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Phenolic acid profile of oat cultivars, and their suppressive effect on intracellular reactive oxygen species

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Abstract

Oat (*Avena sativa* L.) has rich phenolic contents with nutritional and therapeutic health benefits. The objective of the present work was to perform a cell cytotoxicity assay of three South Asian oat cultivars to determine their suppressive effect on intracellular reactive oxygen species (ROS). Oat cultivars were firstly examined for total phenolic content, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, and phenolic acid profiling through HPLC. Total phenolic contents of oat cultivars ranged from 167.57 to 198.41 mg GAE/100 g, and DPPH scavenging activity was IC_{50} = 18.81 to 13.18 mg/mL. Oat cultivars displayed average content of phenolic acids such as vanillic acid (0.33 µg/g), syringic acid (6.70 µg/g), caffeic acid (4.16 µg/g), ferulic acid (2.06 µg/g), *p*-coumaric acid (20.22 µg/g), with the highest being gallic acid (74.32 µg/g). Cell cytotoxicity assay of oat polyphenolic extracts revealed that 10 and 15 µg/mL concentrations had non-significant differences when compared with their respective control treatments. Oat extracts suppressed ROS in Caco-2-cells, with or without hydrogen peroxide stimulation from 65.20 - 86.13%, and 56.36 - 79.56% with 10 and 15 µg/mL concentrations, respectively. In conclusion, oat is a rich source of polyphenols which have strong antioxidant behaviour to suppress the ROS activity.

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Introduction

Oxidative stress in the human body is the cause of destructive cellular effects linked with pathophysiology, thus leading to numerous health issues like atherosclerosis, cancers, aging, inflammations, cataracts, and many others (Shahidi and Zhong, 2015). Oxidative stress is well-known to participate in neurodegenerative diseases, as well as triggers and stimulates the development of various chronic disorders (Afify *et al.*, 2012). Defence systems (enzymatic and non-enzymatic) in organisms are developed to deal with oxidative stress. Several enzymes like catalase, superoxide dismutase, thioredoxin, and glutathione peroxidase are responsible for detoxifying reactive oxygen species (ROS) in the enzymatic system. In the non-enzymatic system, different antioxidants are present in the human body like bilirubin, glutathione, uric acid, melanin, α-tocopherol, coenzyme Q, lipoic acid, melatonin, and estrogenic sex hormones. Antioxidants in the human body play a pivotal role in delaying, controlling, or preventing the process of oxidation (Yilmaz-Ozden *et al.*, 2020).

Antioxidants are free radical scavengers, peroxide inactivators, singlet oxygen quenchers, secondary oxidation product quenchers, metal ion chelators, and ROS inhibitors (Shahidi and Zhong, 2015). One of the major groups of natural antioxidants is the phenolic compounds produced as

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secondary metabolite phytochemicals in dietary plants. They support the body and protect it from fatal diseases. These bioactive compounds are widely studied following the discovered potential positive effects of polyphenol-rich foods (Jakobek, 2015).

Polyphenols are generally divided into two broad classes; flavonoids and phenolic acids (Abbas *et al.*, 2017). In nature, a group of phenolic acids was found to be the most potent antioxidants, and among this group, each compound has a particular antioxidant and biological action (Yamuangmorn *et al.*, 2021). Antioxidant behaviour and stabilisation of phenolic acids are due to the hydroxyl substituents on the aromatic ring (phenol moiety); it affects their free radical quenching ability. Variation in antioxidant activity also depends on the source of diet, *i.e.*, whole grains, fruits, vegetables, spices, tea, and coffee (Niroula *et al.*, 2019).

Whole grains are acknowledged as a source of bioactive compounds including polyphenols and other antioxidants, which work synergistically to optimise human health (Calinoiu and Vodnar, 2018). Oats are a famous and commonly consumed grain, and research nowadays are focussed towards their impact on health and overall high nutrient content. Metabolic regulation is one of the important pathways, for which, the oat is an effective food. Dietary oats have been stated to prevent cancers, control coronary heart diseases, reduce the level of serum cholesterol, and improve the symptoms of obesity and eventually diabetes. These attributes of oats are due to the high dietary fibre and polyphenolic content (Tong *et al*., 2014).

