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Safety assessment of the innovative functional food ingredient from *Cannabis sativa* L. wastes

Fatmanur Gönce[1], Elmas Ersöz[2], Meryem Kara[3], Gökhan Kars[4], Saliha Dinç[5], Serpil Edebali[6], Manuel Roman[7,8] and Meltem D. Kars[9]*

Abstract

Xylooligosaccharides (XOS) are the oligomers of β-1,4 linked xylose monomers and they have health promoting effect by modulating the beneficial microorganisms in intestine. In this study, hydrolysate obtained from hemp (*Cannabis sativa*) shives was investigated in terms of its in vitro toxicological impacts at cellular and genetic levels and antioxidant activity. The hydrolysate was found to contain 0.264 mg mL^{-1} of xylose, 0.789 mg mL^{-1} of xylobiose and 0.171 mg mL^{-1} of xylotriose in addition to hydroxymethlyfurfural (HMF) and furfural (F) at concentrations of 0.545 mg mL^{-1} and 0.107 mg mL^{-1}, respectively. The cells, colon epithelial cells (CoN) and colon cancer cells (Caco-2), exposed to 5.00 mg mL^{-1} or lower XOS hydrolysate showed very similar growth profiles to the untreated control cells. At the genetic level, the oxidative responses of the cell types to XOS hydrolysate were different as measured by *NFE2L2* (Nuclear factor, erythroid-derived 2-like 2) gene expression. Regarding antioxidant activity, the amount of XOS hydrolysate (IC_{50}) that cleared 50 % of the 2,2-diphenyl-l-picrylhydrazyl (DPPH) in the medium was calculated as 0.12 mg mL^{-1}. To conclude, based on in vitro studies, XOS hydrolysate obtained from lignocellulosic hemp shives emerges as an innovative, alternative and safe functional food candidate.

Keywords: Colon cancer; functional food; hemp shives; cytotoxicity; *Cannabis sativa* L.; xylooligosaccharides

[1]Department of Nanotechnology and Advanced Materials, Institute of Science, Selçuk University, Konya, Turkey

[2]Department of Nutrition and Dietetics, Akşehir Kadir Yallagöz School of Health, Selçuk University, Konya, Turkey

[3]Department of Food Technology, Çumra Vocational School, Selçuk University, Konya, Turkey

[4]Department of Molecular Biology and Genetics, Faculty of Science, Necmettin Erbakan University, Konya, Turkey

[5]Department of Organic Agricultural Management, Çumra School of Applied Sciences, Selçuk University, Konya, Turkey

[6]Department of Chemical Engineering, Faculty of Natural Sciences and Engineering, Konya Technical University, Konya, Turkey

[7]Contactica S.L., Madrid, Spain

[8]Isanatur S.L., Navarra, Spain

[9]Department of Animal and Herbal Production, Meram Vocational School, Necmettin Erbakan University, Konya, Turkey

*Corresponding author: M. D. Kars
E-mail: dmeltem@yahoo.com

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Introduction

The source of food is becoming insufficient in some parts of the world. In addition to this, there is an increasing demand among the people towards the use of healthier foods. Therefore, there is a need to find out sustainable and innovative food sources. At this stage research and innovation for production of value added food products from renewable raw materials from agriculture, forestry, fisheries and aquaculture and the valorization of lignocellulosic bioresources for the production of functional foods could help to satisfy the demands of the consumers (1). The use of lignocellulosic biomass augments both the diversity and abundance of food sources. Moreover, healthier food sources with additional functions could possibly be generated. In this context, the use of renewable sources is inevitable for the sustainability of the production process. This will in turn enhance the sustainability of agriculture and bio-based economy. One of the potent lignocellulosic sources is hemp (*Cannabis sativa* L.) which is claimed to be one of the oldest plants (2). It can be cultivated throughout the world. The ease of cultivation techniques, its capability to cope with biotic and abiotic stress conditions, requirement of lower amount of water and fertilizer and its positive environmental impacts increase the agro-economical value of hemp (3, 4). The hemp crop has been cultivated for many years for conventional purposes like textile manufacturing but it was started to be used as a feedstock in challenging fields like biofuel, essential oil and nutraceutical productions, recently (4).

It is already known for a long time that there is a close connection between dietary habits and health. In this regard, there is an increasing demand for foods which are both nutritious and promoting health. These types of foods named as functional foods are generally fortified with antioxidants, amino acids, prebiotics or other physiologically-active

components so that they reduce the risk of disease and optimize health (5, 6). Xylooligosaccharides (XOS) are among the well-known prebiotics functioning as modulating the beneficial microorganisms in gastrointestinal tract (6). As the name implies, XOS are the oligomers of β-1,4 linked xylose monomers and depending on the existing number of monomers, these oligomers are classified as xylobiose, xylotriose, xylotetrose, xylopentose and so forth (7). These xylose oligomers specifically promote the beneficial bacteria such as bifidobacteria and lactobacilli so that a healthy microbiota is established in the digestive system by suppressing the pathogenic and other opportunistic microbes. Considering the sources of XOS, they could naturally be found in fruits and vegetables but thermochemical and enzymatic productions of XOS from xylan are also possible (8). For instance, Buruiana et al. succeeded to get 11.7 g L^{-1} of xylooligosaccharides from corn stover by hydrothermal treatment (9). Various sources like agricultural and forestry lignocellulosic materials could be used to produce XOS at high scales through thermochemical and enzymatic processes. One important functional food ingredient produced from xylose is a polyol, xylitol. Xylitol is a natural sweetener with low calorie and there are intensive applications on its usage in food industry, especially for diabetic consumers (10). The production of XOS and xylitol from lignocellulosic biomass in bio-refinery concept attracts interest in the field of food research and food industry. Depending on the production process, there occur various types of byproducts like hydroxymethylfurfural (HMF) and its derivative furfural (F) in biomass hydrolysate. In addition, high concentration of these chemicals may pose unintentionally harm to the health of consumers. Unfortunately, HMF and F are the inevitable ingredients of the carbohydrate containing foods as they are formed as a result of heat treatment in sterilization and cooking processes (11).

