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Antioxidant profiling of indigenous oat cultivars with special reference to avenanthramides

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Abstract

Oat (*Avena sativa*) of the South Asian region, particularly Pakistan, remains neglected for decades. Oat is unique in terms of the presence of exceptional polyphenolic compounds known as avenanthramides (AVAs). AVAs have therapeutic effects and behave as antioxidative, anti-proliferative, and vasodilatory agents. The present work was designed to explore the nutritional and antioxidant aspects of oat, especially of their phenolic content, including AVAs. Three cultivars of oat (S-2000, S-2011, and L-632) were examined for their proximate composition and mineral profile. Conventional solvents (acetone, ethanol, and methanol) and supercritical carbon dioxide (SC-CO_2) were used for polyphenol extraction. Extracts were spectrophotometrically analysed for their phytochemical profile and antioxidant activity. Vital avenanthramides (AVA-A, AVA-B, and AVA-C) were quantified through HPLC/UV-Vis detector. The data reported highest total phenolic content (222.72 mg GAE/100 g) and total flavonoid content (137.13 mg QE/100 g) in S-2011 with maximum antioxidant activity (1,1-diphenyl-2-picrylhydrazyl IC_{50} = 12.38 mg/ml and ferric reducing antioxidant power = 39.98 µmol/g). This highest antioxidant potential was examined in supercritical extract while methanol evaluated the best among conventional solvents. Best extractions were further analysed with HPLC for the quantification of AVAs. SC-CO_2 extraction recorded maximum concentrations of AVA-A (137.84 µg/g), AVA-B (105.10 µg/g), and AVA-C (119.86 µg/g) in S-2011 cultivar. In conclusion, oat is the rich source of antioxidants with the presence of exceptional AVAs.

Introduction

Oat in Pakistan is planted both in spring and winter seasons on irrigated as well as rain-fed areas (Ahmad *et al.,* 2014; Shah *et al.,* 2016). Oat and its products are beneficial for vital organs of the body and as a source of energy. Oat also has long been considered as a cure for different ailments, and recent research proved its role even against poison ivy, sunburn, eczema, and psoriasis (Meydani, 2009). Oat is sufficient in essential nutrients with appreciable content of soluble fibre and phytochemicals. Oat is also palatable, succulent, and nutritious crop rich in protein and carbohydrate (Chauhan *et al.,* 2018). In oat composition, the major constituents are 65 - 85% starch, 15 - 20% protein (including enzyme), 3 - 11% lipids, and 5% fibre including beta-glucan (Sur *et al.,* 2008). The worth of oat is unique among cereals because of its exceptional health benefits with an ample amount of natural antioxidants, such as phenolic acids and their derivatives such as alk(en)ylresorcinols and tocopherols (Mir *et al.,* 2018).

Oat also uniquely possesses avenalumic acids (ethylenic homologues of cinnamic acids) and avenanthramides (AVAs) (N cinnamoylanthranilate alkaloids), as compared to the other cereals (Ahmad *et al.,* 2014). AVA group consists of anthranilic acid and hydroxycinnamic acid derivatives. AVA-A, AVA-B, and AVA-C are the three most abundant AVAs in oat which constitute hydroxyanthranilic acid with *p*-coumaric acid, ferulic acid, caffeic acid as hydroxycinnamic acid, respectively. These three AVAs have concentration of more than 300 ppm with antioxidant activity of 10 - 30 times greater than other widely occurring phenolic acids (Boz, 2015; Ninfali *et al.,* 2019).

Presently, oat is utilised in different industries to make valuable products, as a source of high-value bioactive compounds (Orozco-Mena *et al.,* 2014). Important properties of oat i.e. antioxidant, anti-inflammatory, and anti-atherogenic, are the basic roles of vital AVA compound of oat. These antioxidants increase the production of nitric oxide and decrease blood pressure by dilating blood vessels. They also increase the activity of superoxide dismutase (SOD) in the heart, liver, kidney, and skeletal muscles, and lessen the

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production of reactive oxygen species (ROS) (Guo *et al.,* 2008; Boz, 2015). The intake of avenanthramide-enriched mixture proved its curative and/or prophylactic effect on the oxidation of low density lipoproteins in humans and hamsters (Chen *et al.,* 2007). Molecular evidence of the colloidal extracts of oat AVAs has been reported to be effective against histamine and exhibited anti-irritation and anti-itching properties (Meydani, 2009; Pridal *et al.,* 2018). Likewise, AVAs present in oats and oatmeal proved their effect against allergic and respiratory diseases (Orozco-Mena *et al.,* 2014; Chen *et al.,* 2018). Phenolic compounds from oat have been extracted with different solvents like methanol, ethanol, or acetone (Verardo *et al.,* 2011). Variation of solvents used for extraction has different effects on particular food and its constituents, and gives different results.

