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Preparation of Bioactive Oligosaccharides from Mallow Residues by Enzymes Mixture of Isolated *Aspergillus flavus* B2 and Evaluation of their Biological Activities

Shireen A.A. Saleh[1], Samia A. Ahmed[1,*] , Faten A. Mostafa[1], Asmaa A. Abd El-Galil[1], Hanan A.A. Taie[2], Walaa A. Abdel Wahab[2]

[1]*Chemistry of Natural and Microbial Products Department National Research Centre, Dokki, Cairo, Egypt ;* [2]*Plant Biochemistry Department, National Research Centre, Dokki, Cairo, Egypt*

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Abstract

The preparation of biologically active oligosaccharides from the stem residues of the mallow plant (*Corchorus olitorius*) was evaluated by the enzymatic hydrolysis method. The isolated strain *Aspergillus flavus* B2 (accession number OL655454) was selected as a potential producer of the enzymes mixture. The factors affecting the hydrolysis of Mallow stems (MS) by enzyme mixture were tested using Plackett-Burman and Central Composite statistical designs. Different production conditions yielded 13 bioactive oligosaccharides (MSOS) that differed in their yield, components, monosaccharides constitution, percentage, and degree of polymerization. The optimized conditions yielded 525 milligrams of oligosaccharide per gram of dry MS using enzymes- mixture containing cellulase 4.9 U/mL, xylanase 6.8 U/mL, and pectinase 12.9 U/mL. Oligosaccharide no. 4 showed higher fibrinolytic activity than Hemoclar. All tested oligosaccharides (1 %) improved the growth of both the probiotics *Bifidobacterium lactis* and *Lactobacillus plantarum*. The antioxidant activity of the produced oligosaccharides varies depending on their characteristics and method of evaluation. Of all the samples tested, MSOS no. 11 showed the highest level of DPPH, reducing power, ABTS, and FRAP activity. Moreover, the highest reduction in tumor cell viability and the inhibited ascites were obtained by MSOS no. 11 (37.24±0.31 %).

Keywords: *A. flavus* B2, bioactivities, Jew's Mallow, enzymes-mixture, oligosaccharides, statistical method

1. Introduction

Oxidative stress caused by free radicals is one of the factors in tumor development and pathogenesis of many human diseases (Rivas *et al*., 2021). Antioxidants can act as cancer chemo-preventives substances to protect cells from oxidative stress (Xiong *et al*., 2021). Chemotherapy drugs still have significant toxicity and critical side effects on healthy cells. This pushes us to demand new treatments for this insidious disease such as natural anticancer substances (Grigalius and Petrikaite, 2017). Natural anticancer drugs are low cost, have several mechanisms of action, and are often active toward cancer cells. Recently, there is a growing demand for functional foods that have health benefits. Prebiotics as functional food components are known as selectively, non-digestible fermented components. This allows certain changes in the activity and/or composition of the intestinal flora resulting in the host's well-being and good health (Desai *et al*., 2020). Prebiotics are carbohydrates with a short chain that improve the activity and the growth of intestine probiotics (Gibson *et al*., 2004).

Oligosaccharides are polymers with short-chain (2 to 10 monosaccharide units) by glycosidic linkage with low molecular weight, which have attracted great interest for their enforcement in different fields. Oligosaccharides have some biological activities such as antioxidant, antitumor, aiding the proliferation of bifidobacteria, protecting the liver, regulating gastrointestinal functions, and reducing cardiovascular risks (Yang *et al*., 2020; Xiong *et al*., 2021). Oligosaccharides are widely and abundantly distributed and have some advantages over other natural antioxidants such as fewer side effects and better biocompatibility (Xiong *et al*., 2021).

The solubility, triple-helical chain structure, and the existence of uronic acid can affect the bioactivity of oligosaccharides (Ahmed, 2021). The biological activities of oligosaccharides appear to be related to their chemical properties. Furthermore, extraction methods may influence the composition and/or type of oligosaccharide extracted. Moreover, the end groups of oligosaccharides (OH^- of C-2 and C-6) can inhibit the oxidative process (Kang *et al*., 2014).

Currently, 37 megatons of agricultural waste are produced worldwide, causing serious economic and environmental problems related to improper disposal (Rivas *et al*., 2021). Using solid residues to produce useful compounds is a new approach to (a) valorize the great amount of the generated byproducts, (b) control the negative effects of accumulated residue on the environment, (c) benefit humans by preventing disease,

* Corresponding author. e-mail: dr_sa_ahmed@ yahoo.com; sa.sayed@nrc.sci.eg.

and (d) extending the foods shelf life through their known antimicrobial and antioxidant activity (Rivas *et al*., 2021).

Since ancient times, plants are important in pharmacology and medicine. Herbs are often used in the development of novel anticancer drugs (Grigalius and Petrikaite, 2017). Bataineh *et al*. (2023) indicated that pomegranate peel not only kills *Helicobacter pylori*, but also has protective effects on the gastric mucosa. Oligosaccharides may be gained by direct extraction or by hydrolyzation with enzymes or acids (Kang *et al*., 2014; Xiong *et al*., 2021). However, enzymatic hydrolysis is the most appealing method because of its advantages: no need for special equipment, environmentally friendly, good selectivity, and product improvement. As well as reduced production of monosaccharides and their unwanted derivatives (Li *et al*., 2016; Rivas *et al*., 2021).

Among different technologies utilized to produce valuable compounds from agricultural and industrial residues is solid-state fermentation (SSF). SSF has the characteristics of economy and sustainability, high (productivity and efficiency), low consumption (water and energy), and minimal concerns about solving disposal problems (Yazid *et al*., 2017). SSF can be utilized to produce valuable compounds because enzymes produced by microorganisms release large amounts of valuable compounds with high bioactivities (Dey and Kuhad, 2014). Sadik *et al*. (2021) used agro-industrial waste in SSF to produce value-added byproducts. In particular, filamentous fungi are suitable for SSF because this technique mimics their natural environment such as low requirements. Fungal multi-enzyme mixtures were used in the production of oligosaccharides (Li *et al*., 2016).

Corchorus olitorius L. (Jew's Mallow) is an annual herbaceous plant in Middle Eastern countries. Mallow is a highly nutritious vegetable, and its green leaves are consumed as a viscous soup (Ahmed, 2021). In addition, it contains high nutrients such as carbohydrate (44%), protein (22%), fiber and vitamins (11%), ash (16%), moisture (5%), and fat (2%) as reported by Ahmed *et al*. (2021). Hence, it is considered a suitable substrate for the production of bioactive compounds (Al-Yousef *et al*., 2017). This work aims to statistically optimize the preparation of bioactive oligosaccharides from Mallow stems residues (MSOSs) by hydrolysis with enzymes mixture from isolated fungus (*A. flavus* B2, OL655454). The prepared MSOSs were chemically characterized and their biological activities (anticoagulant, fibrinolytic, antitumor, antioxidant, and prebiotic) were estimated.