In this research, the particular objective was to explore in vitro toxicological impacts of XOS-containing hydrolysate, obtained from hemp shives, at cellular and genetic level in addition to its antioxidant activity. In vitro toxicological assessments were done using both colon epithelial cells and colon cancer cells. The expression level of *NFE2L2* (Nuclear factor, erythroid-derived 2-like 2) gene was also evaluated to reveal if XOS supplementation led to any oxidative stress activity in normal and colon cancer cells. *NFE2L2* gene encodes a transcription factor regulating oxidative stress-responsive genes and it is expressed when a cell is exposed to an oxidative stress condition (12). Monitoring the changes in the expression levels of *NFE2L2* gene provided insights into the oxidative stress response of the cells to the treatment in vitro. Collectively, in-vitro safety assessment of the innovative functional food ingredient from *Cannabis sativa L.* was evaluated.

There are no sufficient researches on the production of innovative functional food supplements from hemp shives and on the safety of the products in the literature. Therefore, the recent findings reported in this paper will contribute to the literature and will inspire new investigations on the development of industrial products from hemp and on valorizing herbal waste materials.

Material and Methods

Lignocellulosic material source

This research was conducted within the scope of an EU funded FP7 project named 'Ingredients for Food and Beverage Industry from a Lignocellulosic Source, FP7-SME-606073' with the acronym LIGNOFOOD. In the project, hemp shives were utilized as a source of lignocellulosic biomass that was the waste of industrial plant *Cannabis sativa* from Spain. The hydrolysate consisting of xylooligosaccharides (XOS) was produced by the project partners and provided to the project (13). The XOS hydrolysate was then used for in-vitro toxicological assessment to accomplish one of the work packages of the project and the results were presented in this research paper.

Characterization of lignocellulosic XOS hydrolysate

The lignocellulosic hydrolysate was analyzed in terms of its XOS content by high-performance anion exchange chromatography (HPAEC, CS2500 Dionex system, Dionex Corporation, Sunnyvale, CA) equipped with pulsed amperometric detector (PAD). CarboPac PA-1 column (250 × 4 mm) in combination with CarboPac PA-1 (50 × 4 mm) guard column was used as stationary phase. Gradient elution (A: 100 mM NaOH, B: 500 mM NaOAc) was performed for separation at 25 °C and eluent B was increased from 0% to 70% in 45 min. A flow rate of 1 mL min^{-1} was applied. After the completion of each operation, the column was cleaned by washing with A and 1 M NaOAc for 10 min and then re-equilibrated for 15 min. All eluents were washed with helium gas for 25 min for degassing. Xylose, xylobiose, xylotriose, xylotetrose, xylopentose and xylohexose were used as standards. The standards were quantified through external calibration where high regression coefficient values were achieved. The analyses were performed at least two times, and results were presented as mean values. Standard deviations were lower than 5%.

Furthermore, HMF and F contents of lignocellulosic XOS liquor were revealed using high performance liquid chromatography (HPLC, Shimadzu, Japan, Model SPD-20A/20AV) equipped with UV-VIS detector at 283 nm wavelength using the method reported by Ariffin et al. (2014) with some modifications (14). Stationary phase was Inertsil ODS-3 (5µm 4.6 x 250) and mobile phase was methanol/water (50% v/v). Separation of HMF and F was realized at 40 °C by applying a flow rate of 1 mL min^{-1}. The mobile phase was filtered using 0.22 µm filter (Millipore FH membrane Bedford, MA) before use. All eluents and standards were prepared in ultra-pure water (Milli-Q Synthesis A10 system; Billerica, MA). The analyses were carried out at least two times, and results were given as the mean values. The standard deviations were lower than 5%.

In-vitro assays to investigate the functional and toxicological effects of XOS hydrolysate in colon cells

In this part of the study, experiments were designed for in-vitro safety assessment of the XOS hydrolysate. The media in which normal colon epithelial and colon cancer cell models were propagated were supplemented with different doses of hydro-

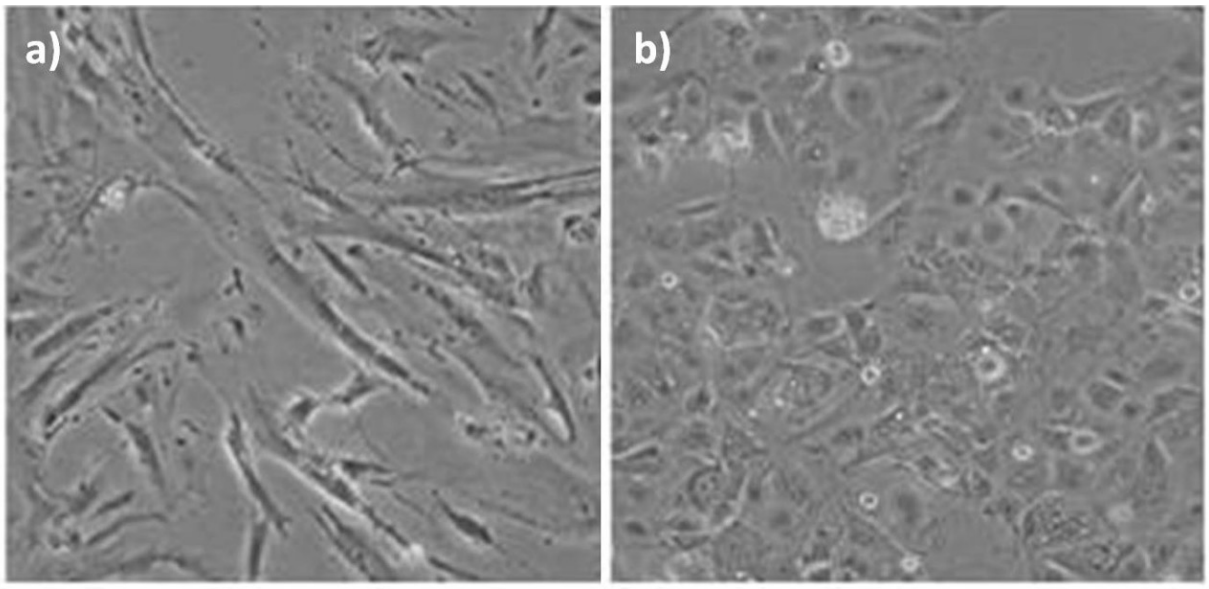

Figure 1. Microscopic appearence of the cells **a)** CoN normal colon epithelial cells, **b)** Caco-2 colon cancer cells.