Physiochemical properties and phenolic compounds of oat have already been quantified. However, no comparison or detailed facts has been published on Pakistani oat cultivars. Solvents were also required to be compared for their extraction efficiency. In view of the studies about numerous health benefits of oat polyphenols, the present work was designed to explore the regional oat cultivars for the first time in terms of their antioxidant capacity. The present work also compared the conventionally used solvents (acetone, ethanol, and methanol) and supercritical CO_2 extractions. All of the oat extracts were analysed for their total phenolic content (TPC), total flavonoid content (TFC), free radical scavenging activity through 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) to select the best extracts for AVAs' quantification through HPLC analysis.

Materials and methods

Materials

Oat cultivars S-2000 (C1), S-2011 (C2), and L-632 (C3) were procured from Fodder Research Institute, Sargodha, Pakistan. Standards of minerals, Folin-Ciocalteu reagent, DPPH, TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), AVA-A, AVA-B, and AVA-C were purchased from Sigma-Aldrich Co. (Missouri, USA). All chemicals and reagents used in the present work were of analytical grade.

Methods

Sample preparation

Oat grains were cleaned to remove foreign materials, and hull from the grains were removed with impact disc-based oat dehuller (Model 15D impact huller, Forsbergs, Inc. Minnesota, USA). Dehulled grains were further processed to flour using a laboratory mill (Perten laboratory mill 3310, PerkinElmer, Hagersten, USA).

Proximate analysis

Flour samples were analysed for moisture content, ash content, crude protein, crude fat, and crude fibre content following the approved methods of AACC, methods no. 44-15A, 08-01, 46-10, 30-10, and 32-10, respectively (AACC, 2000).

Mineral profile

Minerals including magnesium (Mg), calcium (Ca), iron (Fe), zinc (Zn), and copper (Cu) were analysed through Atomic Absorption Spectrometer (AAS) (AA240, Varian), while potassium (K) and sodium (Na) were determined through Flame Photometer (Sherwood Scientific Ltd., Cambridge, Model 410). Prior to analyses, flour sample (1 g) was ignited, followed by digestion on the hot plate with nitric acid and perchloric acid (7:3), following method no. 965.17 - 968.08 of AOAC (2006). Digested sample was diluted with deionised water and filtered by using Whatman filter paper No. 1 for further analysis at AAS and Flame Photometer.

Extraction of polyphenols

Polyphenols were extracted from the oat flour with conventional solvents and supercritical carbon dioxide.

Polyphenol extraction with conventional solvents

Acetone/water, ethanol/water, and methanol/water solvent extraction of oat flour was carried out following the protocol of Verardo *et al.* (2011) with some modifications. Sample-solvent mixture was shaken for 30 min at 300 rpm before sonication and then centrifugation was performed at 1,500 *g* for 15 min.

Supercritical fluid extraction of polyphenols

Supercritical Fluid Extraction (SFE) of polyphenol was performed with equipment model no. SFT-150 system (SuperCritical Technologies, WA, USA) as described by Escobedo-Flores *et al.* (2018) with some modifications. 30 g flour was filled in a 100 mL extractor with co-solvent (ethanol). The extraction was carried out at 35°C and 30 MPa. The extracted polyphenols were recovered after complete removal of CO_2 from the extractor.

Phytochemical analysis

Total phenolic content (TPC)

The TPC was determined with Folin-Ciocalteu reagent following the method of Verardo *et al.*

(2011). The absorbance was read on UV-Vis Spectrophotometer at 750 nm. The calibration curve was plotted with gallic acid to assess the TPC in extractions of oat cultivars.

Total flavonoid content (TFC)

The TFC was determined following the method proposed by Žilić *et al.* (2011). The absorbance was measured at 510 nm to assess the TFC against the standard curve of quercetin prepared in different concentrations (0 - 1000 µg/g).