2. Materials and Methods

2.1. Materials

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate); 2,4,6-tripyridyl-s-triazine (TPTZ); 1,1-diphenyl-2-picrylhydrazyl (DPPH); potassium ferricyanide ($C_6N_6FeK_3$); ferrozine; Trolox; ferrous and ferric chloride [$FeCl_2$ and $FeCl_3$]; and 2,2′-azinobis-(3-ethylbenzothiazoline- 6-sulfonate) (ABTS) were obtained from Sigma Chemical Co [St. Louis, MO, USA]. Other chemicals in the present work were obtained from Sigma-Aldrich and Merck.

2.2. Methods

2.2.1. Waste material collection and preparation

Jew's Mallow stems (MS) residues were obtained from the local market (in Egypt), washed with H_2O, cut into ~1cm pieces, and dried for 1 day in an oven at 50°C. The dried sample was separated by a sieve (1 cm) and packed in airtight containers.

2.2.2. Microorganism and maintenance

The isolated strain from sugar cane bagasse *A. flavus* B2 (accession number OL655454) was selected as a potential enzymes- mixture producer as previously reported (Abdel Wahab *et al*., 2023). *A. flavus* B2 was maintained at 4°C on potato dextrose agar medium. Probiotic bacterial strains *B. lactis* and *L. plantarum* were maintained in DeMan, Rogosa, and Sharpe broth medium MRS (De Man *et al*., 1960). The strains were stored in 30 % glycerol at -80°C and then propagated for 24 h at 37°C in a suitable medium before use.

2.2.3. Utilization of MS residues for fungal enzymes and oligosaccharides (OSs) production

Fungal enzymes and oligosaccharides were produced by adding 2 g of MS to 10 mL distilled H_2O in 250 mL Erlenmeyer. After sterilization, each flask was inoculated with 1 mL of cell suspension containing 6×10^8 spores/mL of 5-day-old in 20 mL H_2O of the fungus (*A. flavus* B2). Flasks were incubated at 35°C under static conditions for 7 days. At the end of the fermentation period, the biomass was separated by adding 50 mL H_2O and centrifuged at 10,000 x*g* at 4 °C for 15 min to obtain the clear supernatant, then the enzyme activities were determined and the MSOSs were extracted.

2.2.4. Enzymes activity

2.2.4.1. Xylanase assay

Enzyme solution (0.5 mL) and 0.5 mL of 1 % xylan solution in acetate buffer (0.05 M, pH 5.0) were incubated for 0.5 h at 50°C (Warzywoda *et al*., 1983). The released reducing sugars were estimated as xylose (Neish, 1952). One unit of enzyme activity (U) was specified as the amount of enzyme releasing 1 µ mol of reducing sugars/min.

2.2.4.2. Carboxy methyl cellulase assay

The reaction mixture contained 0.5 mL enzyme solution and 0.5 mL of 1 % carboxy methyl cellulose (CMC) solution in citrate phosphate buffer (0.05 M with pH 5.0) (Hernández-Domínguez *et al*., 2014). The reaction was incubated for 0.5 h at 50°C then analyzed for the reducing sugars released as glucose (Neish, 1952). One unit (U) of enzyme activity was specified as the amount of enzyme releasing 1 µ mol of reducing sugars/min.

2.2.4.3. Pectinase assay

This was done by adding 0.2 mL of enzyme solution to 0.8 mL of 0.5 % citrus pectin in 0.05 M and pH 5.0 of acetate buffer (Silva *et al*., 2005). The reaction was incubated for 10 min at 50°C, and the released reducing sugars as galacturonic acid was estimated (Neish, 1952). One unit of pectinase activity was specified as the amount of enzyme needed to release 1 µ mol of reducing sugars/min.

2.2.5. First statistical design (Plackett-Burman PB) for MSOSs production

We tested the influence of 11 factors on the production of OSs including A is Mallow stems (MS) weight (g/flask), B is baker's yeast (g/L), C is $(NH_4)_2SO_4$ (g/L), D is glucose, E is $MgSO_4$ (g/L), F is K_2HPO_4 (g/L), G is NaCl (g/L), H is $CuSO_4$ (g/L), J is CaCl (g/L), K is Tween 80 (mL/L), and L is incubation time (day). Each of these factors was tested with high level (+1) and low level (−1) resulting in 12 runs. The statistical significance was estimated by F-value, and the proportion of variance explained by the model obtained was given by the multiple coefficients of determination, R^2.

2.2.6. Second statistical design (Central Composite CC) for MSOSs production

We tested the quantitative effect of the most 2 effective factors obtained from the PB design for OSs production including, A: $(NH_4)_2SO_4$ (g/L) and: K_2HPO_4 (g/L). Variables were investigated with 5 levels -1.414, -1, 0, and +1, +1.414 giving 13 runs. Analysis of variance (ANOVA) was used for Statistical analysis of the model.

2.2.7. Extraction of the produced oligosaccharides (OSs)

Extraction of the produced OSs was achieved by mixing fermentation broth (20 mL) with 100 mL pure ethanol, and the mixture was shaken for 1 h at 50 rpm. The collected extracts were vaporized under vacuum, weighed (designated as numbers), and presented to preparative PC on Whatman No.3 filter paper and n- butanol- acetone-water in the ratio 4: 5: 1 (v/v) as a solvent mixture (Jayme and Knolle, 1956). The developed chromatograms were air dried, and two longitudinal narrow strips (from the two sides of each chromatogram) were cut off and sprayed with aniline phthalate reagent (Partridge, 1949). The colored strips were then reassembled as markers, and areas of the unsprayed central portions, which included the individual OSs, were cut out and eluted with a suitable amount of 50 % ethanol. The eluted OSs were concentrated under vacuum.

2.2.8. Chemical characterization of the produced MSOSs

2.2.8.1. Oligosaccharides yield

After the fermentation period, the culture filtered was dried, weighed (W2), and re-dissolved in 10 ml distilled water, then treated with ethanol (3 volumes). After centrifugation, the resulting precipitate was dried, weighed (W1), and designated as numbers. The OSs yield is calculated (Dubois *et al*., 1956) as follows Eq. (1):

$$\text{Yield (\%)} = (W1/W2) \times 100 \quad (1)$$

2.2.8.2. Total carbohydrates

The total carbohydrates of the various MSOSs were determined by adopting the method of phenol-sulfuric acid (Dubois *et al*., 1956). Sample (1 mL) was added to 1 mL phenol solution (5 %), thereafter concentrated H_2SO_4 (5 mL), was added quickly. The reaction was shaken and kept at room temperature for 10 min and then at 25-30°C for 20 min in a water bath. The color density was estimated at 480-490 nm. Quantities were determined using standard curves resulting from measurements on a solution containing proper sugars in appropriate ratios for each extract of OSs.

