Bioactive Carbohydrates and Dietary Fibre 24 (2020) 100238

Contents lists available at ScienceDirect

Bioactive Carbohydrates and Dietary Fibre

journal homepage: http://www.elsevier.com/locate/bcdf

Fructooligosaccharides synthesized by fructosyltransferase from an indigenous coprophilous *Aspergillus niger* strain XOBP48 exhibits antioxidant activity

Jeff Ojwach [a,*], Ajit Kumar [a], Samson Mukaratirwa [a,b], Taurai Mutanda [a,c]

[a] Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal (Westville Campus), Private Bag X54001, Durban, 4000, South Africa
[b] One Health Center for Zoonoses and Tropical Veterinary Medicine, Ross University School of Veterinary Medicine, Basseterre, West Indies
[c] Centre for Algal Biotechnology, Department of Nature Conservation, Faculty of Natural Sciences, Mangosuthu University of Technology, P.O. Box 12363, Jacobs, 4026, Durban, South Africa

ARTICLE INFO

Keywords:
Fructosyltransferase
Biotransformation
Fructooligosaccharides
Antioxidants
Nutraceutical

ABSTRACT

Fructooligosaccharides (FOS) produced via sucrose biotransformation by partially purified Fructosyltransferase (Ftase) from an indigenous coprophilous *Aspergillus niger* strain XOBP48 (*An*XOBP48) are reported to have antioxidant and nutraceutical properties in the present study. The Ftase activity on sucrose yielded FOS identified as monomeric glucose, 1- kestose (GF_2), and 1,1– kestotetraose (GF_3) which were further purified and quantified using HPLC-RI system. The antioxidant activities of purified FOS at concentrations of 1, 1.5, 3, 6, 12, 24 and 48 μg/ml were determined by applying three experimental models and comparing their properties with FOS standards and vitamin C. The free radical scavenging activity measured by 1,1 - diphenyl-2-picryl hydroxyl (DPPH) assay, ferric reducing antioxidant power (FRAP) assay and nitric oxide (NO) radical inhibition assay, yielded IC_{50} values of 2.6 μg/ml, 3.9 μg/ml and 3.4 μg/ml, 3.8 μg/ml and 0.69 μg/ml, 0.74 for purified GF_2 and GF_3, respectively. The free radical scavenging and inhibition activities showed a concentration dependent antioxidant activity of purified FOS with no significant differences as compared to the standards $p < 0.01$ and vitamin C. In conclusion, the results demonstrated that purified FOS could be exploited in biotechnological applications and/or as to be used as functional ingredients in commercial products such as the production of nutraceutical compounds due to their potential antioxidant properties.

1. Introduction

The biofunctional foods with prebiotic effects have received great attention due to their health-promoting properties in the human gut besides their basic nutrition (Witschinski et al., 2018). Fructooligosaccharides (FOS) are the most studied prebiotics because they are non-digestible carbohydrates that promote the growth of beneficial bacteria in the host gut leading to improved health while concurrently suppressing the proliferation of potentially pathogenic bacteria (Gibson et al., 2010; Guerreiro et al., 2016; Patel & Goyal, 2011; Roberfroid & Slavin, 2000). Moreover, a panel of experts in nutrition, clinical research, and microbiology recently reviewed the scope of the prebiotic definition to a non-viable food component or a substrate that is selectively utilized by host microorganisms conferring a health benefit on the host associated with modulation of the microbiota (Gibson et al., 2017). Prebiotics target human-associated and animal-associated microbiota to improve health. Besides nutritional benefits, they are also found to modulate the immune status by decreasing oxidative stress or increase the antioxidant potential and improve colonic morphology by scavenging for free radicals (Abasubong et al., 2018; Choudhary et al., 2018; Guerreiro et al., 2016; Kumar, Jain, & Sardar, 2018).

Dietary antioxidants are considered essential nutraceuticals due to their health-promoting properties. This is the major reason why they are widely assimilated in food industries as inhibitors of lipid peroxidation, and their ability to scavenge for free radicals. They also prevent other malignant that are cytotoxic including scavenging for reactive oxygen species (ROS) that may trigger redox homeostatic disturbance leading to cell degeneration (Czarnocka & Karpiński, 2018; Guerreiro et al., 2016;

* Corresponding author.
E-mail addresses: ojwachj@gmail.com (J. Ojwach), ajitkanwal@yahoo.com (A. Kumar), mukaratirwa@ukzn.ac.za (S. Mukaratirwa), mutanda.taurai@mut.ac.za (T. Mutanda).

https://doi.org/10.1016/j.bcdf.2020.100238
Received 9 December 2019; Received in revised form 7 August 2020; Accepted 10 August 2020
Available online 18 August 2020

Poprac et al., 2017). ROS are produced either endogenously or exogenously (Galano et al., 2018). In the endogenous mechanism, the mitochondrion is the source where incomplete oxygen metabolism produces ROS such as hydroxyl radicals (HO^-), superoxide radicals, anion, and nitric oxide as by-products (Poprac et al., 2017). The exogenous mechanisms are whereby anthropogenic activities which may be carcinogenic such as cigarette smoking, an excess diet with iron or copper, industrial effluent pollution, and UV irradiation are predisposing to oxidation (Bouayed & Bohn, 2010; Shibamoto, 2017).

Hydroxyl radicals play a key role in the degradation and biotransformation of organic compounds (Das & Roychoudhury, 2014; Takeda et al., 2017). This is because the hydroxyl radicals lead to a more toxic reactive oxygen species in biological systems causing adverse pathological response (DeFeudis et al., 2003; Lucas-Abellán et al., 2011; Molyneux, 2004; Sanchez-Moreno, 2002). Therefore, it is imperative to propose FOS as antioxidant remedies to address this fundamental challenge. Several synthetic and natural antioxidants from plants such as flavonoids and phenolic compounds have been widely reported in the literature. These antioxidants scavenge for free radicals and inhibit nitric oxide (Ak & Gülçin, 2008; Benzie & Devaki, 2017; Chen & Yan, 2005; de Francisco et al., 2018; DeFeudis et al., 2003; Hou et al., 2015; Kellett et al., 2018; Kumaran & Karunakaran, 2006; Lim & Quah, 2007; Motohashi et al., 2017; Rao, 1997; Russo et al., 2015; Sumanont et al., 2004; Valko et al., 2007).

The synthetic antioxidants like butylated hydroxyanisole and butylated hydroxytoluene (have been found to have deleterious effects on human health such as damage to the liver and carcinogenesis in animals (Lobo et al., 2010). Therefore, natural non-toxic antioxidants are produced by fructosyltransferase (Ftase) enzyme via sucrose biotransformation yielding FOS which are highly compatible with dietary fiber. Several attempts have been made to determine the antioxidant activity of FOS from *Helianthus tuberosus,* a plant rich in polydisperse inulin. From the various genetic variants of FW for the variety 'Bergly' 20 mg g^{-1} to 37 mg g^{-1} for 'Kirkeoy' tested, 30%–40% of total carbohydrates were FOS and the rest were disaccharides and low levels of fructose (Seljåsen & Slimestad, 2005). In another *in vitro* study, the antioxidant potential of *Cichorium glandulosum* seed extracts for radical scavenging activity of 1, 1 - diphenyl- 1-picryhl-hydrozyl (DPPH), ABTS, hydroxyl radicals was investigated (Yao et al., 2013).