In vitro antioxidant activity

DPPH assay

Antioxidant activity through 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay was performed following the method of Tong *et al.* (2014). The results were reported using half-maximal inhibitory concentration (IC_{50}) value.

FRAP assay

FRAP (Ferric Reducing Antioxidant Power) assay was performed following Cheng and Bhat (2015). The results were expressed as µmol TE (Trolox equivalent)/g of antioxidant capacity.

Quantification of phenolics through HPLC

Based on phytochemical potential and antioxidant activity, extractions of best solvents were examined through HPLC for the quantification of AVAs. RP-HPLC system (Perkin Elmer, 200 series, CT, USA) was installed with UV-VIS detector and C_{18} column (PerkinElmer, Spheri-5, 220 mm × 4.6 mm, particle size 5 µm).

Quantification of avenanthramides (AVAs)

The quantification of the three important AVAs; AVA-A, AVA-B, and AVA-C were carried out by HPLC following the method of Tong *et al.* (2014) with some modifications. The mobile phase consisted of 2% acetic acid in HPLC grade water v/v (solvent A) and acetonitrile (solvent B). The flow rate was kept at 1.0 mL/min with injection volume of 20 µL for a total run time of 40 min. The gradient program for AVA quantitation started with 0.5 min pre-run, with 95% solvent A followed by its decrease to 87% (8 min), 80% (8 min), 73% (7 min), and 65% (7 min) that again increased to 75% (2 min), 85% (2 min), and 95% for 6 min.

Statistical analysis

All analyses was performed in triplicate experiment independently. The experimental data were reported as means ± standard deviation. Data were analysed using Analysis of Variance (ANOVA) under Completely Randomized Design (CRD) with two factorial. The ANOVA was determined using Tukey's significant difference test (HSD) at $\alpha = 0.05$ (Steel *et al.,* 1997).

Results

Proximate composition

The proximate composition of oat cultivars is presented in Table 1. All parameters varied significantly among the cultivars except NFE that showed non-significant behaviour. S-2011 result was found the highest in crude protein and crude fibre content.

Mineral profile

Seven mineral elements were analysed in the three oat cultivars. Mean contents of respective minerals found in each cultivar expressed in mg/100 g of oat grain flour are shown in Table 1. Highly significant differences ($p < 0.01$) have been noticed in all minerals except in sodium and zinc that differed at $p < 0.05$ between the cultivars.

Total phenolic content (TPC)

The TPC measured through the Folin-Ciocalteu method was expressed as mg gallic acid equivalent per 100 gram (mg GAE/100 g). The results (Table 2) showed that cultivars, extracts, and their interaction exhibited highly significant difference from each other ($p < 0.01$). The value for TPC was found the lowest in oat acetone extract of L-632 (117.32 mg GAE/100 g) while the highest was recorded in oat supercritical extract of S-2011 (222.72 mg GAE/100 g).

Total flavonoid content (TFC)

The TFC of oat cultivars with different extracts was measured and revealed as highly significant between cultivars, extracts, and interaction on TFC. The effect of different extracts on TFC of oat cultivars is shown in Table 2. The TFC values in all treatments ranged from 56.77 mg QE/100 g in acetone extract of L-632 to 137.13 mg QE/100 g in the supercritical extract of S-2011.

DPPH assay

The results in Figure 1 show that L-632 exhibited significantly the lowest free radical scavenging activity (IC_{50} = 20.74 mg/mL) with acetone extract, while maximum (IC_{50} = 12.38 mg/mL) was observed in S-2011 with supercritical oat extract.

FRAP assay

Highly significant differences ($p < 0.01$) were

Table 1. Proximate composition and mineral profile of oat.