Various phenolic acids present in oat play their role in combating syndromes and aiding in the overall antioxidant capacity of oat grains. The most important phenolic acids in oats are *p*-coumaric, vanillic, caffeic, ferulic, gallic, and hydroxybenzoic acids (Verardo *et al.*, 2011). Hamster-based research study proved that oat phenolics protect LDL during oxidation and interact synergistically in the presence of vitamin C (Chen *et al.*, 2004). Research on oat phenolics has shown that it possesses anti-hypertensive and anti-inflammatory activity (Chen *et al.*, 2018).

In addition to analysing antioxidant potential with the chemical method based on hydrogen atom transfer, in the present work, an intracellular antioxidant activity assay was also adopted to evaluate the oat polyphenolic potential. The analysis was developed to quantify the antioxidant potential of bioactive compounds from the selected source in the cultured cells. The popularity of assays based on cells is rising due to their excellent authentication (Wolfe and Liu, 2007). Although the Hep G2 cell line is used in many studies, the human Caco-2 cell line is said to be more representative as they have marked resemblance with epithelial cells of the small intestine (Kellett *et al.*, 2019). Oat cultivars of the selected region (South Asia, particularly Pakistan) have never been analysed for their cellular antioxidant potential. Therefore, the present work was focused on exploring the suppressive effect of indigenous oat cultivars on intracellular ROS, for increasing the worth of oat grains to be used as human food.

Materials and methods

Chemicals and materials

Chemicals

Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), gallic acid, *p*-coumaric acid, caffeic acid, vanillic acid, syringic acid, and ferulic acid were purchased from Sigma-Aldrich Co. (Missouri, USA). All other chemicals and reagents used were of analytical grade.

Oat samples

Three commercial oat cultivars namely S-2000, S-2011, and L-632, which originated from Pakistan, were grown at Fodder Research Institute Sargodha, Pakistan, and were procured from there for the present work. Oat grains were cleaned to separate foreign materials, and dehulling was performed with an 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, Perkin Elmer, Hagersten, USA).

Intestinal epithelial cells

The human colonic epithelial cell line (Caco-2 cells) was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultivated on Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 10% Foetal Bovine Serum (FBS) and 50 U/mL penicillin-streptomycin, and then maintained at 37°C in a humidified chamber of 5% CO_2 at the School of Food Science, Washington State University, Pullman, Washington, USA.

Extraction of phenolic compounds

Oat flour extraction was carried out with methanol as mentioned previously (Manzoor *et al*., 2020), separately for each cultivar, by following the protocol of Verardo *et al.* (2011) with some modifications. Briefly, 1 g of flour samples was extracted by adding 100 mL of solvent/water mixture in the 4/1 ratio. Sample mixtures were shaken for 30 min at 300 rpm and 30°C in an incubator shaker (KS 4000I i control IKA, Wilmington, US). After that, each sample was placed in an ultrasonic sonicator (USC0440D, Bioevopak Co., Ltd. WA, US) for 20 min at 30°C, followed by centrifugation in benchtop centrifuge (5804R Eppendorf International, MA USA) at 1,500 rcf for 15 min. Supernatants were collected separately, and extraction was repeated with leftover residue by following the same protocol. Supernatants from both extractions were mixed and stored well after nitrogen evaporation. The dried extract was diluted with measured distilled water, and used in further analysis and calculation.

Total phenolic content analysis

The total phenolic content (TPC) of flour extractions was estimated with Folin-Ciocalteu reagent following the method of Verardo *et al.* (2011). Briefly, polyphenol extract (100 µL), Folin-Ciocalteu reagent (500 µL), and distilled water (6 mL) were mixed in a test tube, and shaken for 1 min. Next, 15% (w/v) Na_2CO_3 (2 mL) was added, and the mixture was shaken again for 0.5 min. Finally, distilled water was added to make up the end volume (10 mL). The absorbance was taken after 2 h at 750 nm on a spectrophotometer (160 UV-VIS Thermo Fisher Scientific, MA, USA). The TPC of samples was calculated as gallic acid equivalent.