2.2.8.3. Soluble protein

The soluble protein was estimated by the colorimetric method using pure bovine albumin as the standard curve (Lowry *et al*., 1951).

2.2.8.4. Monosaccharide constituents

For acid hydrolysis, 0.5-1.0 mg of MSOSs were heated in a sealed glass tube at 100°C with 2 mL H_2SO_4 (2 M) for 6 h followed by neutralization using $BaCO_3$ (Perila and Bishop, 1961). The hydrolysates were centrifuged, filtered, neutralized (with Dowex 50 resin H^+), concentrated under vacuum, and presented to quantitative paper chromatographic analysis (Whatman no.1) (Wilson, 1959) using n-butanol- acetone- water in the ratio 4: 5: 1 (v/v/v) as running solvent. The components of monosaccharides were clarified after spraying the chromatogram with aniline phthalate (Partridge, 1949).

2.2.8.5. Degree of Polymerization (DP)

One ml (100 µg/ mL) of each OS was added to sodium borohydride solution (0.5 mL, 1 %), and reduction was allowed at room temperature (in a dark place) for 1 h. Another similar set of samples was added to 2 N H_2SO_4 acid (0.5 mL) at the same time. Add to both groups 1mL of aqueous phenol (3 %) followed by concentrated H_2SO_4 acid (5 mL). After careful mixing, the solutions were kept at room temperature for 10 min, and then left to cool for 25 min. Absorbance measurements at 480 nm were done, and the average was used for calculating the DP of the OSs (El Azm *et al*., 2019) as follows in Eq. (2):

$$Q = A1/A2 \quad (2)$$

Where: Absorbance of the OS hydrolysate before reduction (A1) and after reduction (A2) as follows in Eq. (3):

$$DP = Q/(Q-1) \quad (3)$$

2.2.9. Biological activity of the extracted MSOSs

2.2.9.1. Anticoagulation efficiency

The anticoagulation activity of the MSOSs was estimated according to the clot formation time and compared with standard heparin sodium preparation (USA Pharmacopoeia, 1960). Glass test tubes (dimensions 31×100 mm) were cleaned by immersion for 1 day in chromic acid. To each tube add 0.8 mL of 0.01 % MSOS solution, saline solution (0.9 %), or standard Heparin solution (1.4 U/mL). Next, to each prepared tube add 1 mL of human plasma and 0.2 mL of 1 % $CaCl_2$ solution and incubate in a water bath at 37°C. Heparin (Sodium heparin) was used as a standard. For control, the experimental extract solution was replaced with the same volume of saline. The blood clot samples were then observed visually for 1 h at room temperature for any noticeable changes. The time period required for clot formation was recorded by a stopwatch by tilting the test tubes every 5 seconds. Each result is representative of at least three separate experiments. Values represent the mean of these experiments, and the results were expressed as the average of three readings.

2.2.9.2. Fibrinolytic efficiency

This was performed by exposing the plasma clot to the effect of MSOSs, an aqueous solution with proper concentration (USA Pharmacopoeia, 1960). Three glass test tubes (dimensions 31×100 mm) were cleaned by

immersion overnight in chromic acid. First, 1 mL of human plasma was mixed with 0.2 mL of 1 % $CaCl_2$ and 0.8 mL of saline solution (0.9 %) and incubated at 37°C in a water bath for fibrin clot formation. When coagulation was completed, 1 mL of Hemoclar (2 mg/tube), saline solution (0.9 %), or the MSOS (2 mg/tube) was individually added. Plasma fibrin clot with saline was used as control. Percentages (%) of plasma clots lysis at 37°C were determined with each MSOS and compared with those of standard Hemoclar (pentosane sulfuric polyester).

2.2.9.3. Prebiotic activity

The growth-enhancing effect of MSOSs on *B. lactis* and *L. plantarum* was performed by cultivating strains in MRS broth medium (10^7 CFU/ mL) fortified with filter sterilized MSOSs at different concentrations (0.1, 1, and 10 %) instead of glucose (as C-source) at 37°C for 24 h under anaerobic conditions. Then, the turbidity (bacterial growth) was determined by a spectrophotometer at 620 nm against a blank composed of an un-inoculated medium (Riaz *et al*., 2010).

2.2.9.4. Antioxidant activity (In vitro)

2.2.9.4.1. DPPH free- radical scavenging

It was estimated by adding 1 mL of methanolic DPPH solution (freshly prepared 20 µg/ mL) to 0.5 mL (2.5 mg) of the tested MSOSs (Williams *et al*., 1995). After 5 min of reaction, the color removal process was estimated at 517 nm. The ability to scavenge the DPPH radical (%) was calculated using the following Eq. (4):

DPPH (%) = [Absorbance of control − Absorbance of sample / Absorbance of control] ×100 (4)

2.2.9.4.2. Reducing power

The tested MSOS (2.5 mg/ 0.5 mL) was added to 2.5 mL of phosphate buffer (0.2 M and pH 6.6), and 2.5 mL of 1 % potassium ferricyanide. The reaction was incubated for 20 min at 50°C. 2.5 mL of aliquots of 10 % trichloroacetic acid were placed into the reaction and centrifuged at 1000 rpm for 10 min. 2.5 mL of the upper layer of solution was added to an equal volume of (0.5 mL of freshly prepared 0.1 % $FeCl_3$ solution and distilled H_2O). The intensity was estimated at 700 nm of the blue-green color (Oyaizu, 1986). According to the tested MSOS reducing power, the yellow color changes to green in the solution.