To date, studies on microbial Ftase to produce bioactive FOS for biotechnological applications like antioxidants are scanty and require more plausible explanations (Mano et al., 2018; Pejin et al., 2014). This study aimed to determine the antioxidant properties of FOS produced by a partially purified Ftase extracted from a coprophilous fungus, *Aspergillus niger* strain XOBP48. Three known and validated methods of 1, 1 - diphenyl- 1-picryhl-hydrozyl (DPPH) assay (Molyneux, 2004; Oyaizu), ferric reducing antioxidant power (FRAP) assay (Kumaran & Karunakaran, 2006; Shaikh et al., 2018; Veenashri & Muralikrishna, 2011) and nitric oxide radical scavenging activity (Hofseth, 2008; Nakagawa & Yokozawa, 2002; Nambiar et al., 2017; Sumanont et al., 2004) were evaluated for their antioxidant effectiveness in determining oxidative inhibition, and free radical scavenging potential of FOS.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), trichloroacetic acid (TCA), potassium ferricyanide, potassium hexacyanoferrate (111), $C_6FeK_3N_6$, methanol, PDA, Czapex Dox agar, fructooligosaccharides (FOS) standards GF_2, GF_3, GF_4 (Megazyme), sucrose (anhydrous), D-fructose and KCl, $MgSO_4{\cdot}7H_2O$ were purchased from Sigma-Aldrich (MI, USA). Ferric chloride hexahydrate, $FeCl_3{\cdot}6H_2O$, (*N*-(1-naphthyl) ethylenediamine dihydrochloride (NED or Griess reagent), 10% tricarboxylic acid and ethanol were purchased from Merck (NJ, USA). All other chemicals and reagents were supplied by reputable scientific suppliers and were of analytical grade unless stated otherwise.

2.2. Isolation and identification of coprophilous fungus

Fresh herbivorous dung samples from various terrestrial habitats in and around KwaZulu-Natal province, the Republic of South Africa (GPS coordinates: 29°39′45.5″S 30°24′17.9″E) were collected and incubated in laboratory conditions for fungal growth and isolation. After isolation and screening of sixty-one axenic fungal cultures, the strain named XOBP48 was selected for further studies, based on its performance for the highest transfructosylating ability of sucrose to fructooligosaccharides (FOS). The fungus was identified morphologically and by sequencing 18S rDNA internally transcribed spacer regions (ITS 4 and ITS 5) (Ramnath et al., 2014; White et al., 1990). The culture was identified as *Aspergillus niger* assigned with the GenBank accession number MH445969 from NCBI database and name as *Aspergillus niger* strain XOBP48 (*An*XOBP48). The pure axenic isolate was preserved on potato dextrose agar (PDA), Czapex dox agar (CDA), and malt extract agar (MEA) at 4 °C (Ademakinwa et al., 2017; Xu et al., 2015) (unpublished data).

2.3. Cultivation, preparation of extracellular enzyme extract and partial purification of Ftase

The pure *An*XOBP48 isolate maintained on PDA at 28 °C was inoculated in a sterilized 100 ml pre-culture fermentation medium comprising of (g/L): sucrose 30, yeast extract 10, $NaNO_3$ 10, KCl 0.5, K_2HPO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, NH_4Cl 0.05, pH 6.5. Further, 10 ml of pre-culture medium was inoculated in fresh 100 ml of the medium in a 250 ml flask and incubated at 28 °C for 72 h at 200 rpm. Uninoculated medium served as control and was incubated under the same condition as the culture (Farid et al., 2015; Park et al., 2001; Sánchez et al., 2010). For the preparation of extracellular enzyme extracts, the fungal mycelia were harvested by filtration using a mutton cloth after 72 h of cultivation. The remaining cell debris was collected by using a Whatman No.1 filter paper and washed twice with 100 mM citrate-phosphate buffer, pH 6.5. All the filtrate broths were pooled together and homogenized by centrifugation at 10,000×*g* for 15 min, and the supernatant was used as the source of the extracellular crude enzyme (Alvarado-Huallanco & Maugeri Filho, 2011; Nguyen, Rezessy-Szabó, Bhat, & Hoschke, 2005). The Ftase was purified and characterized as described previously (unpublished data).

2.4. FOS production and purification

The FOS production was carried out by incubating 1 ml partially purified Ftase (1 mg total protein) with sucrose (5%, w/v) suspended in 100 mM citrate-phosphate buffer (pH 6.0) as a substrate in 250 ml Erlenmeyer flasks for 6 h. All experiments were carried out in triplicate and reaction mixture end products were analyzed by high-performance liquid chromatography coupled with a refractive index detector (HPLC-RI, YL 9100 system, Techno Lab System, column: Pinnacle II Amino 3 μm, 150 × 4.6 mm) run at a flow rate 1.0 ml/min using mobile phase acetonitrile – 70%: H_2O – 30% with (0.04% w/v) ammonium hydroxide and isocratic elution (Correia et al., 2014; Manosroi et al., 2014; Tihomirova et al., 2016). The retention times of FOS produced by the reaction of the partially purified Ftase were compared with pure standards of 1- kestose, 1,1- kestopentaose, and 1,1,1- kestotetraose (Mussatto et al., 2009b). The individual peaks observed during the run were manually collected in clean sterilized tubes and lyophilized. The dried samples were resuspended according to the required concentrations, confirmed by HPLC-RI, and used for further studies. The reconstituted concentrations of the pure FOS were validated by comparing the peak area of the known concentrations of the standards.

2.5. DPPH radical scavenging activity

To determine the antioxidant potential, the total free radical scavenging activity of purified FOS and standard FOS (GF_2 and GF_3) was carried out by modified DPPH radical scavenging activity assay (Ak & Gülçin, 2008; Oyaizu) and compared to vitamin C as a standard antioxidant. Briefly, an aliquot of 500 µl of a 0.3 mM solution of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) dissolved in methanol (analytical grade 99%) was added to 1 ml of resuspended purified FOS and standards at different concentrations from (1, 1.5, 3, 6, 12, 24 and 48 µg/ml). The solutions were mixed with ethanolic solution of DPPH and incubated for 30 min in the dark at room temperature. The absorbance (Abs) was measured spectrophotometrically at wavelength 517 nm against a blank lacking the free radical scavengers (Manosroi et al., 2014; Shah et al., 2016). The DPPH results were expressed as a percentage of the control (blank) by applying Eq. (1):

$$\% \text{ Inhibition} = [(\text{Abs of control - Abs of sample})] / \text{Abs of control}] \times 100 \quad (1)$$

The IC_{50} value (amount of FOS produced in the reaction mixture required to reduce the initial concentration of DPPH radical by 50%) was calculated from a curve obtained by plotting log values of FOS concentration vs % scavenging activity (Lim & Quah, 2007).

