cheaper antioxidant assay in use. This method of stable DPPH free radical scavenging is used to assess the antioxidant activity of compounds or extracts in a less time consuming manner [16]. Antioxidant activity of juneberries (Table 1) (Fig. 2) ranged from 50.08 ± 0.77 to $98.60 \pm 1.12\%$ overall. Flavonoids are strong antioxidants against free radicals, as they are efficient "radical-scavengers". This scavenging activity is due to the phenol functional group that acts as a source of hydrogen atoms, such that when radicals are produced, electrons can be delocalize over the flavonoid structure [17]. From Fig. (2) demonstrates that majority of the juneberry samples showed a higher antioxidant activity than the reference compound quercetin dihydrate ($86.20 \pm 0.05\%$). A similar study by Poina *et al.*, investigated the antioxidant activity of cherry, strawberry, bilberry, red gooseberry, raspberry and blackberry and discovered that bilberry showed the highest antioxidant activity of 40.60% [18]. Juneberries from ND cultivars show higher scavenging activity than bilberry extracts used in the study. Highest antioxidant activity reported in the study of 2012, KH1, KH2, Velva Park Hill, Velva Lea 8, Velva Thiesen, KH3, KH4,; 2013, M5, FB2, NT3 and 2014; WC1A, WC2B, WC3A. Juxtaposing total phenols

and antioxidant activity results from our studies, a significant correlation can be seen Figs. (S12-S14). This implies that the antioxidant activity seen is possibly due to the high levels of phenolic content in juneberries.

Orthophenolic Content Previous work conducted by Torres *et al* shows that orthophenolic compounds are better antioxidants in comparison to other phenol constituents [19]. Additionally, a study conducted on antitumor activity showed a positive correlation with the orthophenolic content in wheat samples given to Min mice [20]. As a result we investigated the orthophenolic content in juneberries to test for any correlation with the other assays. Orthophenolic content was recorded in Caffeic Acid Equivalents (CAE), mg.g⁻¹ of FW. Orthophenolic content in juneberries ranged from 0.12 ± 0.03 to 2.38 ± 0.15 mg.g⁻¹ of FW (Fig. 1). Overall highest orthophenolic content was shown in 2012 juneberries; KH2, KH3, and KH4. 2013 juneberry crops show a moderate level of orthophenolic content with the highest amount in Martin 4 WC and lesser amounts in M5 and NT3. In 2014, WC1A, WC2B, and WC3A cultivars showed the highest orthophenolic content. Comparing results across the three years, a decline of orthophenolic content in juneberries can be observed. A notable correlation also exists between CAE levels and antioxidant activity in this study (Figs. S15-S17).

ABTS Radical Cation Decolorization Assay

The ABTS radical cation decolorization assay present the free radical scavenging response to ABTS of Juneberry extracts (Tables 1 and 2). Highest ABTS inhibition by juneberries of 2012 was seen by KH3 and KH4, while M5, FB2 and NT3 had the highest inhibition in 2013. 2014 Juneberry samples showed much lower ABTS inhibition percentages with the highest by WS 1A and WC 1A. In comparison to the positive control, Trolox which displayed an inhibition of 94.03 ± 0.21%, juneberries from wild type KH3 displayed an inhibition of 60.25 ± 1.65%. ABTS inhibition data correlates closely with finding of TPC, antioxidant activity, ABTS radical inhibition, and orthophenolic content. This suggests that phenolic content of juneberries plays an important role in inhibitory activity and in overall antioxidant activity. Many other studies as well assert that polyphenols highly correlate with the antioxidant activity in fruits [21, 22].