2.2.9.4.3. Metal chelating

For chelating of ferrous ions of MSOSs, 0.5 mL of each MSOSs extract (2.5 mg) was added to 0.05 mL of 2 mM $FeCl_2$ solution (Dinis *et al*., 1994). The reaction was started by adding 0.2 mL of 5 mM ferrozine, then shaking the mixture vigorously and leaving for 10 min at room temperature. The absorbance of the solution was estimated at 562 nm by spectrophotometer. The inhibition percentage (%) of the ferrozine-ferrous complex of each MSOS sample was determined as following Eq. (5):

Inhibition % = [Absorbance of control − Absorbance of sample / Absorbance of control] ×100 (5)

2.2.9.4.4. ABTS radical scavenging

ABTS radical scavenging assay was performed by the ABTS cation decolorization test with some modifications (Re *et al*., 1999). The stock solutions contained 7 mM of ABTS solution and 2.4 mM of $K_2S_2O_8$ solution. The working solution was made ready by mixing equal quantities of the two stock solutions and kept to react for 12 h in a dark place at room temperature. Then, 60 mL of methanol was added to 1 mL ABTS radical solution to obtain an absorbance of 0.706±0.001 units at 734 nm. 0.5 mL of 0.1 % tested MSOSs was reacted with 2.5 mL of ABTS reagent for 7 min and the absorbance was estimated at 734 nm using a spectrophotometer. The ABTS radical cation color removal ability of the MSOS and percentage inhibition were estimated as following Eq. (6):

ABTS (%) = [AC − AS / AC] × 100 (6)

Where: AC, absorbance of the ABTS radical cation methanol and AS, absorbance of the ABTS radical cation sample extract.

2.2.9.4.5. FRAP

The FRAP (Ferric ion Reducing Antioxidant Power) assay was done with some modifications (Benzie and Strain, 1999). The stock solutions contained 300 mM of acetate buffer pH 3.6, 10 mM of TPTZ solution in 40 mM of HCl, and 20 mM of $FeCl_3.6H_2O$ solution. The working solution was ready by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of $FeCl_3.6H_2O$ solution and heating it before using it at 37°C. The tested MSOSs (2.5 g/0.5 mL) was reacted with 2.5 mL of FRAP solution in a dark place for 30 min. The produced color of the ferrous TPTZ complex was estimated at 593 nm and the results are expressed in µ mol Trolox/100 g dry matter.

2.2.9.4.6. 2.2.9.5. Antitumor activity In Vitro (Trypan blue exclusion test)

The trypan blue exclusion test was used to detect cell viability. The tumor cell suspension was obtained from peritoneal cavities of tumor-bearing mice and then diluted with phosphate-buffered saline pH 7.0, so the final preparation included 2.5×10^5 cells/ 0.1 mL. In a set of sterile test tubes, 0.1 mL/tube of the cell preparation was dispersed, followed by the addition of 0.8 mL aliquots of phosphate-buffered saline (/tube). The tested MSOSs (0.5mg) were used in tubes at 0.1 mL/ tube. The sample tubes were incubated for 2 h at 37°C under 5% CO_2 for 2 h. After that, centrifuged the tubes (5 min at 1000 rpm) the separated cells were suspended in saline. For each tube (control and examined), a new clean, dry small test tube was used, and 0.1 mL of cell suspension, 0.8 mL of saline, and 0.1 mL trypan blue were added and mixed. Then, the number of living cells was estimated by a hemocytometer slide. Viable cells appeared as unstained bodies, whereas nonviable cells appeared as blue bodies (El-Merzabani *et al*., 1979).

3. Results and discussion

Industrial applications of enzymes have been restricted by their high production cost (Ahmed *et al*., 2022). The plant cell wall structure is a complex polysaccharides composed mainly of hemicellulose, cellulose, and pectic substances. Therefore, hemicellulases, cellulases, and pectinases enzymes play an essential role in the hydrolysis of agricultural residues (Abdel Wahab *et al*., 2023). The enzymes- mixture of pectinase 3.33 U/mL, cellulase 1.74 U/mL, and xylanase 1.31 U/mL was produced by *A. flavus* B2 using MS residues as substrate under SSF as previously reported (Abdel Wahab *et al*., 2023), in addition to 150.2 mg of oligosaccharides. In the current study, the statistical

improvement of MSOSs production was evaluated by enzymes- mixture hydrolysis.

3.1. First statistical design (Plackett-Burman PB) for MSOSs production

As presented in Table 1, the interaction between the 11 factors has remarkable and different effects on oligosaccharides production.

Table 1. Plackett-Burman (PB) design for Mallow stems oligosaccharides (MSOSs) production.

Run	A MS weight , g/flask	B Baker's yeast , g/L	C $(NH_4)_2SO_4$, g/L	D Glucose, g/L	E $MgSO_4$, g/L	F K_2HPO_4, g/L	G NaCl, g/L	H $CuSO_4$, g/L	J CaCl, g/L	K Tween 80, mL/L	L Incubation time, day	MSOS weight, mg
1	1	10	10	10	0	0	0	0.2	0	1	7	349.99
2	2	0	0	0	0.5	0	2	0.2	0	1	7	205.00
3	1	0	0	0	0	0	0	0	0	0	4	115
4	2	10	10	0	0	0	2	0	0.5	1	4	405
5	1	10	0	10	0.5	0	2	0.2	0.5	0	4	190
6	1	10	10	0	0.5	5	2	0	0	0	7	408
7	2	0	10	10	0.5	0	0	0	0.5	0	7	280
8	2	10	0	0	0	5	0	0.2	0.5	0	7	284.99
9	1	0	0	10	0	5	2	0	0.5	1	7	325.00
10	2	0	10	10	0	5	2	0.2	0	0	4	350
11	1	0	10	0	0.5	5	0	0.2	0.5	1	4	410
12	2	10	0	10	0.5	5	0	0	0	1	4	295

Maximum MSOSs production was obtained in run 11 (410 mg), causing a 2.73-fold increase in production. Nine factors (B: baker's yeast, C: $(NH_4)_2SO_4$, D: glucose, F: K_2HPO_4, G: NaCl, H: $CuSO_4$, J: $CaCl_2$, K: Tween 80, L: incubation time) of the eleven tested factors have a significant effect on OSs production. The maximum MSOSs production (run 11) is related to the action of enzymes- mixture (U/mL) pectinase 2.97, xylanase 2.33, and cellulase 0.69.

As seen in the Pareto chart (Figure 1), the other two factors (A: MS weight and E: $MgSO_4$) did not have a significant effect. The seven factors that enhanced MSOSs production included baker's yeast, $(NH_4)_2SO_4$, K_2HPO_4, NaCl, $CaCl_2$, Tween 80, and incubation time, while glucose and $CuSO_4$ showed a negative effect on MSOSs production.

Oligosaccharide weight (mg) = +303.75 +22.92 * baker's yeast +67.92 * $(NH_4)_2SO_4$ -5.42* glucose +46.25 * K_2HPO_4 +14.58 * NaCl -5.42 * $CuSO_4$ +12.08 * $CaCl_2$ +27.92* Tween 80 + 9.58 * incubation time.