2.6. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed as described previously (Ahmed et al., 2017; Serpen et al., 2012) with some modifications (Lim & Quah, 2007). Briefly, the resuspended purified FOS and standards (500 µl) for each concentration (1, 1.5, 3, 6, 12, 24 and 48 µg/ml) were incubated with 500 µl of 0.2 M sodium phosphate buffer (pH 6.6) and 1% (w/v) potassium ferricyanide at 50 °C for 30 min and the reacting products were acidified by adding 500 µl 10% (w/v) TCA. Subsequently, 500 µl of the acidified sample was mixed with 500 µl distilled water and 200 µl of 0.1% $FeCl_3$ and absorbance was measured at 700 nm. The antioxidant activity was estimated by measuring the increase in absorbance as a result of the formation of ferrous ions from the FRAP reagent containing TPTZ and $FeCl_3 \cdot 6H_2O$ (Veenashri & Muralikrishna, 2011). The results were expressed as vitamin C equivalent antioxidant activity. The FRAP results were expressed as a percentage of the control (blank) by applying Eq. (1) as shown above. The IC_{50} value (amount of FOS produced in the reaction mixture required to reduce the initial potassium ferricyanide concentration by 50%) was calculated from a curve obtained by plotting log values of FOS concentration vs % inhibition activity (Lim & Quah, 2007).

2.7. Nitric oxide (NO) scavenging assay

The NO scavenging activity of the produced FOS was estimated according to the modified method as described previously (Lalhminghlui & Jagetia, 2018). Briefly, sodium nitroprusside (5 mM) in saline phosphate buffer (pH 7.2, 100 mM) was mixed with different concentrations of chloroform, ethanol, or resuspended purified FOS and standards were incubated at 50 °C for 2 h. The samples were withdrawn and mixed with Griess reagent 1% sulfanilamide, 2% H_3PO_4, and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Measurement of absorbance at 546 nm for the intense pink color that formed (chromophore) during diazotization of nitrite with sulfanilamide and the coupling with NED was monitored (Nambiar et al., 2017). The total NO inhibition by the FOS was determined by comparing the NO inhibition by vitamin C as a standard. The % NO scavenging activity was calculated using Eq. (2):

$$\text{NO scavenging activity } (\%) = (A_0 - A_1) / A_0 \times 100 \quad (2)$$

Where A_0 and A_1 are the absorbances of the control and sample, respectively.

The IC_{50} value (amount of FOS produced in the reaction mixture required to reduce the initial sodium nitroprusside concentration by 50%) was calculated from a curve obtained by plotting log values of FOS concentration vs % scavenging activity (Lim & Quah, 2007).

2.8. Statistical analysis

All the experiments were carried out in triplicate unless otherwise specified. The data obtained was presented as mean ± standard deviation using one-way ANOVA in MS Excel, with $p < 0.05$ significance level. The calibration curves of the standards used were considered significant if $R^2 \geq 0.99$.

3. Results and discussion

In this study, a partially purified fructosyltransferase (Ftase) enzyme from *Aspergillus niger* sp. XOBP48 (*An*XOBP48) was used for fructooligosaccharides (FOS) production by hydrolyzing sucrose. The end products were analyzed by HPLC-RI as depicted on the chromatogram profiles (Fig. 1A). The end products of enzymatic assay involving the partially purified Ftase showed FOS at peak of retention time of 4.983, 8.408 and 11.767 min for glucose, 1- kestose and 1, 1 – kestoteraose, respectively (Fig. 1A, Table 1). The standards of oligosaccharides exhibited the retention time for 1, 1, 1 – kestopentaose, 1- kestose and 1,1- kestotetraose at 3.892, 8.800 and 12.767 min, respectively (Fig. 1b, Table 2). The peak area and retention time shown in Table 1 and Table 2 were used to determine and validate the identity and concentrations for the purified FOS. A similar method to quantify the FOS using HPLC-IR was employed in the fortification of fruit juices containing FOS (Renuka et al., 2009).

3.1. DPPH radical scavenging activity assay

The DPPH radical scavenging activity assays confirmed the antioxidant activity of the purified FOS as it was able to reduce DPPH (Fig. 2). FOS standards showed no significant difference in DPPH radical scavenging activity as compared to the pure synthesized FOS ($p < 0.05$). IC_{50} of purified GF_2 and GF_3 samples were found to be 2.6 µg/ml and 3.9 µg/ml while standard GF_2 and GF_3 showed a relatively higher IC_{50} of 2.9 µg/ml and 5.0 µg/ml, respectively, but not significantly different (Table 3). The purified FOS showed good free radical inhibition even at a lower concentration of 3 µg/ml. Although the antioxidant activity of purified FOS was observed lower than vitamin C, it is vital to remark that they are interesting sources of dietary fiber that can supplement fiber-enriched food.

The other fiber sources compared to dietary antioxidants such as cellulose, glucomannans, and wheat bran lack intrinsic antioxidant activity and bioactive compounds (Fuentes-Alventosa et al., 2009). These facts, together with functional properties reported such as stimulation of gut enzyme, bifidogenic effect, and prebiotic effects make FOS synthesized by using industrial enzymes more attractive. In this study, the DPPH assay reveals that GF_2 and GF_3 have scavenging properties like a standard antioxidant vitamin C. The acute administration of FOS could overcome the body's reaction to exogenous antioxidants during appropriate therapies in certain pathophysiological conditions associated with oxidative stress. Therefore, herein we provide compelling *in vitro* evidence that purified FOS along with other oligomers can scavenge OH. These prebiotics have the potential to offer protection against oxidative stress.

3.2. Ferric reducing antioxidant power (FRAP) assay

FRAP assay has been used to evaluate the antioxidant ability of purified FOS to reduce Fe^{3+} to Fe^{2+} (Uppin et al., 2018). FRAP activity of the purified FOS (Fig. 3) has shown effective reducing power but the FOS standards showed slightly higher activity with no significant difference ($p < 0.01$). At different concentrations from (1.0 µg/ml – 48 µg/ml),