Property	Parameter	Oat cultivar		
		S-2000	S-2011	L-632
Proximate analysis (%)	Moisture	8.73 ± 0.22^{a}	7.89 ± 0.31^{b}	8.05 ± 0.18^{b}
	Ash	3.17 ± 0.10^{b}	3.23 ± 0.14^{b}	3.71 ± 0.11^{a}
	Crude protein	13.43 ± 0.16a^{b}	13.69 ± 0.12^{a}	13.08 ± 0.19^{b}
	Crude fat	8.31 ± 0.08^{a}	7.73 ± 0.11^{c}	8.04 ± 0.09^{b}
	Crude fibre	2.64 ± 0.06^{b}	2.83 ± 0.09^{a}	2.70 ± 0.05^{ab}
	NFE	63.72 ± 0.41	64.62 ± 0.69	64.41 ± 0.24
Mineral profile (mg/100 g)	K	289.63 ± 5.50^{b}	315.32 ± 7.25^{a}	251.36 ± 3.77^{c}
	Mg	138.11 ± 4.56^{b}	129.26 ± 3.75^{b}	171.36 ± 3.26^{a}
	Ca	43.23 ± 1.77^{c}	51.31 ± 3.03^{b}	69.41 ± 2.71^{a}
	Na	8.32 ± 0.28^{b}	8.84 ± 0.24^{ab}	9.13 ± 0.30^{a}
	Zn	3.25 ± 0.13^{ab}	3.39 ± 0.16^{a}	2.89 ± 0.14^{b}
	Fe	4.06 ± 0.15^{a}	3.26 ± 0.09^{b}	2.99 ± 0.06^{c}
	Cu	0.41 ± 0.02^{b}	0.45 ± 0.03^{b}	0.53 ± 0.05^{a}

Data are means ± standard deviation.

found between cultivars, extracts, and their interactions in FRAP assay (Figure 1). Acetone extract depicted the lowest antioxidant activity while supercritical extracts showed the highest FRAP activity for each cultivar among different extracts (Figure 2). However, the reducing power ranged from 30.23 µmol/g (L-632) to 39.98 µmol/g (S-2011).

Avenanthramide content

Based on phytochemical potential (TPC and TFC) and antioxidant activity (DPPH and FRAP), oat supercritical extract and oat methanol extract were

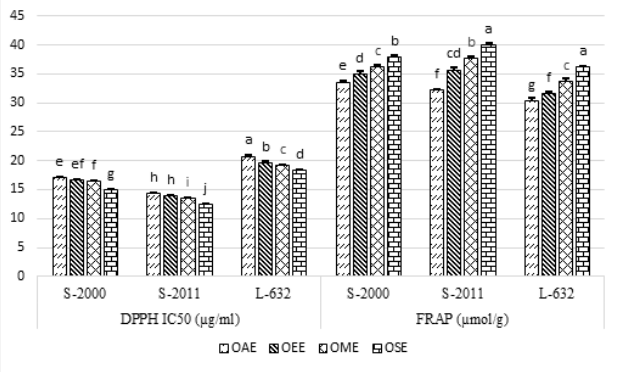

Figure 1. DPPH and FRAP of oat cultivars with different extracts. OAE = oat acetone extract; OEE = oat ethanol extract; OME = oat methanol extract; and OSE = oat supercritical extract.

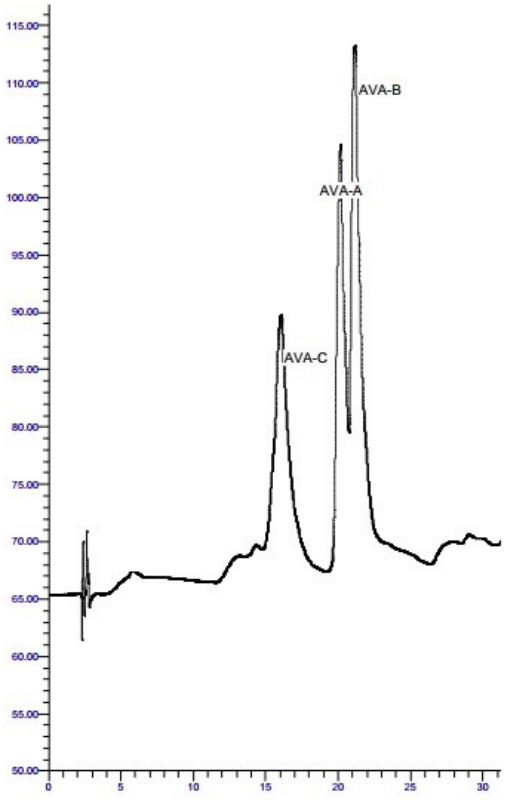

Figure 2. HPLC chromatogram of avenanthramide standards (*x*-axis = minutes, *y*-axis = mAU). Retention times: AVA-A = 15.50 min, AVA-B = 19.63 min, and AVA-C = 20.63 min.

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), and avenanthramides (AVAs) of oat with different extracts.