lysate and then the effects of the XOS hydrolysate were determined. For this, healthy colon epithelial CCD 841 CoN (ATCC® CRL-1790™) and colon cancer Caco-2 (ATCC® HTB-37™) cell lines were used. Time and dose dependent responses of the cell lines were figured out by real time cell analysis system-RTCA (xCelligence, ACEA Biosciences Inc., USA). Additionally, transcriptional analyses were carried out by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) to reveal if the XOS hydrolysate supplementation exerted any alterations in the related gene expression levels.

In-vitro toxicological assessment of XOS hydrolysate on colon cells

CoN is a normal human colon epithelial cell line that was established from 21-week female fetus while Caco-2 is a model cell line for colon cancer that was established from 72-year male colon cancer patient (**Fig.1**). CoN cell line was cultured in 20% FBS supplemented DMEM-F12 (BioChrom FG-4815, Germany) and Caco-2 cell line was cultured in RPMI-1640 (BioChrom FG-1215, Germany). Different concentrations of XOS hydrolysate filtered with a 0.22 µm filter were applied to the cell culture media (20 mg mL^{-1}, 10 mg mL^{-1}, 5 mg mL^{-1}, 2.5 mg mL^{-1}, 1.25 mg mL^{-1}). Then, time and dose dependent responses of the cell lines in terms of proliferation were revealed by RTCA system. The previously established method was employed for this analysis (15). The effects of XOS hydrolysate on cell proliferation and on cell doubling time were analyzed by the software (xCELLigence RTCA Software Pro - ACEA Biosciences Inc.).

Effect of XOS hydrolysate on the expression level of NFE2L2 gene in colon cells

The normal and colon cancer cells were cultured in media supplemented with 10 mg mL^{-1}, 5 mg mL^{-1} and 1.25 mg mL^{-1} XOS hydrolysate for five hours. These concentrations were determined according to the results obtained in toxicological assessment of XOS hydrolysate on colon cells. The total RNA extraction was performed with TRI Reagent (Sigma, MO, USA) and purification was completed using Qiagen (mini-prep) purification columns. The qualities of the total RNAs were determined by Nanodrop-2000 (Thermo Scientific, Massachusetts, USA) and agarose gel electrophoresis (70V, 90 min, Biorad, California, USA). cDNA synthesis was performed using 5 µg total RNA sample, 2 pmol *NFE2L2* and *β-ACTIN* specific reverse primers. The synthesis reaction was performed by the activity of 200 units of M-MuLV Reverse Transcriptase enzyme at 37 °C, for 50 min in a thermal cycler (Biorad). After cDNA synthesis, PCR was performed using each of the cDNA samples to identify the *NFE2L2* gene expression levels in each cell line treated with various concentrations of XOS hydrolysate. In the reactions, one unit of Taq DNA polymerase was used per 25 µL

Table 1. Oligonucleotide sequences and PCR conditions

Primer pairs	Sequence	Product size	Annealing T (°C)	Cycle
NFE2L2 Sense	5'GCGACGGAAAGAGTATGAGC3'	181 bp	60	25
NFE2L2 Antisense	5'GTTGGCAGATCCACTGGTTT3'			
β-actin Sense	5'TGAGCGCGGCTACAGCTT3'	120 bp	56	35
β-actin Antisense	5'TCCTTAATGTCACGCACGATTT3'			