Antioxidant profiling through DPPH free radical scavenging assay

Antioxidant activity through 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (half-maximal inhibitory concentration, IC_{50}) was performed following the method of Tong *et al*. (2014). DPPH (Sigma-Aldrich, USA) solution was prepared in a concentration of 100 µmol/L methanol. Next, 2 mL polyphenolic extract was transferred into 15 mL centrifuge tube, followed by DPPH solution (2 mL), and incubated for 30 min in the dark at room temperature. The sample was run at 517 nm using a spectrophotometer (160 UV-VIS Thermo Fisher Scientific, MA, USA) to determine the decrease in absorbance due to the activity of free radicals.

Quantification of phenolic acids through HPLC

Oat flour extracts were examined using the RP-HPLC system (Perkin Elmer, 200 series, CT, USA) installed with UV-VIS detector and C_{18} column (PerkinElmer, Spheri-5; 220 × 4.6 mm, particle size = 5 µm). Phenolic acids like gallic, *p*-coumaric, caffeic, vanillic, syringic, and ferulic acids were quantified according to Kim *et al.* (2006) at 260 nm with UV-VIS detector. In mobile phases, 2% acetic acid in water was used as solvent A, and acetonitrile was used as solvent B. The flow rate was kept at 1.0 mL per min with an injection volume of 10 µL for a total run time of 70 min. The gradient program for solvent A was as follows: 100 - 85% in initial 30 min, 85 - 50% in 20 min, 50 - 0% in 5 min, increasing 0 - 100% in the next 5 min, and at the end, 10 min of post-run for reconditioning.

Cell cytotoxicity analysis

The cytotoxicity activity of oat flour extracts was measured using MTT assay (Mosmann, 1983) with slight modifications. Caco-2 cells were cultivated in a 96-well plate (1×10^4 cells/well). Next, 100 µL of growth medium containing this cell suspension was added to each well, and incubated for 12 h at 37°C in 5% CO_2 incubator; outer line wells were left blank to decrease the drying effect on absorbance taken at the end. The medium was exchanged with growth medium containing different concentrations of 10, 15, 20, 25, 30, 35, and 40 µg/mL oat flour extracts, narrowed down from concentration of 10 to 200 µg/mL. Cells were kept in a growth medium containing oat flour extract for 48 h by changing it after 24 h. Wells containing medium without oat flour extract served as control. After 48 h, 10 µL of MTT/PBS solution (5 mg/mL) was added to each well without removing extract-containing medium, and incubated for 4 h at the same conditions. The medium was removed with aspiration, DMSO (100 µL) was added in each well, and plates were placed on shaker for 10 min. The absorbance was determined at 560 nm and background at 670 nm by using a microplate reader (Infinite M200, Tecan US, Inc.). One row of the well was taken as a blank without seeding the cells but treated with an extract-containing medium, PBS, and DMSO. The final value at each concentration was obtained by subtracting the

control's and blank's absorbance from its particular absorbance.

Effect of oat flour extracts on intracellular ROS production

Intracellular ROS level was measured using a fluorescent probe, *i.e.*, cell membrane permeable, 2,7-dichlorofluorescein diacetate (DCFH-DA), as described previously (Bellion *et al.*, 2009) with some modifications. Caco-2 cells with a density of 5×10^5 cell/mL for seeding in 96-well plate was prepared in a medium containing FBS and antibiotic in DMEM. Next, 100 µL of cell suspension containing growth medium was added to each well, and incubated for 12 h at 37°C in the 5% CO_2 incubator. At the end of the seeding period, cell suspension medium was exchanged with culture media containing two different concentrations of oat flour extracts, *i.e.*, 10 and 15 µg/mL (100 µL/well). The cells were kept in medium containing extract for 12 h; after that, wells were washed with PBS, and incubated with 100 µL of 10 µM fresh 2,7-dichlorofluorescein diacetate (DCFH-DA) prepared in PBS for 20 min. After that, the solution was discarded carefully with aspiration, and wells were again washed with PBS. Then, it was incubated again with 100 µL of PBS as control, and 1 mM of H_2O_2 was prepared in HBSS for oxidative stress. After 2 h of incubation, the fluorescence of each well at an excitation wavelength of 485 nm and an emission wavelength of 530 nm was measured. The 96-well microplates were placed in the incubator for 2 h. All values were normalised with the control (*i.e.*, Caco-2 cells with no extract and H_2O_2).