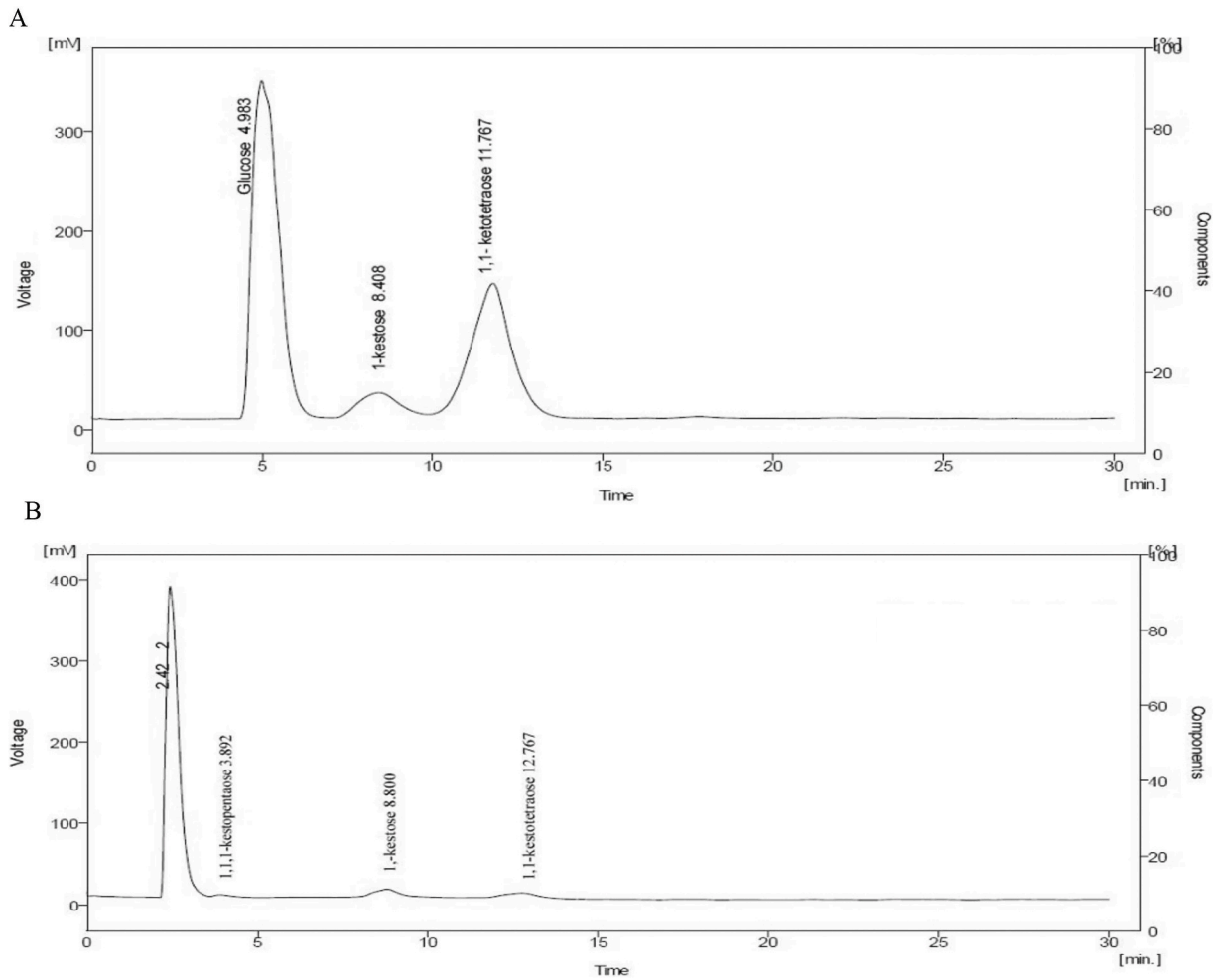

Fig. 1. HPLC-RI chromatograms showing retention time of FOS. (A) FOS liberated after the hydrolysis of sucrose by the partially purified Ftase, (B) standard FOS.

Table 1
The retention times and peak areas of FOS products after hydrolysis of sucrose by the partially purified Ftase from *Aspergillus niger* strain XOBP-48.

Peak	Retention Time (Min)	Area (mV.s)	Height (mV)	Area (%)	Height (%)	W05 (min)	Compound name
1	0.233	6.494	0.539	0.0	0.1	0.15	
2	1.092	80.259	0.640	0.2	0.1	1.03	
3	4.983	1847.493	340.436	55.6	66.6	0.90	Glucose
4	8.408	2376.360	26.676	7.1	5.2	1.43	1-kestose
5	11.767	11,708.143	136.784	35.2	26.8	1.27	1,1-kestoteraose
6	16.308	59.604	1.037	0.2	0.2	1.02	
7	17.817	280.755	2.482	0.8	0.5	1.38	
8	22.008	199.654	1.190	0.6	0.2	3.32	
9	25.500	39.853	0.683	0.1	0.1	1.13	
10	27.133	21.251	0.370	0.1	0.1	0.78	

Table 2
The retention times and peak areas of FOS standards.

Peaks	Retention Time (Min)	Area (mV.s)	Height (mV)	Area (%)	Height (%)	W05 (min)	Compound name
1	0.217	4.62	0.374	0.0	0.1	0.21	
2	2.425	10,076.80	382.532	83.5	93.6	0.42	
3	3.892	149.582	3.511	1.2	0.9	0.64	1,1,1-kestopentaose
4	6.233	133.357	1.241	1.1	0.3	2.08	
5	8.800	852.318	11.300	7.1	2.8	0.92	1-kestose
6	12.767	678.768	7.435	5.6	1.8	1.23	1,1-kestoteraose
7	21.308	24.453	0.382	0.2	0.1	0.57	
8	23.758	100.456	0.814	0.8	0.2	2.04	
9	27.458	24.190	0.524	0.2	0.1	0.93	
10	28.142	17.719	0.491	0.1	0.1	0.63	

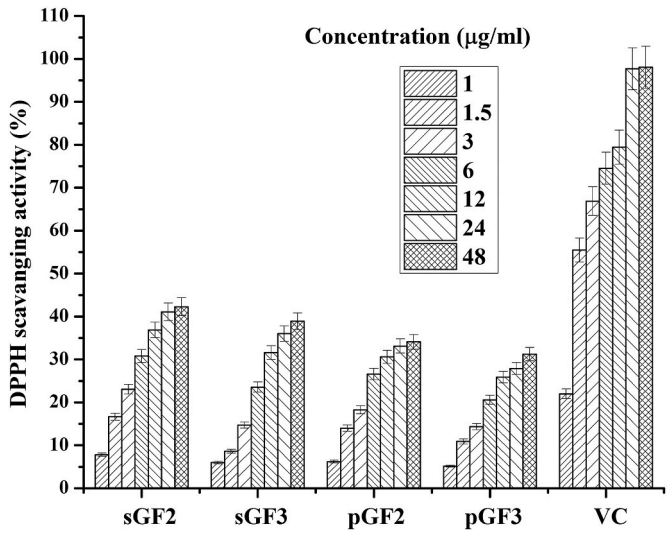

Fig. 2. Percent DPPH radical scavenging activity of standard 1-Kestose (sGF2), 1,1-Kestotetrose (sGF3), purified 1-Kestose (pGF2), purified 1,1-Kestotetrose (pGF2) and vitamin C (VC). The values are the mean percentage ± S. D from three independent experiments.

Table 3
IC_{50} values exhibited by the standards, purified FOS and vitamin C for DPPD, FRAP and NO scavenging activity.

	IC_{50} (μg/ml)		
	DPPH	FRAP	NO
sGF2	2.9	2.9	1.1
sGF3	5.0	3.4	0.87
pGF2	2.6	3.4	0.69
pGF3	3.9	3.8	0.74
VC	1.7	5.4	0.71

sGF2, standards GF2; sGF3, standards GF3; pGF2, purified GF2; pGF3, purified GF3; VC, vitamin C.

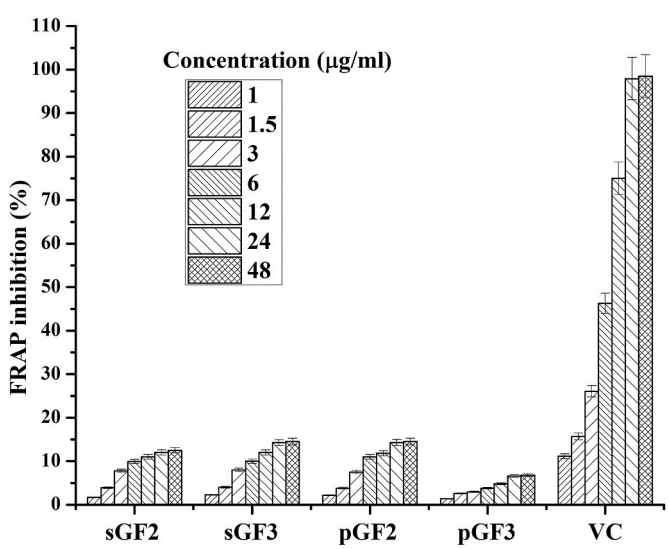

Fig. 3. Percent FRAP inhibition by standard 1-Kestose (sGF2), 1,1-Kestotetrose (sGF3), purified 1-Kestose (pGF2), purified 1,1-Kestotetrose (pGF2) and vitamin C (VC). The values are the mean percentage ± S. D from three independent experiments.