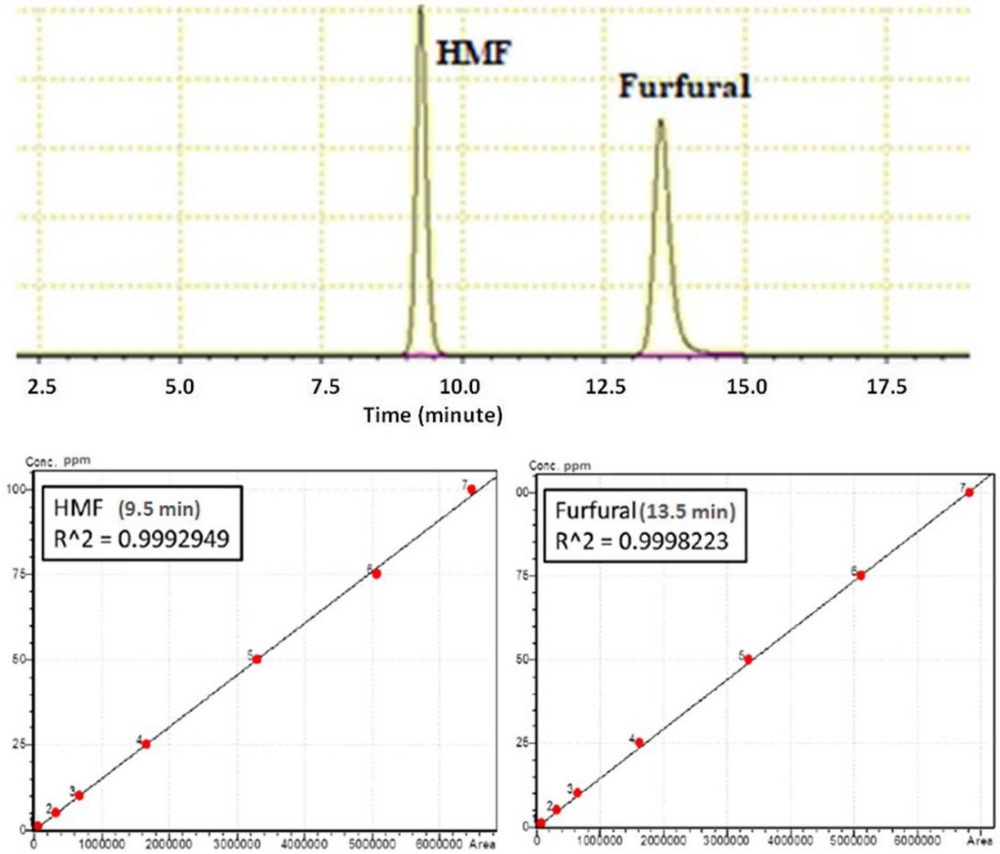

Figure 2. Schematic representation of **a)** HPLC chromatogram of HMF and furfural and b) Calibration curves of HMF and furfural standards.

of reaction volume. The primer pairs specific for *NFE2L2* and β-actin genes were used in PCR reactions for relative expression analysis (16). The β-*ACTIN* gene was used as a common internal control. Primer sequences, optimal PCR cycle numbers and annealing temperatures are presented in **Table 1**.

Agarose gels (1.5%) were prepared and then RT-PCR products were run in the gels for 60 min at 90 V. Following electrophoresis, the gels were stained with ethidium bromide and visualized using Vilber Lourmat Gel Documentation System (Cedex, France). Densitometry analyzes were done by analyzing the band intensities using Image J program (National Institute of Health, USA). The band intensities of *NFE2L2* gene products were normalized with that of β-*ACTIN* PCR products. Any alterations in *NFE2L2* gene expression levels were expressed as fold change values. The analysis was repeated at least three times and the variations between treatment groups were examined. Finally, the groups were compared regarding *NFE2L2* gene expressions to see if XOS hydrolysate supplementations gave rise to oxidative stress in the cells.

The antioxidant activity of XOS hydrolysate

In-vitro antioxidant effect of XOS hydrolysate was determined by DPPH free radical clearance test as previously performed (17). Antioxidant activity of XOS hydrolysate and L-Ascorbic acid (L-ASA) were calculated by using serially diluted XOS hydrolysate (7.50 mg mL^{-1} - 0.015 mg mL^{-1}) and L-ASA (200 μg mL^{-1} - 0.098 μg mL^{-1}). The DPPH solution was added on to the sample solutions as 1:3 (v/v) ratio. After 30 min incubation, clearance of the dark color of DPPH was identified by spectrophotometric measurements at 517 nm. Spectrophotometric value of each hydrolysate dilution was subtracted from that of the corresponding analysis result to eliminate the background of the yellowish color of the hydrolysate. Concentrations of samples that cleared 50% of the color of DPPH were calculated from the equation of logarithmic curve of DPPH inhibition vs XOS and L-ASA graphs.

Statistics

The analyses regarding toxicological assessments, gene expression analysis and DPPH analysis were repeated at least three times and the variations between treatment groups were examined by applying Student's t-test ($p<0.05$).

Results

Characterization of XOS hydrolysate

Xylooligosaccharide (xylobiose, xylotriose, xylotetrose, xylopentose, xylohexose) content of the hydrolysate was revealed by HPAEC analysis. Consequently, XOS hydrolysate was shown to contain 0.264 mg mL^{-1} of xylose, 0.789 mg mL^{-1} of xylobiose and 0.171 mg mL^{-1} of xylotriose. The xylooligosaccharides with 4, 5 and 6 monomers were not detected in the hydrolysate. The analysis revealed that major oligosaccharide was xylobiose in the