Statistical analysis

Data for all analyses were presented as mean ± SD (standard deviation). The experimental data were analysed statistically using one way ANOVA (analysis of variance). The level of significance was taken as *p*-value less than or equal to 0.05.

Results and discussion

Total phenolic content (TPC) and antioxidant activity

Total phenolic content varied significantly among oat cultivars, with the highest being in S-2011 (198.41 mg GAE/100 g), and the lowest in L-632 (167.57 mg GAE/100 g). Antioxidant activity was determined through 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. The IC_{50} values of S-2000, S-2011, and L-632 were 16.03, 13.18, and 18.81 mg/mL, respectively. This agreed with Manzoor *et al*. (2020).

Methanol has widely been used for polyphenolic extraction among conventional solvents due to its efficiency in optimum extraction which leads to higher extraction rate of phenolic compounds (Magwaza *et al.*, 2016). TPC of oat flour reported by Zdunczyk *et al.* (2006) after extraction with 80% methanol was 117 mg/100 g FAE. Gujral *et al.* (2013) also evaluated the TPC of oat flour after methanol extraction, and reported 202.3 mg FAE/100 g of total phenolic content. In the present work, the antioxidant activity of S-2011 was higher than the other oat cultivars. These results indicated that the antioxidant activity of oat cultivars was directly associated with total phenolic content. The presence of hydroxycinnamic acids and hydroxybenzoic acids also favours the efficiency of antioxidant activity (Xue *et al.*, 2017).

Phenolic acid

Benzoic acid derivatives (gallic, vanillic, and syringic acids) and cinnamic acid derivatives (caffeic, *p*-coumaric, and ferulic acids) of phenolic acids were quantified in the flour of oat cultivars, and chromatogram of their standards is presented in Figure 1. Their values are given in Table 1. Cultivars showed highly significant variation ($p < 0.01$) for all phenolic acids as examined through HPLC.

In oat flours, gallic acid ranged from 17.42 µg/g in L-632 to 145.50 µg/g in S-2011. In different types of oat cultivars, gallic acid followed the order of S-2011 > S-2000 > L-632. Vanillic acid was discovered in minute quantities. The values for vanillic acid in different oat cultivars ranged from 0.05 µg/g in L-632 to 0.75 µg/g in S-2011. Syringic acid values ranged from 2.30 µg/g in L-632 to 13.09 µg/g in S-2000. The values for syringic acid in oat cultivars followed the order of S-2000 > S-2011 > L-632.

Caffeic acid is a hydroxycinnamic acid, and in oat cultivars it ranged from 1.45 to 6.77 µg/g. The caffeic acid content in oat cultivars followed the order of S-2011 > S-2000 > L-632. Ferulic acid was found highest in L-632 (2.59 µg/g) and lowest in S-2011(1.19 µg/g). *p*-coumaric acid in different oat cultivars ranged from 0.07 µg/g in L-632 to 52.67 µg/g in S-2011. The *p*-coumaric acid values followed the order of S-2011 > S-2000 > L-632.