purified FOS demonstrated powerful reducing ability which was found to be concentration-dependent ($p < 0.01$). The IC_{50} value of FRAP extrapolated from the IC_{50} curve were found to be 3.4 μg/ml and 3.8 μg/ml for GF2 and GF3, respectively (Table 3). Ferrous ion reduction of Fe^{3+} complex of tripyridyltriazine (Fe $(TPTZ)^{3+}$) to more intensely coloured Fe^{2+} complex (Fe $(TPTZ)^{2+}$) demonstrated the electron donor property of purified FOS for neutralizing free radicals by forming stable products (Aparadh et al., 2012; Russo et al., 2015). Hence, purified FOS exhibits the potential to donate electrons that scavenge free radicals in the actual biological and food systems. This antioxidant property was also seen in other phenolic compounds *in vitro* (Ak & Gülçin, 2008). In previous studies, the FRAP assay of the single *Lactobacillus plantarum* and mixed fermentation mango pulp demonstrated significantly ($p < 0.05$) increased to 1.47 and 1.49 mM FeSO4, respectively. Moreover, the same study showed that FRAP was increased after fermentation with *L. plantarum* compared to unfermented samples (Jin et al.,2019). Some researchers inferred that FRAP may be related to the TPC, and the presence of phenolic compounds in a sample extract leads to a reduction in the TPTZ-Fe3+ complex to the TPTZ-Fe2+ form. Therefore, the higher TPC in the assays containing *L. plantarum* may help to increase FRAP (Jin et al.,2019).

3.3. Nitric oxide (NO) scavenging assay

The ability of the purified FOS to inhibit nitric oxide production *in vitro* is shown in (Fig. 4). The purified FOS exhibited a relatively higher NO scavenging activity as compared to standards as the IC_{50} was shown slightly lower than standards. Consequently, the results expressed in IC_{50} (μg/ml), equivalent to 0.69 μg/ml and 0.74 μg/ml for purified GF2 and GF3 were lower as compared to 1.1 μg/ml and 0.87 μg/ml shown by standard GF2 and GF3, respectively (Table 3). Standard GF_3 showed higher NO inhibition potential correlated to the previous study where nystose exhibit higher biological activity than 1- kestose and it is crucial to optimize its production (Correia et al., 2014; Mutanda et al., 2015). Incubation of solution of sodium nitroprusside with PBS resulted in linear dependent nitrite production, which was reduced by purified FOS. Structural features are also responsible for scavenging properties for prebiotics like difficulty in cleaving glycosidic linkages in free hydroxyls (Shang et al., 2018). Suppression of released NO may be partially attributed to direct NO scavenging as the purified FOS decreased the amount of nitrite *in vitro*. The nitric oxide radical is toxic after the reaction with oxygen and the purified FOS reduced NO along with the

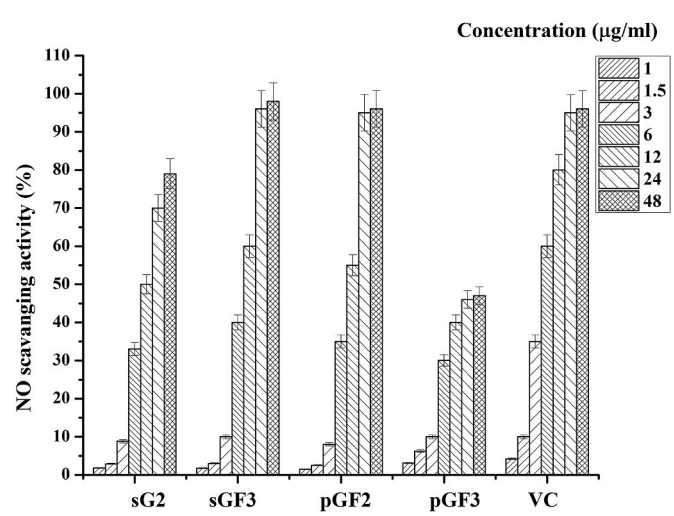

Fig. 4. Percent scavenging for nitric oxide by standard 1-Kestose (sGF2), 1,1-Kestotetrose (sGF3), purified 1-Kestose (pGF2), purified 1,1-Kestotetrose (pGF2) and vitamin C (VC). The values are the mean percentage ± S. D from three independent experiments.

standard antioxidant in a concentration-dependent manner. Similarly in another study investigating FOS antioxidant potential, muffin was incorporated with oligosaccharide from *Emblica officianalis* and exhibited scavenging of NO radicals in a concentration-dependent manner (Nambiar et al., 2017). Nitric oxide is a vital bioregulatory molecule with numerous physiological effects that include neural signal transduction, anti-tumor activity, anti-cancer properties, and antimicrobial activity. Low concentration as presented in Fig. 4 is sufficient to inhibit or scavenge free radicals (Kumaran & Karunakaran, 2006).

3.4. Bioactivities of FOS as antioxidants

FOS are non-digestible oligosaccharides fructans and are sucrose derived as they contain several fructose units and a common glucose residue (Pejin et al., 2014). FOS are prebiotics that has increased health benefits besides basic nutrition where they stimulate the growth of *Bifidobacteria* and *Lactobacilli* in colonic microflora (do Prado et al., 2018; Dominguez et al., 2014; Mutanda et al., 2014). Other FOS benefits that have been claimed include a reduction in serum cholesterol, increase in magnesium and calcium absorption, vitamin B production, and immune stimulation among others (Maiorano et al., 2008; Mussatto et al., 2009a; Mutanda, Wilhelmi, & Whiteley, 2008; Sangeetha, 2003; Silvério et al., 2018; Yan et al., 2018; Zhu, 2017). These health benefits have brought significant attention to interrogate or explore their antioxidant potential. FOS antioxidant property has not been elucidated and information on this nutraceutical remains scanty. Few polysaccharides have been tested and like fructofuranan such as *Plantago lanceolate* were found inactive against peroxidation (Pejin et al., 2014). There is evidence that reactive oxygen species and free radicals formed under physiological conditions and are not being eliminated by the endogenous system induce oxidative stress to the cell (Uppin et al., 2018). These accumulation may lead to cytotoxicity and other malignant neoplasia like atherosclerosis, cancer, cardiovascular disease, mild cognitive impairment, alcohol-induced liver disease, and Parkinson's disease (Dave, 2009; Yamaguchi et al., 1998). Free radicals are a consequence of various metabolic activities and their excess production is a major cause of illness or cellular damage (Lalhminghlui & Jagetia, 2018). Free radicals are atoms with an unpaired number of electrons formed when O_2 reacts with free molecules. These interactions may cause damage to cell membranes poor cell function or even apoptosis a process that can be mitigated by prebiotic antioxidants (Lobo et al., 2010). Free radical scavenging activity is vital due to the harmful effects it causes in biological systems (Huyut et al., 2017). FOS radical scavengers have shown the potential to react with free radicals and this model can be useful in termination of peroxidation chain reaction which is the primary product of lipid oxidation (Ak & Gülçin, 2008). When the end product of transferase assay was added to a medium containing stable DPPH, free radicals were reduced by decolonization to form non-radical DPPH-H upon the donation of H proton (Alam et al., 2013; Huyut et al., 2017). In other studies, overexpression of NO synthase in vascular endothelial cells has been reported to cause iNOS (inducible nitric oxide synthases) expression in macrophages leading to cytotoxicity, autoimmune disease inflammatory responses or organ destruction (Rao et al., 2016). FOS could alter biological activity in the human body as a potential scavenger of free radicals. FOS as an antioxidant may also act directly on nitric oxide radical N = O and related ROS such as H_2O_2 and ferryl ions. In addition, it may indirectly decrease free radicals formation such as OH, N = O probably through inhibition or suppression of expression of genes such as c-FOS, c-JUN, and c-MYC which are implicated in transcriptional induction (Hussain et al., 2003). In this study, purified FOS produced by partially purified Ftase has demonstrated inhibitory activity against NO production and it is a promising candidate for inducing iNOS inhibitory activity. These results can be inferred to recommend FOS prebiotics as inhibitors of NO production creating a therapeutic response in the management of inflammatory diseases. The relationship between *in vivo* and *in vitro* models remains to be established to make the *in vitro* results substantial. More, biological activity *in vitro* and *in vivo* models need to be investigated synchronously to correlate their health claims.