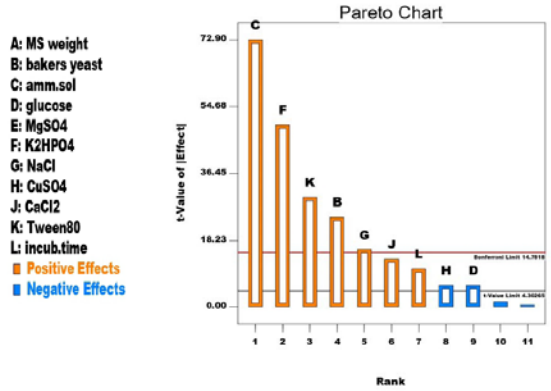


Figure 1. Pareto chart for production of Mallow stems oligosaccharides by PB design.

The enhancing effect of $(NH_4)_2SO_4$, K_2HPO_4, NaCl and $CaCl_2$ on MSOSs can be attributed to the positive significant effect of these factors on *A. flavus* CMCase, xylanase, pectinase production as described in previous work(Abdel Wahab *et al.*, 2023) since MSOSs are the hydrolyzed products of these enzymes. The negative effect of $CuSO_4$ and glucose on MSOSs production can be a result of the more enhancing effect of these factors on *A. flavus* enzymes production causing more hydrolysis for MS polysaccharides producing monosaccharides not oligosaccharides.

Mallow stems are suitable residues, inexpensive, and available abundantly in Egypt and other countries (Ahmed and Mostafa, 2013). This step of factorial production optimization improved MSOSs production by 2.73-fold. The significance of the design for MSOSs production was statically analyzed by ANOVA (Table 2). Values of "Prob

> F" are <0.0500 indicating that bakers yeast, $(NH_4)_2SO_4$, glucose, K_2HPO_4, NaCl, $CuSO_4$, $CaCl_2$, tween 80 and incubation time are significant model terms. Moreover, the R2 value (0.9998) for the production of oligosaccharides, respectively, showed the significance of the model since the R2 value was very close to 1.Also, the value of predicated R^2 (0.9927) was reasonably consistent with the adjective R^2 (0.9989).

Table 2. ANOVA for PB design for Mallow stems oligosaccharides production.

Source	Sum of Squares	df	Men Square	F value	p-value Prob>F	
Model	102785.2117	9	11420.57907	1096.639	0.0009	significant
B-baker's yeast	6301.900001	1	6301.900001	605.1276	0.0016	
C-$(NH_4)_2SO_4$	55351.81167	1	55351.81167	5315.049	0.0002	
D- glucose	352.0833333	1	352.0833333	33.808113	0.0283	
F-K_2HPO_4	25668.75	1	25668.75	2464.792	0.0004	
G-NaCl	2552.200001	1	2552.200001	245.07	0.0041	
H-$CuSO_4$	352.1050003	1	352.1050003	33.81019	0.0283	
J-$CaCl_2$	1752.083333	1	1752.083333	168.2404	0.0059	
K-Tween 80	9352.195	1	9352.195	898.0262	0.0011	
L-incubation time	1102.083333	1	1102.083333	105.8254	0.0093	
Residual	20.82833367	2	10.41416683			
Cor Total	102806.04	11				

R^2 0.9998, Adj R^2 0.9989, Pred R^2 0.9927

3.2. Chemical characterization of produced MSOSs by BP design

As presented in Table 3, the chemical characterization of MSOSs indicated that the interaction between the eleven factors successfully produced 12 oligosaccharides that differed in chemical characteristics.

3.3. Monosaccharide constituents

The produced MSOSs differ in their monosaccharides constituents and percentage as illustrated in Figure 2A. I.e. some oligosaccharides composed of 4 monosaccharides constituents (galacturonic acid, galactose, glucose, and xylose), 3 monosaccharides constituents (galacturonic acid, galactose, and glucose), 2 monosaccharides constituents (galacturonic acid and glucose) or (galactose and xylose) with different percent. Differences in the composition of oligosaccharides can be attributed to the different enzymes produced during fermentation. I.e galacturonic acid as pectin monosaccharide component due to the action of pectinase enzyme, glucose as monosaccharide component of cellulose due to the action of cellulase enzyme and xylose as monosaccharide component of xylan due to the action of xylanase enzyme. Silva *et al*. (2015) reported that hydrolysis of oat spelts xylan by xylanases from by *T. inhamatum* produced larger xylooligosaccharides, xylobiose, xylotriose, and xylotetrose.

Table 3. Chemical characterization of the extracted Mallow stems oligosaccharides by PB design.

Run	Yield (%)	D.P	Analytical characteristics (%)	
			Total carbohydrate	Soluble protein
1	65.57	9	68.70	21.30
2	28.41	6	64.52	25.44
3	28.41	6	64.52	25.44
4	13.58	2	44.30	45.70
5	28.41	6	64.52	25.44
6	28.41	6	64.52	25.44
7	53.57	4	54.00	36.00
8	44.91	6	65.10	25.00
9	28.41	6	64.52	25.44
10	50.57	4	56.10	43.00
11	63.41	4	63.00	37.00
12	75.59	4	56.80	43.00

* D.P: Degree of Polymerization

3.4. Second statistical design (Central Composite CC) for MSOSs production

The quantitative effect of the most two effective factors obtained from the PB design including, (A) $(NH_4)_2SO_4$ (g/L) and (B) K_2HPO_4 (g/L) for oligosaccharides was studied. As presented in Table 4, the interaction between the quantitative effect of the highly effective factors $(NH_4)_2SO_4$ and K_2HPO_4 led to a significant variation in oligosaccharides production resulting in a 3.50-fold increase (run 4) in oligosaccharides production compared to the un-optimized medium. The highest production of MSOSs was due to the action of enzymes-mixture (pectinase 12.89 U/mL, cellulase 4.9 U/mL, and xylanase 6.76 U/mL).

Oligosaccharide weight (mg) can be calculated from the following equation:

Oligosaccharides weight (mg) = +375.00 +122.08* $(NH_4)_2SO_4$ +49.65 * K_2HPO_4 -80.73* $(NH_4)_2SO_4$ * K_2HPO_4 -21.46 * $(NH_4)_2SO_4{}^2$ -39.82 * $K_2HPO_4{}^2$

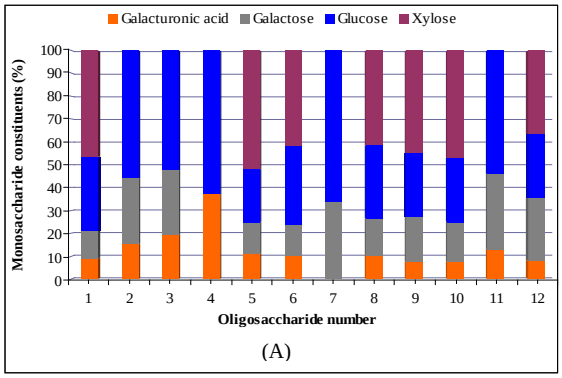
The significance of the design for oligosaccharides production was statically analyzed by ANOVA (Table 5). Values of "Prob > F" are < 0.0500 decided that A, B, AB, A^2, B^2 are significant model terms. Moreover, the value of R^2 (0.9930) indicated the significance of the model since the R^2 value was very near to 1. Also, values of predicated R^2 (0.9506) were reasonably consistent with the adjective R^2 (0.9881).