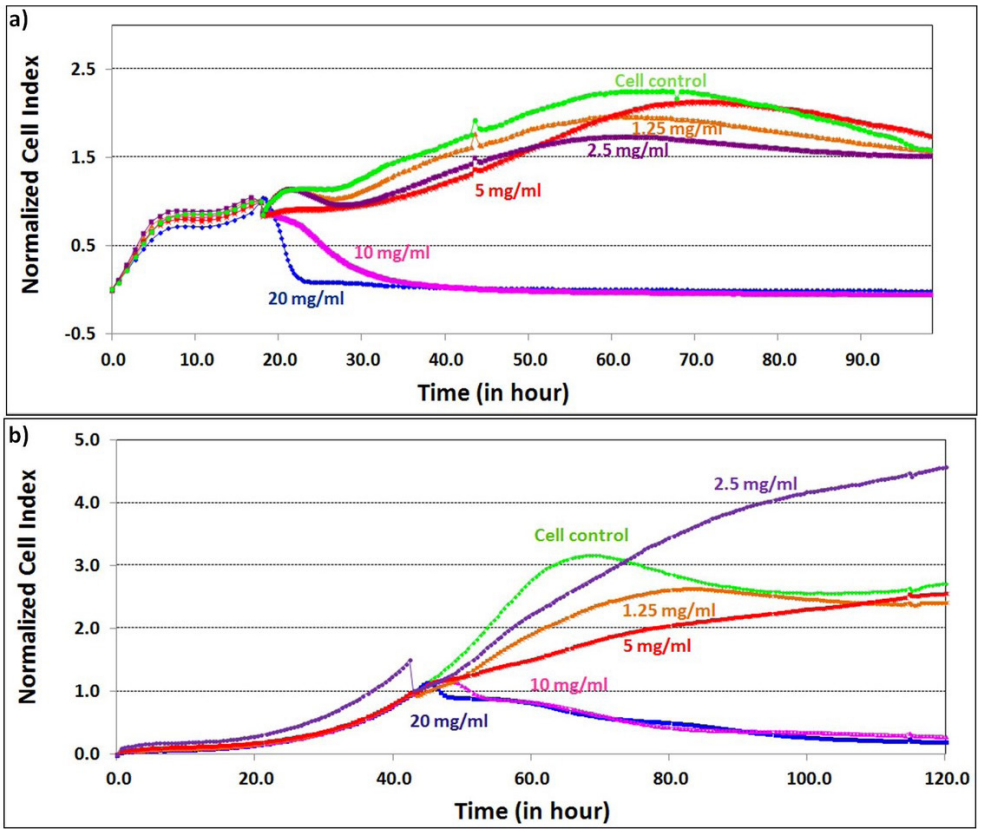

Figure 3. Real-time cell proliferation analysis: Effects of XOS hydrolysate supplementation on proliferation of **a)** CoN cormal colon cells, **b)** Caco-2 colon cancer cells.

hydrolysate. Xylobiose is already known with its prebiotic nature due to its promoting effect on *Bifidobacteria* sp. (18). Thus, XOS hydrolysate is also proved to have prebiotic property because of its comparatively higher xylobiose content. This paper presents the results of particular tasks of the project consortium. The beneficial effects of the hydrolysate were documented in project report by all contributors. Accordingly, XOS types from the hydrolysate have shown prebiotic effect (13).

HMF and furfural are among the major byproducts occurring during the hydrolysis process and therefore they were analyzed in XOS hydrolysate by HPLC. After analyses, 0.545 and 0.107 mg mL^{-1} of HMF and furfural were detected in the hydrolysate, respectively. The standard calibrations curves and chromatogram were presented in **Fig. 2**. In this study, a total amount of 650 mg of HMF and furfural was found to be present in one liter of XOS solution. There is a possibility that HMF and furfural could contribute to the toxicity of high concentration XOS hydrolysate to the model cell lines. And, in the next section, in-vitro toxicological assessment of the hydrolysate was done and possible contributions of these byproducts were also taken into account.

Effects of XOS supplementation on the cell proliferation

According to the results of cell proliferation analysis, 20 mg mL^{-1} and 10 mg mL^{-1} XOS hydrolysate exerted anti-proliferative effect on the normal colon and colon cancer cells. On the other hand, both cell lines displayed very similar growth profile to that of non-treated control cells, in the 5.00 mg mL^{-1}, 2.50 mg mL^{-1}, and 1.25 mg mL^{-1} XOS supplemented media (**Fig. 3**). The IC_{50} values for CoN and Caco-2 cell lines were found by real time cell analyses system at 72 hours treatment as 10 mg mL^{-1} and 15 mg mL^{-1} hydrolysate respectively. According to U.S. National Cancer Institute, for crude extracts; when $IC_{50} \leq 20$ µg ml^{-1}, the extract is highly cytotoxic and if $IC_{50} > 501$ µg ml^{-1}, it means the extract does not exert cytotoxicity. According to the results it was revealed that the crude extract obtained from lignocellulosic waste was not in the limits of toxicity levels even it contains byproducts.

The doubling time of each cell line was calculated to determine the impact of XOS hydrolysate supplementation on proliferation of them. The duplication times of healthy colon epithelial cells and colon cancer cells after treatment with different concentrations of XOS hydrolysate were depicted in **Table 2**. According to the results, the XOS supplements lower than 5 mg mL^{-1} seems to be considerably safe to be used directly on to the cells for long term exposure. Doubling time period of colon cancer cells is higher than that of normal colon cells without any treatment. Specifically, Caco-2 cell doubles once in every 50 hours and CoN cells doubles in 30 hours normally. Caco-2 cells seem to proliferate more slowly than CoN normal colon cells in-vitro. Because of that fact; after treatments, cell growth took more time to reach the logarithmic phase for Caco-2 cells than it occurred in CoN cells. After XOS hydrolysate treatment, doubling rate of the cells increased especially in 2.50 mg mL^{-1} XOS hydrolysate supplied medium.

Table 2. Duplication time (td) of cell lines after XOS hydrolysate supplementation

Treatment	Duplication time (h)	
XOS hydrolysate	**CoN (normal colon cells)**	**Caco-2 (colon cancer cells)**
Control	30.78 ± 0.50	53.65 ± 4.56
1.25 mg mL^{-1}	28.75± 0.67	37.70 ± 1.35
2.50 mg mL^{-1}	25.97± 0.32	26.97 ± 0.54
5.00 mg mL^{-1}	27.05± 0.21	47.19 ± 0.78

Expression analysis of *NFE2L2* gene

Relative quantification of the *NFE2L2* gene was performed by RT-PCR using the total RNA extracted from treated and non-treated cells. The purities (OD_{260nm}/OD_{280nm}) of the RNA samples were between 1.96-2.01. Gel electrophoresis image of the total RNA samples indicated that the total RNA extracted from treated and non-treated groups were intact to proceed to RT-PCR analysis (**Fig. 4a**). RT-PCR products after electrophoresis were presented in **Fig. 4b**. Accordingly, *NFE2L2* (181 bp) and internal control *β-ACTIN* (120 bp) genes were amplified for control and XOS treated groups and bands were visualized. The PCR product bands were subjected to densitometry analysis. The band intensities that were calculated by densitometry analysis of *NFE2L2* gene products were normalized with that of β-ACTIN PCR products (*NFE2L2/ β-ACTIN)*. Any alterations in expression level of the gene encoding the NFE2L2 protein was evaluated by dividing the fold change values of treatment groups with that of non-treated control group. The fold change values were found to be 1.00, 0.99, 1.03 and 1.26 for non-treated control, 1.25 mg mL^{-1}, 5 mg mL^{-1} and 10 mg mL^{-1} groups in CoN cells, respectively. Also the fold change values were found to be 1.00, 0.77, 1.80 and 2.08 for non-treated control, 1.25 mg mL^{-1}, 5 mg mL^{-1} and 10 mg mL^{-1} groups in Caco-2 cells, respectively. It was found that supplementation of 1.25 mg mL^{-1} and lower XOS hydrolysate did not cause any change in expression level of oxidative stress related *NFE2L2* gene in colon cancer model cells ($p>0.05$) (**Fig. 5**). Additionally, 10 mg mL^{-1} and lower XOS hydrolysate treatment did not exert any significant alteration in *NFE2L2* gene expression level in normal colon epithelial cells ($p>0.05$). However, 10 mg mL^{-1} XOS supplemented growth medium induced up-regulation of the *NFE2L2* gene 2.08 folds in colon cancer cells (**Fig. 5**).