4. Conclusion

In conclusion, this study reports the FOS) production via sucrose biotransformation by partially purified Ftase from *An*XOBP48. The enzyme was able to yield monomeric glucose, 1- kestose (GF_2), and 1,1–kestotetraose (GF_3) which further showed antioxidant activities as measured by DPPH, FRAP and NO radical inhibition assay. The ability of prebiotic oligosaccharides to reduce DPPH to DPPH-H by donating protons, reduction of Fe^{3+} to Fe^{2+} through FRAP assay, and reduction of nitrite oxide by scavenging for free radicals indicate a remarkable antioxidant property to relieve oxidative stress and other reactive oxygen species. Further, and more detailed studies on the chemical composition of these prebiotic oligomers using different experimental models like Trolox equivalent antioxidant capacity (TEAC) assay, Liquid peroxidation, Oxygen radical absorbance capacity (ORAC) assay, and metal chelating activity, Superoxide anion radical scavenging (SO) assay and Hydroxyl radical scavenging (HO) assay need to be explored. In addition, *ex vivo* and *in vivo* studies are paramount to fully corroborate and comprehend their potential nutraceutical and biotechnological application, as it will aid in the characterization of fructooligosaccharides as biological antioxidants.

Author contributions

J.O conceived the experiments, performed data curation, analysis, investigation, validation, visualization and writing of original draft. A.K participated in formal data analysis, validation, review and editing. T.M and S.M conceived the study, experiments, analysis, investigation, validation, writing of original draft, review, editing and supervision.

Funding

Department of Science and Technology DST-National Research Foundation, Center in Indigenous Knowledge Systems (CIKS), South Africa.

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

Declaration of competing interest

All the authors declare that they have no conflict of interest.

Acknowledgment

The authors express their sincere gratitude to the Ukulinga research farm, Tala game reserve, and Phinda game reserve, Department of Science and Technology DST-National Research Foundation, Center in Indigenous Knowledge Systems (CIKS), South Africa., for helping, and allowing for the sampling of herbivore dung at their premises.

References

Abasubong, K. P., Liu, W.-B., Zhang, D.-D., Yuan, X.-Y., Xia, S.-L., Xu, C., et al. (2018). Fishmeal replacement by rice protein concentrate with xylooligosaccharides supplement benefits the growth performance, antioxidant capability and immune responses against Aeromonas hydrophila in blunt snout bream (Megalobrama amblycephala). *Fish & Shellfish Immunology, 78*, 177–186.

Ademakinwa, A. N., Ayinla, Z. A., & Agboola, F. K. (2017). Strain improvement and statistical optimization as a combined strategy for improving fructosyltransferase production by Aureobasidium pullulans NAC8. *Journal of Genetic Engineering and Biotechnology, 15*(2), 345–358.

Ahmed, E., Elkhateeb, W., Taie, H., Rateb, M., & Fayad, W. (2017). Biological capacity and chemical composition of secondary metabolites from representatives Japanese lichens. *Journal of Applied Pharmaceutical Science, 7*(1), 98–103.

Ak, T., & Gülçin, İ. (2008). Antioxidant and radical scavenging properties of curcumin. *Chemico-Biological Interactions, 174*(1), 27–37.

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal, 21*(2), 143–152.

Alvarado-Huallanco, M. B., & Maugeri Filho, F. (2011). Kinetic studies and modelling of the production of fructooligosaccharides by fructosyltransferase from Rhodotorula sp. *Catalysis Science & Technology, 1*(6), 1043–1050.

Aparadh, V., Naik, V., & Karadge, B. (2012). Antioxidative properties (TPC, DPPH, FRAP, metal chelating ability, reducing power and TAC) within some Cleome species. *Annali di Botanica, 2*, 49–56.

Benzie, I. F., & Devaki, M. (2017). *The 5 ferric reducing/antioxidant power (FRAP) assay for non-enzymatic antioxidant capacity: Concepts, procedures, limitations and applications. Measurement of antioxidant activity and capacity: Recent trends and applications, 77*.

Bouayed, J., & Bohn, T. (2010). Exogenous antioxidants—double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxidative medicine and cellular longevity, 3*(4), 228–237.

Chen, H.-M., & Yan, X.-J. (2005). Antioxidant activities of agaro-oligosaccharides with different degrees of polymerization in cell-based system. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1722*(1), 103–111.

Choudhary, S., Singh, M., Sharma, D., Attri, S., Sharma, K., & Goel, G. (2018). Principal component analysis of stimulatory effect of synbiotic combination of indigenous probiotic and inulin on antioxidant activity of soymilk. *Probiotics and antimicrobial proteins*, 1–7.

Correia, D. M., Dias, L. G., Veloso, A. C. A., Dias, T., Rocha, I., Rodrigues, L. R., et al. (2014). Dietary sugars analysis: Quantification of fructooligossacharides during fermentation by HPLC-RI method. *Frontiers in Nutrition, 1*, 11.

Czarnocka, W., & Karpiński, S. (July 2018). Friend or foe? Reactive oxygen species production, scavenging and signaling in plant response to environmental stresses. *Free Radical Biology and Medicine, 22*, 4–20. https://doi.org/10.1016/j.freeradbiomed.2018.01.011.

Das, K., & Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science, 2*, 53.

Dave, R. (2009). In vitro models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. *African Journal of Microbiology Research, 3*(13), 981–996.

DeFeudis, F. V., Papadopoulos, V., & Drieu, K. (2003). Ginkgo biloba extracts and cancer: A research area in its infancy. *Fundamental & clinical pharmacology, 17*(4), 405–417.

Dominguez, A. L., Rodrigues, L. R., Lima, N. M., & Teixeira, J. A. (2014). An overview of the recent developments on fructooligosaccharide production and applications. *Food and Bioprocess Technology, 7*(2), 324–337.

Farid, M. A.-F. M., Kamel, Z., Elsayed, E. A., & El-Deen, A. M. N. (2015). Optimization of medium composition and cultivation parameters for fructosyltransferase production by Penicillium aurantiogriseum AUMC 5605. *Journal of Applied Biological Chemistry, 58*(3), 209–218.

de Francisco, L., Pinto, D., Rosseto, H., Toledo, L., Santos, R., Tobaldini-Valério, F., et al. (2018). Evaluation of radical scavenging activity, intestinal cell viability and antifungal activity of Brazilian propolis by-product. *Food Research International, 105*, 537–547.