Table 4. Central Composite (CC) design for Mallow stems oligosaccharides production.

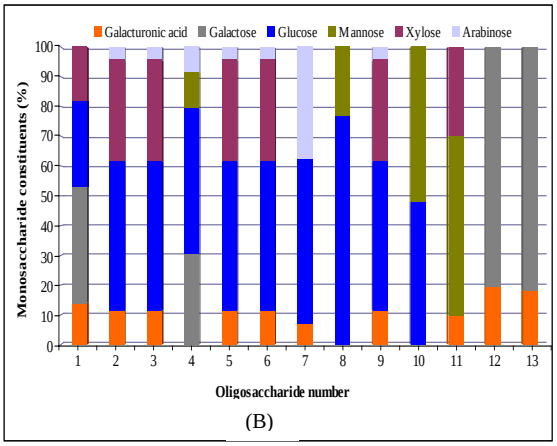
Run	Factor 1 A: $(NH_4)_2SO_4$ [g/L]	Factor 2 B: K_2HPO_4 [g/L]	Oligosaccharide Weight [mg]
1	5	2	70
2	10	6	375
3	10	6	375
4	17.071	6	525
5	10	6	375
6	10	6	375
7	5	10	322.5
8	15	2	455
9	10	6	375
10	2.929	6	150.55
11	10	11.657	377.11
12	15	10	384.6
13	10	0.343	225

3.5. Chemical characterization of MSOSs extracted by CC design

As shown in Table 6, the chemical characterization of MSOSS reported that the interaction between the highly effective factors (two) successfully produced 13 oligosaccharides that differed in their chemical characteristics.



(A)



(B)

Figure 2. Monosaccharides constituents (A) in MSOSs produced by PB design **(B)** in MSOSs produced by Central Composite design.

Table 5. ANOVA for CC design for Mallow stems oligosaccharides production.

Source	Sum of Squares	df	Mean Square	F Value	p-value prob>F	
Model	177922.8	5	35584.56	199.990561	< 0.0001	significant
A-$(NH_4)_2SO_4$	119231.2	1	119231.2	670.097185	< 0.0001	
B-K_2HPO_4	19722.57	1	19722.57	110.843796	< 0.0001	
AB	26066.1	1	26066.1	146.495388	< 0.0001	
A^2	3203.698	1	3203.698	18.0052616	0.0038	
B^2	11030.49	1	11030.49	61.9929798	0.0001	
Residual	1245.519	7	177.9312			
Lack of Fit	1245.519	3	415.1728			
Pure Error	0	4	0			
Cor Total	179168.3	12				

Table 6. Chemical characterization of the extracted Mallow stems oligosaccharides by CC design.

Run	Yield (%)	D.P	Analytical characteristics (%)	
			Total carbohydrate	Soluble protein
1	1.4	9	88.38	8.75
2	7.5	2	41.72	10.27
3	7.5	2	41.72	10.27
4	10.5	4	30.6	16.73
5	7.5	2	41.72	10.27
6	7.5	2	41.72	10.27
7	3.8	2	73.62	6.08
8	9.1	3	38.7	1.52
9	7.5	2	41.72	10.27
10	4.3	3	79.38	7.98
11	10.7	3	42.3	6.46
12	5.1	2	34.38	5.32
13	4.5	2	58.5	2.28

* D.P: Degree of Polymerization

3.6. Monosaccharide constituents

The produced MSOSs differed in their monosaccharides' constituents and percentage as illustrated in Figure 2B. Some oligosaccharides were composed of 4 monosaccharides, 3 monosaccharides, and 2 monosaccharides with different percent. The difference in oligosaccharide composition can be attributed to the different enzymes produced during fermentation. Galacturonic acid was produced as the monosaccharide component of pectin due to the action of pectinase, xylose as the monosaccharide component of xylan due to the action of xylanase, and glucose as the monosaccharide component of cellulose due to the action of cellulose. The great diversity in oligosaccharides' structure and chemical composition, as well as their safety and biodegradability, make them ideal for diverse applications (Rivas *et al.*, 2021).

3.7. Biological activity of MSOSs

3.7.1. Anticoagulation efficiency

The anticoagulation efficiency of the produced MSOSs was evaluated by estimating the coagulation times for a mixture of plasma-standard heparin, plasma-tested samples, and plasma-saline (as control). The results in Table 7 indicated that most of the MSOSs tested exhibited either weak or even no anti-coagulation activity. The coagulation times recorded for a mixture of plasma with each tested MSOSs ranged from 3 min and 1 sec to 13 min and 31 sec compared to 3 min (in control experiments) and 90 min for standard heparin. From the results in Table 7, MSOSs no. 8, 10, and 12 showed the highest anticoagulant activity. This result may be because MSOSs (8, 10, and 12) inhibit platelet aggregation. Chegu *et al.* (2018) reported that aqueous extract of ginger showed lower anticoagulant activity (4.15 min) than our result. Polysaccharides isolated from clove may have antithrombic effects as reported by Chegu *et al.* (2018). Helal *et al*. (2020) and Abd El-Galil *et al*. (2021) reported that aqueous extracts of some plants (ginger and Pea peel) had no anticoagulant activity.

3.7.2. Fibrinolytic activity

Fibrinolytic activities of the produced oligosaccharide were estimated as lysis (%) of plasma. A commercial sample of Hemoclar, a sulfated pentosan polyester, was set for comparison in a simultaneous experiment as a standard fibrinolytic agent. Amongst the tested materials (Table 7), MSOS no. 4 exhibited fibrinolytic activity (80 % lysis) which is higher than that of Hemoclar the standard sample (50 % lysis). Also, MSOS no. 11 demonstrated fibrinolytic activity equal to that of the standard "Hemoclar" sample. In contrast, Abd El-Galil *et al*. (2021) reported that aqueous oligosaccharide extracts from pea peel had no fibrinolytic activity. It was observed that eleven MSOS products showed lower fibrinolytic activity (Lysis ≤ 25 % of plasma clot) than the standard Hemoclar sample.

Table 7. Anticoagulation and fibrinolytic activity of the Mallow stems oligosaccharides produced by CC design.