Analysis	Extract	Oat cultivar			Mean
		S-2000	S-2011	L-632	
TPC (mg GAE/100 g)	OAE	121.53 ± 0.36^{j}	133.63 ± 0.26^{i}	117.32 ± 0.27^{k}	124.16 ± 8.47^{d}
	OEE	165.47 ± 0.29^{f}	171.06 ± 0.81^{e}	149.47 ± 0.32^{h}	162.01 ± 11.20^{c}
	OME	183.71 ± 0.29^{d}	191.63 ± 0.29d^{c}	160.23 ± 0.37^{g}	178.52 ± 16.33^{b}
	OSE	203.69 ± 0.73^{b}	222.72 ± 0.45^{a}	184.49 ± 0.51^{d}	203.63 ± 19.11^{a}
	Mean	168.60 ± 35.05^{b}	179.76 ± 37.37^{a}	152.88 ± 27.87^{c}	
TFC (mg QE/100 g)	OAE	91.96 ± 0.84^{h}	116.11 ± 0.92^{d}	56.77 ± 0.69^{l}	88.28 ± 29.84^{d}
	OEE	98.03 ± 0.67^{g}	120.74 ± 0.46^{c}	65.07 ± 0.62^{k}	94.61 ± 27.99^{c}
	OME	103.12 ± 0.35^{f}	128.04 ± 0.35^{b}	70.80 ± 0.44^{j}	100.65 ± 28.69^{b}
	OSE	110.66 ± 0.31^{e}	137.13 ± 1.22^{a}	81.25 ± 0.72^{i}	109.68 ± 27.95^{a}
	Mean	100.94 ± 7.92^{b}	125.51 ± 9.17^{a}	68.47 ± 10.28^{c}	
AVA-A (µg/g)	OME	93.51 ± 0.27^{d}	132.47 ± 0.15^{b}	62.73 ± 0.44^{f}	96.24 ± 34.95^{b}
	OSE	99.19 ± 0.13^{c}	137.84 ± 0.39^{a}	66.96 ± 0.09^{e}	101.33 ± 35.49^{a}
	Mean	96.35 ± 4.02^{b}	135.16 ± 3.79^{a}	64.84 ± 2.99^{c}	
AVA-B (µg/g)	OME	78.70 ± 0.25^{d}	99.33 ± 0.18^{b}	37.84 ± 0.09^{e}	71.96 ± 31.29^{b}
	OSE	86.19 ± 0.11^{c}	105.10 ± 0.09^{a}	42.43 ± 0.11^{f}	77.91 ± 32.14^{a}
	Mean	82.45 ± 5.29^{b}	102.22 ± 4.08^{a}	40.14 ± 3.24^{c}	
AVA-C (µg/g)	OME	66.71 ± 0.10^{f}	112.96 ± 0.89^{b}	91.58 ± 0.68^{d}	90.42 ± 23.14^{b}
	OSE	75.21 ± 0.22^{e}	119.86 ± 0.13^{a}	99.21 ± 0.12^{c}	98.09 ± 22.36^{a}
	Mean	70.96 ± 6.01^{c}	116.42 ± 4.88^{a}	95.39 ± 5.39^{b}	

Data are means ± standard deviation. OAE = oat acetone extract; OEE = oat ethanol extract; OME = oat methanol extract; and OSE = oat supercritical extract.

selected for HPLC analyses. AVAs (AVA-A, AVA-B, and AVA-C) were quantified through HPLC.

The HPLC chromatogram for AVAs' standard is presented in Figure 2 with retention times at which AVA-A, AVA-B, and AVA-C were examined in oat cultivars. The values in Table 2 showed a highly significant effect ($p < 0.01$) between cultivar, extract, and interaction on AVAs profile. The extractions with SC-CO_2 showed in Figure 3(a), 3(b), and 3(c) yielded significantly highest AVAs in oat. The values of AVA-A, AVA-B, and AVA-C were found maximum in S-2011 (137.84, 102.22, and 116.42 µg/g, respectively). The lowest content of AVA-A and AVA-B were recorded in L-632 (66.96 and 42.43 µg/g, respectively) while AVA-C was found the lowest in S-2000 (75.21 µg/g).