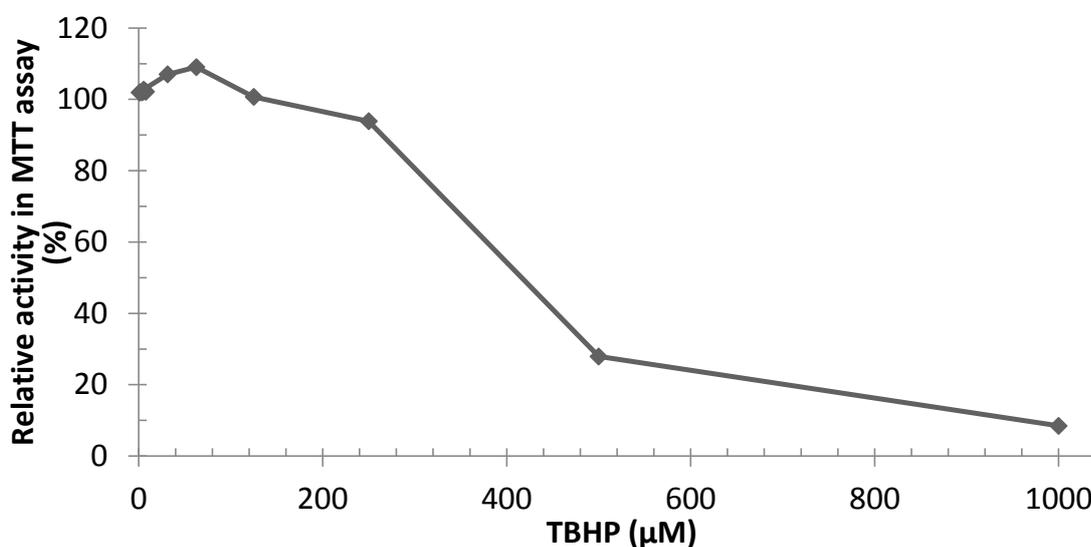


Fig. (3). Cytotoxicity of TBHP in HepG2 cells.

Cell-based Antioxidant Assay

Cytotoxicity assay was carried out on TBHP concentrations ranging from 1000 uM to 0.9 uM on HepG2 cells. While 400 uM showed 50% cell survival this was not sufficient of a concentration to kill cells in the cytoprotection assay. Therefore, a concentration of 500 uM was used, as shown in Fig. (3) this produced a 38% cell survival. The pretreatment of hepG2 cells with 100 µg/µL of 2014 sample WC 2B produced a 56% viability. Quercetin dihydrate control showed 90% cytoprotection under the same treatment. Five samples with the highest cytoprotection activity is shown in Table 2. along with its data for total phenol, antioxidant activity, ABTS radical scavenging activity and

orthophenolic content. Cell-based antioxidant activity showed very high correlation with DPPH radical scavenging activity. A similar study conducted on HeLa cancer cells using various berry extracts show a wide range of cytoprotection of 20% - 98% [23]. Therefore, our findings on the cytoprotection by juneberry extracts in HepG2 cells are well within this range.

CONCLUSION

Wild type KH4 and KH3 of 2012 juneberry crop show higher phenolic content, orthophenolic content, DPPH radical scavenging, ABTS radical inhibition, and few of the highest cell based cytoprotection. Our data suggest that juneberries grown in the wild as opposed to those grown in cultivars are richer in bioactive secondary metabolites. This idea is in accordance with similar studies conducted on various other berries reporting a higher levels of bioactive phytochemicals and total phenols in organically bred fruits than their commercially grown counterparts [15, 24]. Berries grown in the wild have a myriad of positive health benefits and are ideal to study secondary phytochemicals released in response to natural chemical and physical stresses [25]. However, greater knowledge in the environmental conditions, soil quality, and seasonal variations would be beneficial to produce juneberries with higher nutritional value. Additionally our results show that total phenolic content and orthophenolic content in juneberries from 2012 were higher than in juneberries obtained from the 2013 and 2014. Food nutritionists investigating fruits and vegetables grown today and those grown seven decades back have shown a lower amount of calcium, iron, vitamin C and phytochemicals have been quantified in current domesticated crops [25]. Therefore, even among wild juneberries it is possible that the nutrient content may decline over time corresponding to the changes in the environment. The high phytochemical content and bioactivity shown by *A. alnifolia* in this study indicates that juneberries have great therapeutic benefit and is also an excellent source of dietary antioxidants. However, due to the variations in phenolic content in different cultivars and breeding practices, the sources of the juneberries should be carefully assessed when recommended for their positive health benefits.

LIST OF ABBREVIATIONS

ABTS	=	2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DMEM	=	Dulbecco's modified eagle medium
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
FBS	=	Foetal bovine serum
FW	=	Frozen weight
MTT	=	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
ROS	=	Reactive oxygen species
RNS	=	Reactive nitrogen species
TAE	=	Tannic acid equivalents
TBHP	=	Tertiary-Butyl hydroperoxide
TPC	=	Total phenolic content
Trolox	=	6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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