Cell culture analysis and molecular studies showed that it is safe to supply XOS hydrolysate up to 5.0 mg mL^{-1} to the colon cells and up to 1.25 mg mL^{-1} XOS hydrolysate supplementation seems to be safe on colon cancer cells.

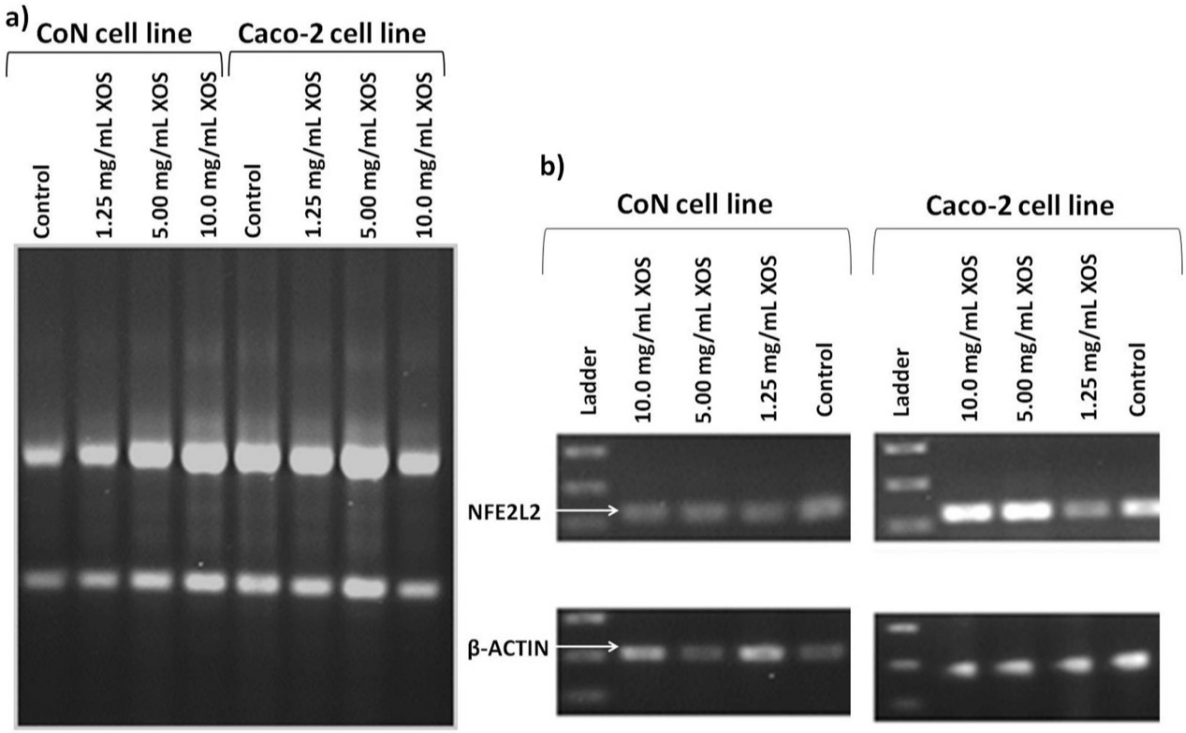

Figure 4. a) CoN and Caco-2 total RNA samples; ribosomal RNA subunits (28S, 18S) on 1% agarose gel. **b)** RT-PCR products for control and treated cells (*NFE2L2* and *β-actin* genes).

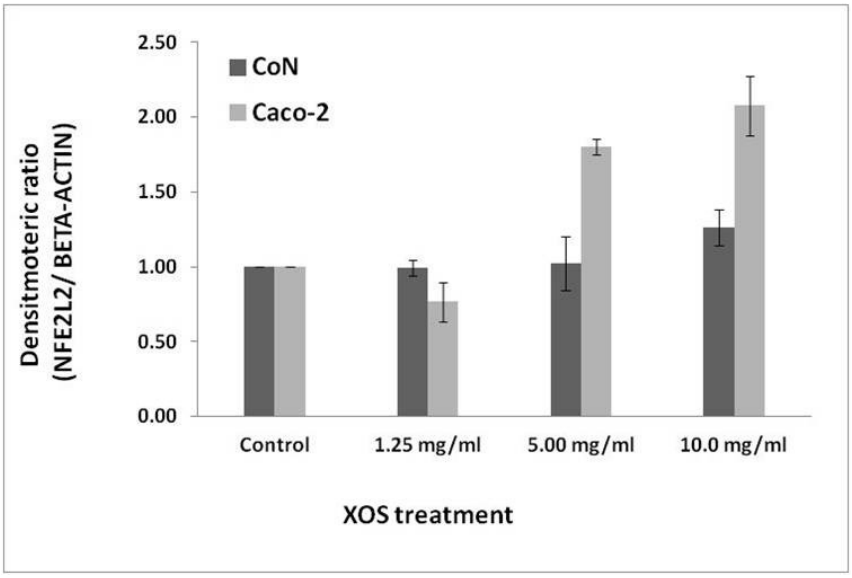

Figure 5. Schematic representation of densitometric analysis of normalized *NFE2L2* gene expression levels in normal (CoN) and colon cancer cells (Caco-2).