Discussion

Oat grains with lower moisture helps in the process of dehulling but it also lowered the moisture content of flour. Chauhan *et al.* (2018) reported higher moisture content of 10.52%, while reported crude protein content of 10.32 - 15.45%, which are in line with the values obtained in the present work. The results for ash content, crude protein content, and NFE were comparable to the values reported by Marmouzi *et al.* (2016) of 2.69 - 3.62, 11.27 - 17.18, and 56.63 - 64.98%, respectively. Crude fibre examined by Biel *et al.* (2014) in oat flour ranged from 2.3 to 3.6%. It is well known that these nutritional parameters might be influenced by environmental conditions, maturity at harvest, time of harvest, and postharvest storage conditions (Martinez *et al.,* 2011).

Mineral profile analysed by Youssef *et al.* (2016) reported that 350 - 362, 112.25 - 120.67, 54.70 - 71.71, 5.35 - 7.03, 3.44 - 3.62, 1.37 - 2.41, and 1.20 - 1.33 mg/100 g of K, Mg, Ca, Na, Zn, Fe, and Cu were found in oat flour, which are in line with those obtained in the present work. The values reported by

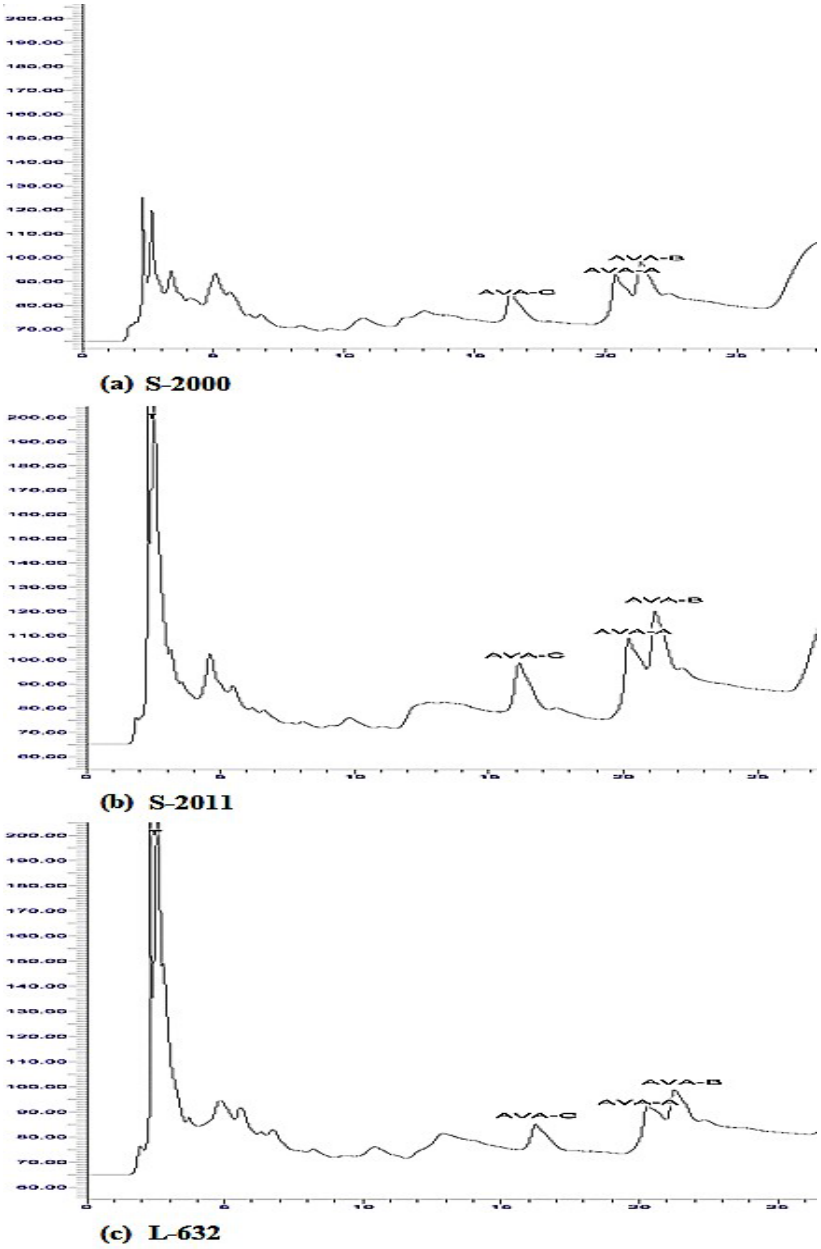

Figure 3. HPLC chromatograms of avenanthramides in oat cultivars (*x*-axis = minutes, *y*-axis = mAU).