Phenolic acids, with their antioxidant potential, activate antioxidative reaction in case of infection in

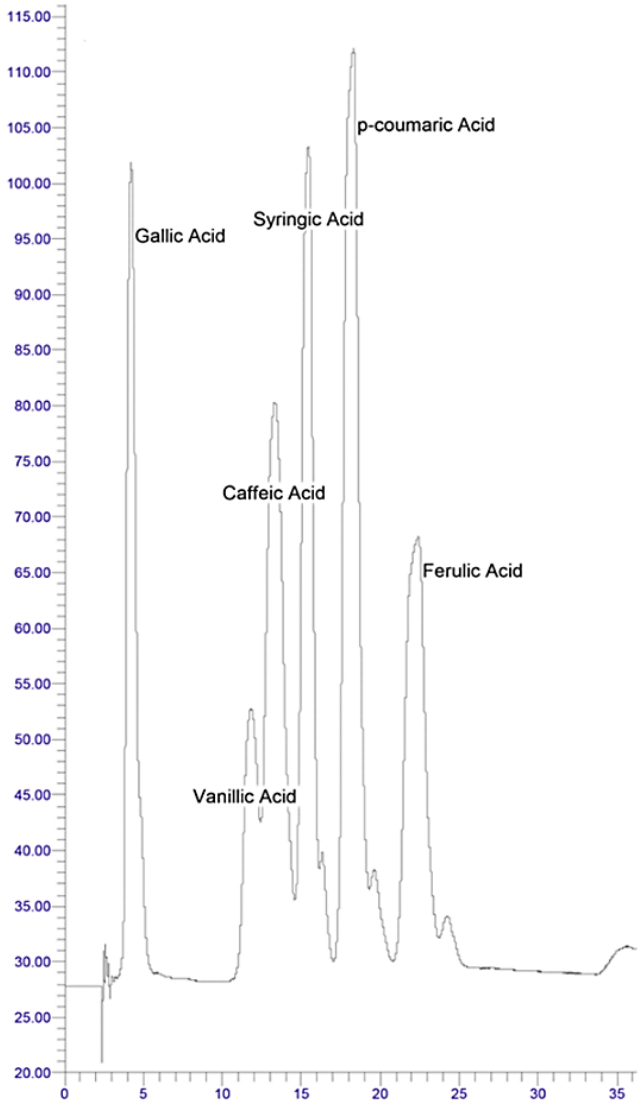

Figure 1. HPLC chromatogram of phenolic acid standards. Retention times: gallic acids = 3.84 min, vanillic acid = 10.81 min, caffeic acid = 12.61 min, syringic acid = 14.65 min, *p*-coumaric acid = 17.53 min, and ferulic acid = 21.70 min.

Table 1. Phenolic acid contents in oat cultivars.

Analysis		Cultivar			Mean
		S-2000	S-2011	L-632	
Phenolic acids (µg/g)	Gallic acid	60.03 ± 0.27^{b}	145.50 ± 0.32^{a}	17.42 ± 0.59^{c}	74.32 ± 65.22
	Vanillic acid	0.17 ± 0.02^{b}	0.75 ± 0.21^{a}	0.05 ± 0.02^{b}	0.33 ± 0.37
	Syringic acid	13.09 ± 0.92^{a}	4.73 ± 0.31^{b}	2.30 ± 0.29^{c}	6.70 ± 5.66
	Caffeic acid	4.27 ± 0.30^{b}	6.77 ± 0.34^{a}	1.45 ± 0.35^{c}	4.16 ± 2.66
	Ferulic acid	2.40 ± 0.19^{a}	1.19 ± 0.07^{b}	2.59 ± 0.10^{a}	2.06 ± 0.76
	p-Coumaric acid	7.92 ± 0.22^{b}	52.67 ± 0.54^{a}	0.07 ± 0.01^{c}	20.22 ± 28.37

Values are mean ± standard deviation. Means followed by different letters in a row are significantly different ($p < 0.05$).

the body (Stuper-Szablewska *et al.,* 2019). Phenolic acid results of the present work agreed with Chen *et al*. (2018), who recorded gallic, caffeic, and ferulic acids in the range of 14.39 - 70.45, 1.23 -5.78, and 1.43 - 2.75 µg/g, respectively. Phenolic acids reported by Verardo *et al.* (2011) through HPLC after being extracted with different solvents, quoted the range of vanillic acid from 0.71 to 2.03 µg/g, in line with the present work. In a recent study, Walters *et al.* (2018) found that caffeic and *p*-coumaric acids in whole oat flour ranged from 4.4 - 6.7 and 6.7 - 12.0 µg/g, respectively. Tong *et al*. (2014) have reported a range of caffeic (0.01 - 1.4 µg/g) and ferulic (1.5 - 4.3 µg/g) acids.