In-vitro antioxidant effect of XOS hydrolysate

In vitro antioxidant effect of hydrolysate was assessed on the basis of DPPH radical scavenging activity. DPPH is a stable reagent at room temperature. So, the assay allows sufficient time to identify even weak antioxidants that react slowly (19). The amount of the XOS solution and L-ascorbic acid that quench half of DPPH free radical (IC_{50}) were determined. IC_{50} concentration of the XOS solution that clear 50% of DPPH was calculated by substituting 50 instead of y in the equation of the logarithmic curve in the graph (**Fig. 6**) and was found to be 0.12 mg mL^{-1}. IC_{50} concentration of L-ASA was found to be 12.01 µg mL^{-1} under the current test condition. The result could be interpreted in a way that 100 g XOS hydrolysate constitutes 10 g L-ASA equivalent antioxidant activity. We found that crude XOS hydrolysate had only 10-fold lower antioxidant capacity that of L-ASA possessed. This result points out that XOS hydrolysate has remarkable antioxidant capacity.

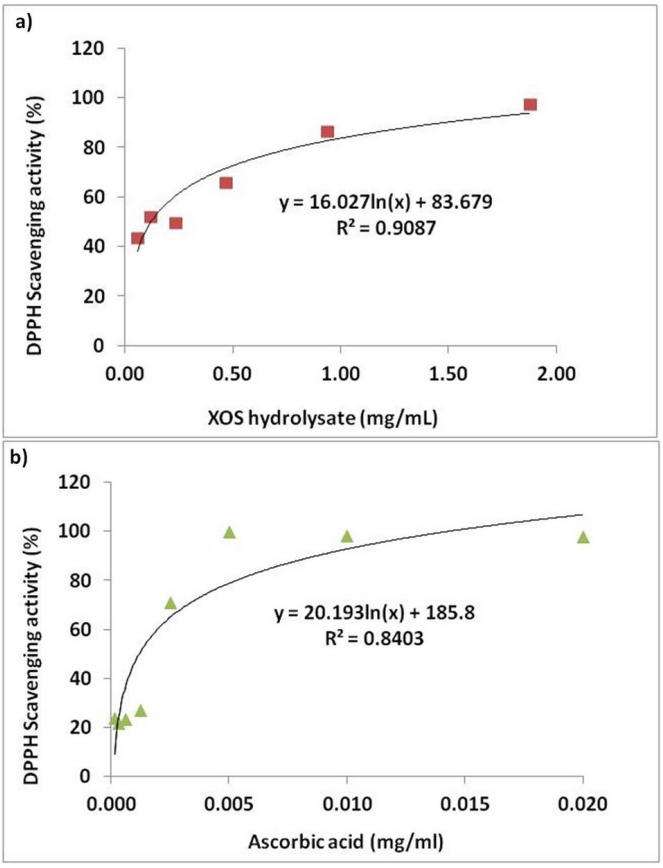

Figure 6. Graphical representations of DPPH scavenging activities of **a)** XOS hydrolysate and **b)** Ascorbic acid (L-ASA).

Discussion

XOS are beneficial for health as they promote gut microbiota thereby help to create a healthy environment in the colon and they could be obtained from various lignocellulosic agricultural sources. In the present research, in vitro toxicological impacts of XOS-containing hydrolysate and its antioxidant activity were explored.

The chromatography results revealed that major oligosaccharide was xylobiose in the XOS syrup. Xylobiose is already known with its prebiotic activity. Godin et al. performed a comprehensive research to establish a method for characterization and quantification of carbohydrates in various lignocellulosic biomasses including hemp (20). Upon hydrolysis and extraction of the hemp biomass with neutral detergent and sulfuric acid, respectively, they obtained a hydrolysate having vast amount of xylan (9.30 g per 100 g dry weight). In this way, it was confirmed that hemp biomass contained significant amount of xylan which was the main source of XOS. In our study, it was found that XOS hydrolysate contained considerable amount of xylose and 0.96 mg mL^{-1} xylooligosaccharides originated from mainly xylan. Braggato et al., reported the yield of xylans from sugarcane differs depending on the hydrolysis process and duration. And, they presented that the major xylooligosaccharide content was in the form of dimer and trimer oligos after

enzymatic hydrolysis (21). In parallel with these findings, the present results also demonstrated that hemp XOS hydrolysate used as supplement in cell culture medium predominantly constituted xylobiose. The findings presented here are mainly in concordance with the literally known concept that lignocellulosic hydrolysate contains xylose and XOS which have potential health uses. Here, it is revealed that hemp shives hydrolysate deserves further studies for the development of prebiotic food supplements.

HMF and furfural were found to be present in XOS syrup and there is a possibility that HMF and furfural could contribute to the toxicity of high concentration XOS hydrolysate to the model cell lines. Janzowski et al. also assessed any toxic effect of HMF on Chinese hamster lung cells and colon cancer cells (11). They found that being exposed to natural HMF from juices and foods does not create any significant health risk. Inherently, HMF is found in the carbohydrate containing foods and beverages and HMF and its derivatives are also formed during heating, cooking and sterilization processes of foods. However, limit for HMF content only for honey is determined in The United Nations Food Codex and Turkish Food Codex as 40-80 mg kg^{-1} honey (22, 23). In this study, a total amount of 650 mg of HMF and furfural was found to be present in one liter of XOS solution. According to the toxicological analysis results, it was revealed that the crude extract obtained from lignocellulosic waste was not in the limits of toxicity levels even it contains byproducts. Doubling time analysis also presents that, the XOS supplements lower than 5 mg mL^{-1} seem to be considerably safe to be used directly on to the cells. Here, when the cells were inspected by real-time cell growth analysis, it was found that doubling time period of colon cancer cells was higher than that of normal colon cells without any treatment. Because of the fact that Caco-2 cells proliferate more slowly than normal colon cells in-vitro; after treatments, cell growth may have taken more time to reach the logarithmic phase for Caco-2 cells than normal colon cells. In parallel to these findings, Maeda et al. found that 1 mg mL^{-1}, 1.5 mg mL^{-1} XOS treatment did not significantly affect the proliferation of normal human skin fibroblast cells, mouse myoblast cells and human breast cancer MCF-7 cells for 24 hours (24). In the study of Ando et al., a mixture of xylose, xylooligosaccharides and water-soluble lignins was used to test the cytotoxicity (25). It was stated that the mixture was containing 25% water soluble lignin and the remaining 75% was containing xylose and xylooligosaccharides (4-6 μg mL^{-1}). Upon application the mixture (150-200 μg mL^{-1}), it was realized that while the proliferation of acute lymphocytic leukemia cell lines was significantly decreased, the proliferation of cell lines derived from myelogenic leukemia and lymphoma cells was not affected. Based on the findings in the literature and in this study, it can be asserted that cell lines behave differently to XOS. And, toxicity might come from high concentrations of XOS as well as other accompanying products like lignin, furfural and HMF.