Marmouzi *et al.* (2016) for K (214.6 - 395.6 mg/100 g) and Ca (42.1 - 86.0 mg/100 g), and examined by Li *et al.* (2014) for Zn (1.87 - 3.05 mg/100 g) and Fe (2.76 - 6.65 mg/100 g) were also comparable to those obtained in the present work. Variation in mineral content of oat flour might be due to different regions and areas used for cultivation of crops (Soetan *et al.,* 2010). Deviation of factors like soil composition and level of fertilisation might also have a direct effect on the mineral composition of the plant.

The present work is based on the evidence that polyphenols are the most abundant antioxidants in oat grains and their products (Gorinstein *et al.,* 2008). Presumably, SC-CO2 depicted higher polyphenolic extraction and antioxidant activity than conventional solvents, and these results were due to its maximum extraction capability (Junior *et al.,* 2010). Among conventional solvents, methanol behaved best and depicted higher total phenolic content and antioxidant activity in its extract (Abbas Ali *et al.,* 2019). The difference in extraction concentration of conventional solvents is due to varied extraction capability in the structure of solvents and their compatibility with phenolic and flavonoid compounds. Higher total phenolic content leads to higher antioxidant efficiency of particular flour in specific extract (Verardo *et al.,* 2011). Gujral *et al.* (2013) reported that the TPC of oat flour was 202.3 mg FAE/100 g. Gujral *et al.* (2013) and Sandhu *et al.* (2017) reported flavonoid concentrations lower than those obtained in the present work.

The applied assays for antioxidant activity (DPPH, FRAP) of oat extracts were based on electron transfer reaction mechanism. DPPH measures the free radical scavenging potential in the food sample. Antioxidant activity examined by either FRAP or DPPH, depends upon the phenolic bioavailability. When phenolics are extracted in high proportion with any method or solvent, the antioxidant activity results of respective media would also be high (Ahmad *et al.,* 2019). The values for DPPH assay in different oat cultivars were lower than the findings reported by Kim *et al.* (2013) who found IC_{50} value of 5.87 mg/mL in oat flour. FRAP is the ferric reducing assay for antioxidant power determination. Different extractions affected the FRAP results, as higher values were observed for supercritical oat extract. Results of the present work were comparable with the earlier finding of Hodzic *et al.* (2009) who examined the influence of phenolic content on antioxidant activity in whole grains, and the ferric reduction calculated in oat cultivars were 28.2 - 33.8 µmol/g.

The study conducted by Tong *et al.* (2014) on Chinese oat cultivars which reported AVA-A (13.7 - 324.8 µg/g), AVA-B (18.6 - 392.5 µg/g) and AVA-C (2.0 - 119.6 µg/g) supported the findings of the present work. Li *et al.* (2017) reported AVA-A (6.1 - 112.3 µg/g), AVA-B (7.3 - 222.8 µg/g), and AVA-C (6.1 - 136.2 µg/g), which are also comparable to those obtained in the present work. Different areas used for cultivation of oat effectively impacted the content of phenolic compounds in their flour due to variation in all factors interacting with them from their sowing to their cultivation (Tong *et al.,* 2014). Ahmad *et al.* (2019) concluded that the variety and cultivar difference, in addition to the technique used for their extraction and quantification, might also affect the level of these compounds. For the same region, variation among variety could be due to the genetic makeup and diversity in their capability to synthesise a specific compound.

Conclusion

For the first time, Pakistani oat cultivars were characterised for their AVAs content by exploring their antioxidant potential. The results of the present work concluded that the S-2011 cultivar yielded the highest antioxidant activity as compared to the other tested oat cultivars. Supercritical carbon dioxide performed as the most effective medium for the extraction of antioxidant compounds, followed by methanol among conventional solvent extractions. S-2011 yielded the highest total phenolic content, total flavonoid content, and antioxidant activities (DPPH and FRAP) in SC-CO_2 extract. The application of HPLC/UV-Vis for AVA analysis depicted significant differences among oat cultivars, extracts, and their interaction, thus concluding that S-2011 is a rich AVA source.

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