Fuentes-Alventosa, J. M., Jaramillo-Carmona, S., Rodríguez-Gutiérrez, G., Rodríguez-Arcos, R., Fernández-Bolaños, J., Guillén-Bejarano, R., et al. (2009). Effect of the extraction method on phytochemical composition and antioxidant activity of high dietary fibre powders obtained from asparagus by-products. *Food Chemistry, 116*(2), 484–490.

Galano, A., Tan, D.-X., & Reiter, R. J. (2018). Melatonin: A versatile protector against oxidative DNA damage. *Molecules, 23*(3), 530.

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., et al. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology, 14*(8), 491.

Gibson, G. R., Scott, K. P., Rastall, R. A., Tuohy, K. M., Hotchkiss, A., Dubert-Ferrandon, A., et al. (2010). Dietary prebiotics: Current status and new definition. *Food Science & Technology Bulletin: Functional Foods, 7*(1), 1–19.

Guerreiro, I., Couto, A., Machado, M., Castro, C., Pousão-Ferreira, P., Oliva-Teles, A., & Enes, P. (2016). Prebiotics effect on immune and hepatic oxidative status and gut morphology of white sea bream (Diplodus sargus). *Fish & Shellfish Immunology, 50*, 168–174.

Hofseth, L. J. (2008). Nitric oxide as a target of complementary and alternative medicines to prevent and treat inflammation and cancer. *Cancer Letters, 268*(1), 10–30.

Hou, Y., Ding, X., & Hou, W. (2015). Composition and antioxidant activity of water-soluble oligosaccharides from Hericium erinaceus. *Molecular Medicine Reports, 11*(5), 3794–3799.

Hussain, S. P., Hofseth, L. J., & Harris, C. C. (2003). Radical causes of cancer. *Nature Reviews Cancer, 3*(4), 276.

Huyut, Z., Beydemir, Ş., & Gülçin, İ. (2017). Antioxidant and antiradical properties of selected flavonoids and phenolic compounds. *Biochemistry research international, 2017*.

Jin, X., Chen, W., Chen, H., Chen, W., & Zhong, Q. (2019). Combination of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* DV10 as starter culture to produce mango slurry: Microbiological, chemical parameters and antioxidant activity. *Molecules, 24*(23). p.4349.

Kellett, M. E., Greenspan, P., & Pegg, R. B. (2018). Modification of the cellular antioxidant activity (CAA) assay to study phenolic antioxidants in a Caco-2 cell line. *Food Chemistry, 244*, 359–363.

Kumar, P., Jain, K., & Sardar, P. (Sep 2018). Effects of dietary synbiotic on innate immunity, antioxidant activity and disease resistance of Cirrhinus mrigala juveniles. *Fish & Shellfish Immunology, 80*, 124–132. https://doi.org/10.1016/j.fsi.2018.05.045.

Kumaran, A., & Karunakaran, R. J. (2006). Nitric oxide radical scavenging active components from Phyllanthus emblica L. *Plant Foods for Human Nutrition, 61*(1), 1.

Lalhminghlui, K., & Jagetia, G. C. (2018). Evaluation of the free-radical scavenging and antioxidant activities of Chilauni, Schima wallichii Korth in vitro. *Future Science OA*, 0), FSO272.

Lim, Y., & Quah, E. (2007). Antioxidant properties of different cultivars of. *Portulaca oleracea. Food chemistry, 103*(3), 734–740.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews, 4*(8), 118.

Lucas-Abellán, C., Mercader-Ros, M., Zafrilla, M., Gabaldón, J., & Núñez-Delicado, E. (2011). Comparative study of different methods to measure antioxidant activity of resveratrol in the presence of cyclodextrins. *Food and Chemical Toxicology, 49*(6), 1255–1260.

Maiorano, A. E., Piccoli, R. M., da Silva, E. S., & de Andrade Rodrigues, M. F. (2008). Microbial production of fructosyltransferases for synthesis of pre-biotics. *Biotechnology Letters, 30*(11), 1867.

Mano, M. C. R., Neri-Numa, I. A., da Silva, J. B., Paulino, B. N., Pessoa, M. G., & Pastore, G. M. (2018). Oligosaccharide biotechnology: An approach of prebiotic revolution on the industry. *Applied Microbiology and Biotechnology, 102*(1), 17–37.

Manosroi, J., Khositsuntiwong, N., & Manosroi, A. (2014). Biological activities of fructooligosaccharide (FOS)-containing Coix lachryma-jobi Linn. extract. *Journal of Food Science and Technology, 51*(2), 341–346.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science and Technology, 26*(2), 211–219.

Motohashi, N., Gallagher, R., Anuradha, V., & Gollapudi, R. (2017). Functional foods and their importance in geriatric nutrition. *Journal of Clinical Nutrition and Metabolism, 2017*.

Mussatto, S. I., Aguilar, C. N., Rodrigues, L. R., & Teixeira, J. A. (2009a). Fructooligosaccharides and β-fructofuranosidase production by Aspergillus japonicus immobilized on lignocellulosic materials. *Journal of Molecular Catalysis B: Enzymatic, 59*(1–3), 76–81.

Mussatto, S. I., Aguilar, C. N., Rodrigues, L. R., & Teixeira, J. A. (2009b). Fructooligosaccharides and β-fructofuranosidase production by Aspergillus japonicus immobilized on lignocellulosic materials. *Journal of Molecular Catalysis B: Enzymatic, 59*.

Mutanda, T., Mokoena, M. P., Olaniran, A. O., Wilhelmi, B. S., & Whiteley, C. G. (2014). Microbial enzymatic production and applications of short-chain fructooligosaccharides and inulooligosaccharides: Recent advances and current perspectives. *Journal of Industrial Microbiology & Biotechnology, 41*(6), 893–906.

Mutanda, T., Wilhelmi, B., & Whiteley, C. (2008). Response surface methodology: Synthesis of inulooligosaccharides with an endoinulinase from *Aspergillus Niger*. *Enzyme and Microbial Technology, 43*(4), 362–368.

Mutanda, T., Wilhelmi, B. S., & Whiteley, C. G. (2015). Biocatalytic conversion of inulin and sucrose into short chain oligosaccharides for potential pharmaceutical applications. *African Journal of Science, Technology, Innovation and Development, 7*(5), 371–380.

Nakagawa, T., & Yokozawa, T. (2002). Direct scavenging of nitric oxide and superoxide by green tea. *Food and Chemical Toxicology, 40*(12), 1745–1750.

Nambiar, S. S., Shetty, N. P., Ravi, R., & Prabhasankar, P. (2017). Changes in properties of muffins incorporated with emblica officinalis fruit osmodehydrated with fructooligosaccharide. *Journal of Food Processing and Preservation, 41*(2).

Nguyen, Q. D., Rezessy-Szabó, J. M., Bhat, M. K., & Hoschke, Á. (2005). Purification and some properties of β-fructofuranosidase from *Aspergillus Niger* IMI303386. *Process Biochemistry, 40*(7), 2461–2466.

Oyaizu, M. (2008). Studies on products of browning reaction: Antioxidative activity of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition, 1986*(44), 307–315.

Park, J.-P., Oh, T.-K., & Yun, J.-W. (2001). Purification and characterization of a novel transfructosylating enzyme from Bacillus macerans EG-6. *Process Biochemistry, 37*(5), 471–476.