The gene expression analysis was conducted to determine the effect of XOS hydrolysate supplementation on the *NFE2L2* gene expression level. The results represented that *NFE2L2* gene expression amount was increased in 10 mg mL^{-1} XOS hydrolysate treated colon cancer cells. Here it may be proposed that, colon cancer cells have experienced oxidative stress in 5 to 10 mg mL^{-1} XOS hydrolysate supplemented culture media. The particular increase in gene expression level in Caco-2 cells may be due to high XOS supplementation that might have switched on the transcription of antioxidant genes by increasing *NFE2L2* expression to eliminate any effects of increased concentration XOS or any byproducts in culture medium. The NFE2L2 pathway may have been activated in response to autophagy that may have been caused by increased osmolarity in culture medium. It was previously reported that various concentrations of sorbitol and mannitol caused hyperosmotic stress and autophagy in human colon cancer cell line HCT116 (26). That mechanism may have promoted expression of *NFE2L2* gene in Caco-2 cells in our case. It is known that colon cancer cells evolve resistance mechanisms to tackle with the effects of any treatment, chemotherapy and radiotherapy and to reach the ultimate goal of survival (27). The molecular and cellular response mechanisms of colon cancer cells, following the supplementation of XOS, HMF and furfural should further be identified. In a study, the antioxidant activity in rat blood upon XOS supplementation was investigated (28). And, they reached to the conclusion that supplementation of diet with XOS may modulate the antioxidant system and lipid metabolism and may alleviate damaging effects of oxidative stress caused by high fat diet (28). In parallel with this study, in the current study, XOS hydrolysate (1.25 mg mL^{-1}) did not trigger *NFE2L2* gene expression in colon cancer cells. Specifically, gene expression was not upregulated significantly in normal colon epithelial cells. Cell culture analysis and molecular studies imply that it is safe to supply XOS hydrolysate up to 5.0 mg mL^{-1} to the colon cells and up to 1.25 mg mL^{-1} XOS hydrolysate supplementation seems to be safe on colon cancer cells. The difference in gene expression levels and responses of the cells to the supplementation seems to be stemmed from the variable responses of source tissues.

In vitro antioxidant power of XOS syrup was assessed through DPPH radical scavenging activity test. It was found out that crude XOS hydrolysate had only 10-fold lower antioxidant capacity than that of L-ASA. It may be proposed that XOS hydrolysate of lignocellulosic waste has non-toxic and good antioxidant nature that its biological activities are worth exploring. Veenashri and Muralikrishna investigated the antioxidant effect of XOS from different sources by DPPH method (29). They found out that 60 μg mL^{-1} XOS exerted the highest antioxidant activity with 70% of the scavenging effect of DPPH radical. In another study, the scavenging effect of the hydrolysate composed of xylotriose, xylotetrose and small amounts of xylopentose and xylohexose on 50% of the DPPH radical was found to be 600 μg mL^{-1} (30). To compare, in this study, the XOS hydrolysate concentration that clears 50% of DPPH activity was found to be 120 μg mL^{-1}. The variations between the findings most probably arise from the differences in the composition of the hydrolysates. The types and amount of XOS and

accompanying chemicals like lignin, phenolic substances, furfural and HMF could influence the overall antioxidant power of hydrolysate. Similarly, Gowdhaman and Ponnusami attributed the reasons for the differences in antioxidant scavenging activities to the saccharide source and to the differences in the yield of extraction and purification methods (31).

Conclusions

Production of XOS from agricultural biomass presents a wide variety options to the food industry since sustainable raw material sources are abundant and accessible. During thermochemical and enzymatic XOS production processes, there occurs different byproducts and these chemicals might still exist in the hydrolysate after purification processes due to the limitations of the techniques. For this reason, in vitro studies like toxicity and antioxidant activity assessments are needed to be done at first. In this regard, in the current study, XOS hydrolysate which was obtained from hemp shives was investigated in terms of its toxicity and antioxidant activity. And, it was revealed that non purified XOS hydrolysate (1.25 mg mL^{-1} and lower supplement concentration) did not exert toxicity and oxidative stress on normal colon cells and colon cancer cells. In addition, hydrolysate demonstrated considerable amount of antioxidant activity. The hydrolysate was shown to contain mainly xylobiose in addition to xylose and xylotriose. However, byproducts (HMF and furfural) also existed in the hydrolysate; therefore, XOS hydrolysate needs to be purified to decrease toxicity observed in this study. The findings of this study will contribute to the further investigations on the development of food supplements for health from hemp, a lignocellulosic source of nature. As a future study, cost-effective purification processes will be employed to remove major contaminants such as HMF and furfural in the hydrolysate and further characterization methods will be conducted.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Ethical compliance

This article does not contain any studies involving human participants or animals performed by any of the authors.

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