Run	Anti-coagulation activity clotting time (min:sec)	Fibrinolytic activity lysis of plasma clot (%)
Control	03:00	< 25
1	03:01	< 25
2	10:33	25
3	10:33	25
4	06:42	80
5	10:33	25
6	10:33	25
7	11:15	< 25
8	13:26	< 25
9	10:33	25
10	13:31	< 25
11	03:12	50
12	12:40	< 25
13	03:13	< 25

***** Control: with saline

3.7.3. Prebiotic activity

The effect of MSOSs on probiotics growth (*L. plantarum* and *B. lactis*) as illustrated in Figure 3 (A and B) differed with the oligosaccharide concentration and probiotic. Firstly, all tested MSOSs had a growth-promoting effect on both probiotics with a 1 % concentration. Gobinath *et al*. (2010) and Pourabedin *et al*. (2015) reported that diets containing oligosaccharides selectively increased the population of lactobacilli and bifidobacteria. Secondly, the growth-promoting effect was higher on *B. lactis* that MSOS no. 8 caused 43 % enhancement in growth compared with 23.71 % on *L. plantarum* with MSOS no. 4 and 12 in contrast to that obtained by Saleh *et al*. (2020). Finegold *et al*. (2014) showed that in the human gut microbiota, xylooligosaccharide increased bifidobacterial but not lactobacilli. Voragen (1998) reported that the difference in the effect of MSOSs on the growth of probiotics may be due to differences in the DP, chemical structure of saccharides, water solubility, and constituent of monosaccharide units.

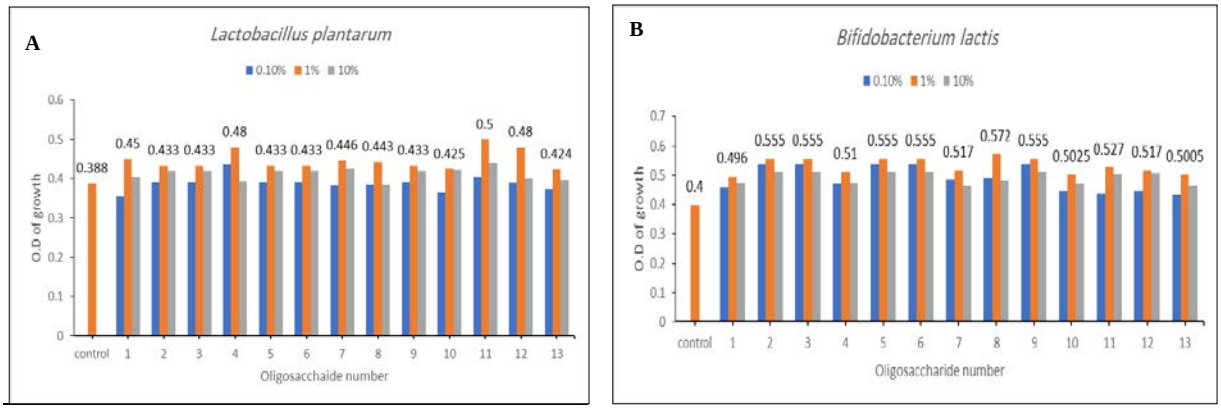


Figure 3. Prebiotic effects of Mallow stems oligosaccharides on probiotics, (A) *L. plantarum*, (B) *B. lactis.*

3.7.4. Antioxidants activity

3.7.4.1. DPPH free- radical scavenging

The antioxidant activity of oligosaccharides attracts much attention. Xiong *et al*. (2013) reported that hydroxyl and special functional groups in the chain are the major sources of polysaccharides' antioxidant activities. Sun *et al*. (2004) found that degradation enhances the oligosaccharide antioxidant activities by increasing the number of activated hydroxyl groups. The antioxidant

ability of oligosaccharides is affected by the assay method, so it cannot be fully qualified by one method. One compounds that contains a free proton radical with an absorbent property is DPPH which is significantly reduced upon exposure to proton radical scavengers. Moreover, it is well accepted that DPPH molecules are scavenged by antioxidants due to their ability to donate hydrogen (Xiong *et al*., 2013). DPPH is a stable free radical concentrated in nitrogen, it changes color from violet by reduction to yellow (by hydrogen capture or electron donation). Results seen in Figure 4A indicate that MSOS no. 11 and 1 possess the highest DPPH at 33.24±0.24 and 29.31±0.21 %, respectively, at concentration (5mg/mL). All the other MSOSs exhibited various activities of DPPH radical scavenging ranging from 10.8±0.17 % for MSOS no. 13 to 26.36 ±0.21 % for MSOS no. 12. Abd El-Galil *et al.* (2021) reported that aqueous oligosaccharide extracts were highly antioxidant as DPPH radical scavenging.

3.7.4.2. Reducing Power

The antioxidants ability to donate electrons is evaluated by reducing power tests (Dorman *et al*., 2003). Antioxidant compounds reduce ferric to ferrous form (Fe^{3+} to Fe^{2+}) due to their reductive abilities. Prussian blue-colored complex is formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. The presence of reductants in the solution reduces the reduction of the compound (ferric/ferricyanide) to the ferrous form which can be determined by estimating the formation of Perls' Prussian blue at 700 nm. All investigated MSOSs exhibited good reducing power activity specially MSOS no. 11 and 7 which recorded 1.169±0.036 and 0.97±0.012 at concentration (5 mg/mL) (Figure 4B). The reducing power ability of MSOSs in the central treatments exhibited 0.95±0.032. MSOS no. 4 recorded the least reducing power activity (0.671± 0.020). Our results are higher than those obtained from *Leucaana leucocephala* leaves extract (Mohammed *et al*., 2015).

3.7.4.3. Metal chelating

For life, iron is necessary for respiration, oxygen transport, and the activity of many enzymes. Moreover, it is a highly reactive metal and will stimulate oxidative changes in proteins, lipids, and other cellular components (Decker and Welch, 1990). Divalent transition metal ions play a pivotal role as catalysts of oxidation processes. These processes can be delayed by iron chelation and deactivation. Transition metal iron generates free radicals from peroxides by Fenton reactions and may have a role in human cardiovascular disease (Halliwell, 1997). Among all investigated MSOSs, only the central 2, 3, 5, 6, and 9 possess iron metal chelating activity (35.47±0.13 %) at concentration (5 mg/mL) (data not shown). Xiong *et al.* (2013) reported that the antioxidant ability of xanthan oligosaccharides is useful in expanding their biomedical applications.