Different analytical procedures and extraction conditions could be the reason for differences in phenolic acid contents obtained in the present work. The phenolic acid yield is particularly affected by the extraction technique. The type of cultivar, use of methanol as a solvent, and double extraction procedure are some variables that could also affect the phenolic acid yield, positively or negatively. In the present work, the use of methanol as a solvent, and double extraction procedure positively affected the phenolic acid values.

Cell cytotoxicity analysis

Cytotoxicity of oat flour extracts was analysed with MTT viability assay to confirm that the reduction in fluorescence during CAA was due to the antioxidant defences and not the cell death. The concentrations of oat flour extracts for performing CAA analysis were selected after performing cell cytotoxicity analysis with a wide range of oat flour extract concentrations. Oat flour extract concentrations were narrowed down from 0 - 120 to 0 - 40 µg/mL in order to optimise the best concentrations for cell viability. Absorbance readings for the cytotoxicity assay with oat flour extracts of different cultivars and control treatment are shown in Figure 2.

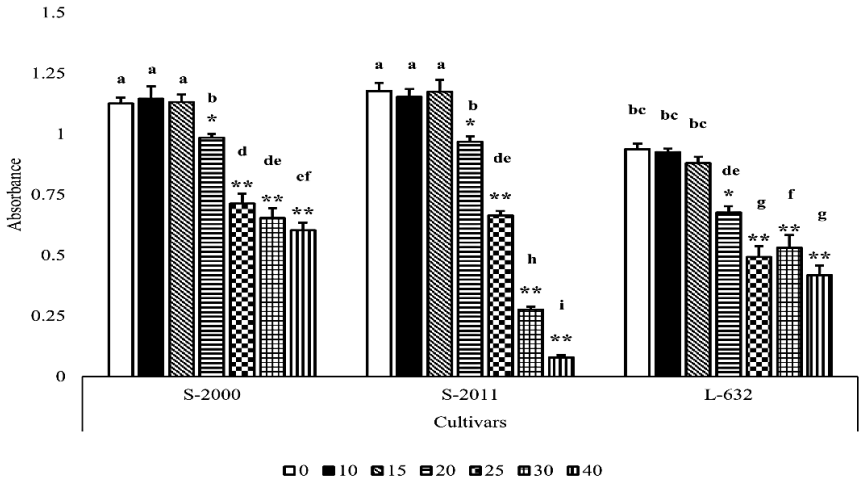

Figure 2. Cytotoxicity of different oat extracts against Caco-2 cells. MTT analysis for cell survival against different doses of oat extracts when compared with control; *significant ($p \leq 0.05$), **highly significant ($p \leq 0.01$).

Results showed that oat flour extracts of 10 and 15 µg/mL concentrations had non-significant differences with control treatments, and were non-toxic to the epithelial cells. The significant difference of 20 µg/mL concentration and highly significant variation beyond this concentration have depicted their negative effect on cell viability. Cultivars having similar letterings depicted non-significant variation among them. 10 and 15 µg/mL concentrations of S-2000 and S-2011 cultivars showed no significant variation in comparison of control treatments and among cultivars, while significantly different than L-632. Furthermore, 10 and 15 µg/mL concentrations in L-632 were also non-significant in comparison to its control, but the fluorescence was reduced overall for this cultivar's treatments.