Patel, S., & Goyal, A. (2011). Functional oligosaccharides: Production, properties and applications. *World Journal of Microbiology and Biotechnology, 27*(5), 1119–1128.

Pejin, B., Savic, A. G., Petkovic, M., Radotic, K., & Mojovic, M. (2014). In vitro anti-hydroxyl radical activity of the fructooligosaccharides 1-kestose and nystose using spectroscopic and computational approaches. *International journal of food science & technology, 49*(6), 1500–1505.

Poprac, P., Jomova, K., Simunkova, M., Kollar, V., Rhodes, C. J., & Valko, M. (2017). Targeting free radicals in oxidative stress-related human diseases. *Trends in Pharmacological Sciences, 38*(7), 592–607.

do Prado, D. Z., Capoville, B. L., Delgado, C. H. O., Heliodoro, J. C. A., Pivetta, M. R., Pereira, M. S., et al. (2018). Chapter 4-nutraceutical food: Composition, biosynthesis, therapeutic properties, and applications. In *Alternative and replacement foods* (pp. 95–140). Academic Press.

Ramnath, L., Bush, T., & Govinden, R. (2014). Method optimization for denaturing gradient gel electrophoresis (DGGE) analysis of microflora from Eucalyptus sp. wood chips intended for pulping. *African Journal of Biotechnology, 13*(3), 356.

Rao, M. (1997). Nitric oxide scavenging by curcuminoids. *Journal of Pharmacy and Pharmacology, 49*(1), 105–107.

Rao, U. M., Ahmad, B. A., & Mohd, K. S. (2016). *In vitro* nitric oxide scavenging and anti inflammatory activities of different solvent extracts of various parts of Musa paradisiaca. *Malaysian Journal of Analytical Sciences, 20*(5), 1191–1202.

Renuka, B., Kulkarni, S., Vijayanand, P., & Prapulla, S. (2009). Fructooligosaccharide fortification of selected fruit juice beverages: Effect on the quality characteristics. *LWT-Food Science and Technology, 42*(5), 1031–1033.

Roberfroid, M., & Slavin, J. (2000). Non-digestible oligosaccharides. *Critical Reviews in Food Science and Nutrition, 40*.

Russo, D., Valentão, P., Andrade, P. B., Fernandez, E. C., & Milella, L. (2015). Evaluation of antioxidant, antidiabetic and anticholinesterase activities of Smallanthus sonchifolius landraces and correlation with their phytochemical profiles. *International Journal of Molecular Sciences, 16*(8), 17696–17718.

Sanchez-Moreno, C. (2002). Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Science and Technology International, 8*(3), 121–137.

Sánchez, O. F., Rodriguez, A. M., Silva, E., & Caicedo, L. A. (2010). Sucrose biotransformation to fructooligosaccharides by Aspergillus sp. N74 free cells. *Food and Bioprocess Technology, 3*(5), 662–673.

Sangeetha, P. (2003). *Microbial production of fructooligosaccharides*. University of Mysore.

Seljåsen, R., & Slimestad, R. (2005). Fructooligosaccharides and phenolics in flesh and peel of spring harvested Helianthus tuberosus. In *I international symposium on human health effects of fruits and vegetables 744* (pp. *447–450*).

Serpen, A., Gökmen, V., & Fogliano, V. (2012). Total antioxidant capacities of raw and cooked meats. *Meat Science, 90*(1), 60–65.

Shah, C., Mokashe, N., & Mishra, V. (2016). Preparation, characterization and in vitro antioxidative potential of synbiotic fermented dairy products. *Journal of Food Science and Technology, 53*(4), 1984–1992.

Shaikh, A., Mohammed, I., & Khan, M. (2018). Comparative in vitro antidiabetic and antioxidant activity of various extracts of Ficus species. *Pharmacognosy Journal, 10*(2).

Shang, H.-M., Zhou, H.-Z., Yang, J.-Y., Li, R., Song, H., & Wu, H.-X. (2018). In vitro and in vivo antioxidant activities of inulin. *PloS One, 13*(2). e0192273.

Shibamoto, T. (2017). Novel 11 methods of antioxidant assay combining various principles. *Measurement of Antioxidant Activity and Capacity: Recent Trends and Applications, 209*.

Silvério, S. C., Macedo, E. A., Teixeira, J. A., & Rodrigues, L. R. (2018). New β-galactosidase producers with potential for prebiotic synthesis. *Bioresource Technology, 250*, 131–139.

Sumanont, Y., Murakami, Y., Tohda, M., Vajragupta, O., Matsumoto, K., & Watanabe, H. (2004). Evaluation of the nitric oxide radical scavenging activity of manganese complexes of curcumin and its derivative. *Biological and Pharmaceutical Bulletin, 27*(2), 170–173.

Takeda, K., Fujisawa, K., Nojima, H., Kato, R., Ueki, R., & Sakugawa, H. (2017). Hydroxyl radical generation with a high power ultraviolet light emitting diode (UV-LED) and application for determination of hydroxyl radical reaction rate constants. Journal of Photochemistry and Photobiology A: *Chemistry, 340*, 8–14.

Tihomirova, K., Dalecka, B., & Mezule, L. (2016). Application of conventional HPLC RI technique for sugar analysis in hydrolysed hay. *Agronomy Research, 14*(5), 1713–1719.

Uppin, J. B., Chandrasekhar, V., & Naik, G. R. (2018). Evaluation of in vitro antioxidant and anti-inflammatory activities of cassia auriculata linn. *Extracts. International journal of pharmaceutical sciences and research, 9*(2), 575–581.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39*(1), 44–84.

Veenashri, B. R., & Muralikrishna, G. (2011). In vitro anti-oxidant activity of xylo-oligosaccharides derived from cereal and millet brans – a comparative study. *Food Chemistry, 126*(3), 1475–1481.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: A Guide to Methods and Applications, 18*(1), 315–322.

Witschinski, F., Demartini, D., Kilian, J., Dallago, R. M., Rosa, C. D., Cansian, R. L., et al. (2018). Development and characterization of light yoghurt elaborated with Bifidobacterium animalis subsp. Lactis Bb-12 and fructooligosaccharides. *Ciência Rural, 48*(3).

Xu, Q., Zheng, X., Huang, M., Wu, M., Yan, Y., Pan, J., et al. (2015). Purification and biochemical characterization of a novel β-fructofuranosidase from Penicillium oxalicum with transfructosylating activity producing neokestose. *Process Biochemistry, 50*(8), 1237–1246.

Yamaguchi, T., Takamura, H., Matoba, T., & Terao, J. (1998). HPLC method for evaluation of the free radical-scavenging activity of foods by using 1, 1-diphenyl-2-picrylhydrazyl. *Bioscience, biotechnology, and biochemistry, 62*(6), 1201–1204.

Yan, Y. L., Hu, Y., & Gänzle, M. G. (2018). Prebiotics, FODMAPs and dietary fiber—conflicting concepts in development of functional food products? *Current Opinion in Food Science, 20*, 30–37.

Yao, X., Zhu, L., Chen, Y., Tian, J., & Wang, Y. (2013). In vivo and in vitro antioxidant activity and α-glucosidase, α-amylase inhibitory effects of flavonoids from Cichorium glandulosum seeds. *Food Chemistry, 139*(1), 59–66.

Zhu, F. (2017). Coix: Chemical composition and health effects. *Trends in Food Science & Technology, 61*, 160–175.