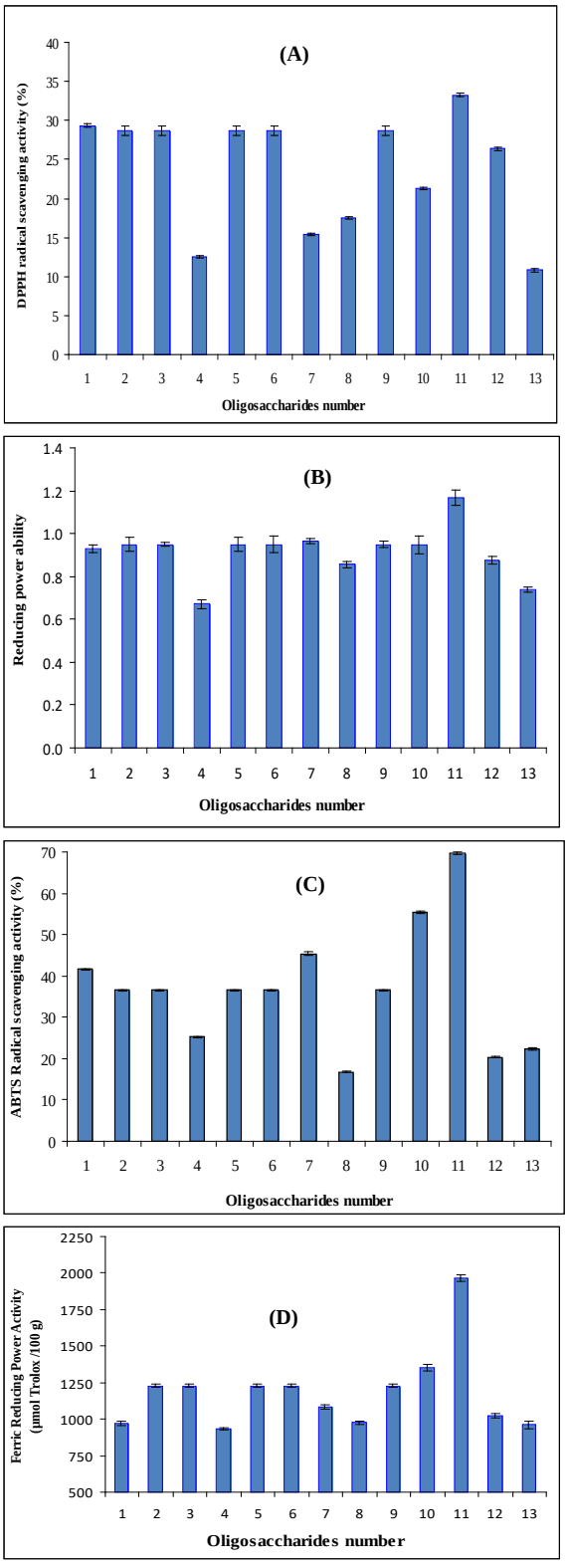


Figure 4. Antioxidant activity of Mallow stems oligosaccharides DPPH radical scavenging (A), Reducing power (B), ABTS radical scavenging (C), and FRAP (D).

3.7.4.4. ABTS radical scavenging

The ability of the MSOSs to scavenge free radicals was quantified by the ABTS test. A sample with ABTS free radical-scavenging activity confirms that it is a hydrogen donor and stopped the process of oxidation by converting the free radicals into more stable products (Suganya *et al*., 2007). Re *et al*. (1999) found that the lack of absorbance is measured after combining ABTS with the antioxidant for a few minutes, after which absorbance stabilizes. Figure 4C displays the ABTS activities of MSOSs extracts and confirms that all investigated treatments possess ABTS radical scavenging ability. As shown in the results, MSOS no. 11 followed by 10 recorded the highest ABTS activity (69.67± 0.24 and 55.41± 0.24 %, respectively). At the same concentration, MSOS no. 8 exhibited the lowest ABTS activity (16.75± 0.34 %). Thetsrimuang *et al*., (2011) reported that reducing sugar in medicinal plants is an important factor possessing antioxidant activity, and higher reducing sugar content means higher antioxidant ability.

3.7.5. Antitumor activity (In Vitro)

Ehrlich's ascites carcinoma cells are one of the most common experimental tumors used for modeling because they resembles undifferentiated human tumors and have a rapid growth rate (Ozaslan *et al*., 2011). As seen in Figure 5, a similar approach was noted in the investigated MSOSs antitumor activity using a viability test of Ehrlich ascites carcinoma cells. The viability of tumor cell was decreased and ascites was inhibited by MSOS no. 11 (37.24±0.31 %), which is considered to have the highest effect on the viability of the cells among all investigated MSOSs, followed by MSOS no. 8 and 10 which exhibited 30.14±0.24 and 25.38±0.23 % dead cells, respectively at concentration 5mg/mL. The recorded results agreed with the hypothesis that the investigated MSOSs exert their antitumor effects as they have antioxidant activity which can be attributed to the radical activity of the substituted sugar (concentration and type).

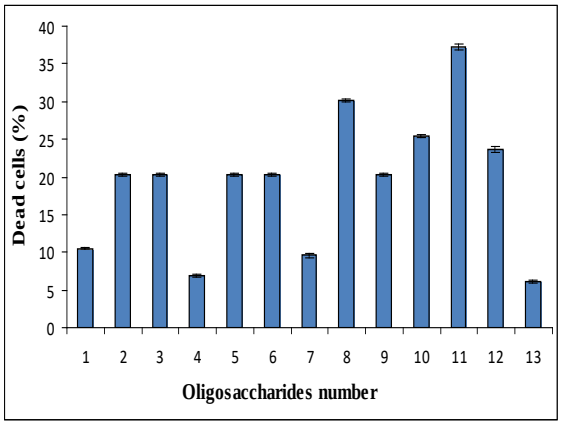


Figure 5. Effect of different Mallow stems oligosaccharides on the EACC viability.

4. Conclusion

It is an economical and eco-friendly way to use Mallow stems which are agro-industrial residues, as an inexpensive substrate for enzymes-mixture and oligosaccharides production. Thirteen oligosaccharides (with a 3.5-fold increase) were successfully obtained from MS by enzymatic hydrolysis produced by an isolated strain (*A. flavus* B2) via RSM. MSOSs extracts can represent a source of bioactive materials. All MSOSs had a growth-stimulating effect on both probiotics *B. lactis* and *L. plantarum* (highest stimulation at a concentration of 1 %), so it can be used in the food industry. In addition, anticoagulation and fibrinolytic activities were evaluated for the MSOSs. MSOS no. 4 and 11 exhibited fibrinolytic activity higher than and equal to that of Hemoclar. Furthermore, MSOSs showed antitumor activity by lowering the Tumor cell viability by 37.2% (MSOS no. 11) without side effects. Based on the results, it can be concluded that sugar content and type may have a role in the antitumor and antioxidant activities of the investigated oligosaccharide.

Declaration of competing interest

There is no conflict of interest.

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