Effect of oat extracts on intracellular ROS production

The protective effect of oat flour extracts in scavenging ROS production of human epithelial cells was measured. 15 µg/mL oat phenolic extracts were found to have more inhibitive effect on ROS

production than 10 µg/mL (Figure 3). With relevance to the oxidation level of control samples of each treatment taken as 100%, S-2000 and L-632 extracts depicted a significant suppressive effect on ROS production in Caco-2 cells without stimulation of H_2O_2. At the same time, S-2000 showed a highly significant difference with H_2O_2 stimulation that enhanced the production of ROS in Caco-2 cells (Figure 3). Oat flour extract of S-2011 at 10 and 15 µg/mL showed the highest inhibiting effect than other oat cultivars on the surge of ROS production in Caco-2 cells, with or without H_2O_2 stimulation, *i.e.*, 65.20:56.36% and 76.19:66.24%, respectively, for 10:15 µg/mL (Figure 3). Moreover, Tukey's' analysis clearly depicted the significant and highly significant variation among cultivars. Antioxidative effect among cultivars was significantly different with both PBS and H_2O_2 stimulation, while 15 µg/mL concentrations of S-2000 and S-2011 with H_2O_2 stimulation had non-significant difference among each other; however, the values depicted more inhibitive effect with S-2011.

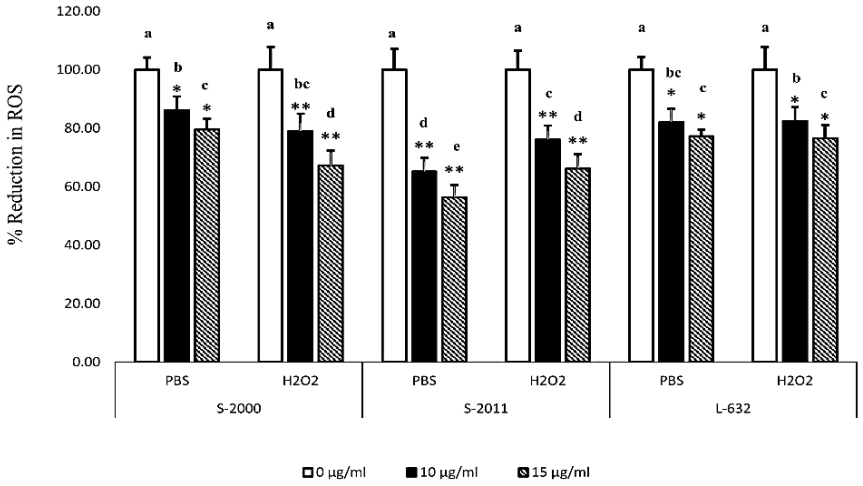

Figure 3. Intracellular reactive oxygen species scavenging activity of oat extract. Caco-2 cells were induced with H_2O_2 for oxidative stress while control Caco-2 cells were induced with PBS. *Significant ($p \leq 0.05$), **highly significant ($p \leq 0.01$).

The effect of oat flour extract on intracellular ROS production was analysed to detect the capacity of extract to inhibit the formation of DCF by H_2O_2-induced free radicals in Caco-2 cells. The extent of fluorescence in Caco-2 cells was relevant to the level of oxidation, thus indicating that decreasing fluorescence in oat samples treated cells when compared to the control was due to the cellular antioxidant property (Wolfe and Liu, 2007). In the present work, the oat cultivars inhibited oxidative stress in agreement with the effectiveness of dandelion leaf extract (chicoric acid; Xue *et al.,* 2017) and chicory seed extracts (Ziamajidi *et al.,* 2013). Oat phenolics and avenanthramide (AVAs) capsules have been found to possess a strong antioxidant effect on the human body (Liu *et al.,* 2011). Altogether, oat extract with the antioxidative function of its high phenolic content protected the intestinal epithelial cells from oxidative stress.

Conclusion

For the first time, oat cultivars of the South Asian region were investigated for intracellular ROS suppression. High phenolic content and antioxidant activity were found in the oat cultivars. Six phenolic acids were quantified through HPLC/UV-Vis detector, and results showed significant differences among the oat cultivars. In cell cytotoxicity assay, oat phenolic extract concentrations were narrowed down to select the concentrations behaving non-significantly with control. The inhibitory effect of oat extract on ROS production was analysed. Oat extracts positively suppressed intestinal oxidative stress in Caco-2 cells, thus could support human health.

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