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Article

Exopolysaccharides from *Klebsiella oxytoca*: anti-inflammatory activity

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Exopolysaccharides (EPS) produced by *Klebsiella oxytoca* are of environmental, pharmaceutical, and medicinal interest. However, studies about the anti-inflammatory activity of EPS produced by this microorganism still remain limited. The aim of this study was to produce, characterize, and evaluate the anti-inflammatory activity of EPS from *K. oxytoca* in a pleurisy model. Colorimetric analysis revealed that precipitated crude exopolysaccharides (KEPSC) and deproteinated exopolysaccharides (KEPS) present high levels of total carbohydrates (65.57% and 62.82%, respectively). Analyses of uronic acid (7.90% in KEPSC and 6.21% in KEPS) and pyruvic acid (3.01% in KEPSC and 1.68% in KEPS) confirm that the EPS are acidic. Gas chromatography-mass spectrometry analyses demonstrated that the EPS consisted of rhamnose (29.83%), glucose (11.21%), galactose (52.45%), and mannose (6.50%). The treatment of an experimental pleurisy model in rats through subcutaneous administration of 50, 100, 200, and 400 mg/kg of KEPS decreased both the volume of inflammatory exudate and the number of leukocytes recruited to the pleural cavity. The present data showed that EPS production by *K. oxytoca* using the method described is easy to perform and results in a good yield. In addition, we show that KEPS exhibit anti-inflammatory activity when administered subcutaneously in rats.

Keywords: Polysaccharides. Inflammation. Pleurisy. Edema. Leukocyte migration. *Klebsiella oxytoca*.

INTRODUCTION

Polysaccharides are renewable, biodegradable, and generally non-toxic materials of biological origin that have been applied in the pharmaceutical, cosmetic, and food industries because of their chemical, physical, and biological properties (Matsumoto, Ohmori, 2001; Manivasagana *et al*., 2015; Tian *et al*., 2015).

Some of the properties that contribute to their broad application and, consequently, the realization of extensive research in this area, includes easy production by bacterial fermentation and manipulation by recombinant DNA technology, the ability to retain water and form films, specific rheological behavior, and the potential to improve the immune response (Barbara *et al*., 2009; Dlamini *et al*., 2007; Dlamini *et al*., 2009; Snapper, 2016; Sun *et al*., 2016; Donot *et al*., 2012).

The carbohydrate polymers secreted by a wide variety of bacteria are known as exopolysaccharides (EPS), which exhibit important biological activities, including immunomodulation, antioxidant, prebiotic, and anti-proliferative properties (Shi, 2016; Zha *et al*., 2015; Sugihara *et al*., 2001; Wang *et al*., 2015).

Klebsiella oxytoca is known as a nitrogen-fixing bacterium, and in the past, a strain was isolated from rice

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rhizosphere (Hirota *et al*., 1978). Strains of *K. oxytoca* are also known to produce EPS of environmental and pharmaceutical interest (Baldi *et al*., 2001; Sugihara *et al*., 2000). Furthermore, according to Sugihara *et al.* (2000; 2002), the AZ9 polysaccharide produced by *K. oxytoca* has been shown to exhibit important immunosuppressive activity in experimental models of chronic inflammatory and allergic diseases.

However, to the best of our knowledge, no studies have described the anti-inflammatory activity of EPS produced by *K. oxytoca*. Thus, the aim of this study was to produce EPS from *K. oxytoca* and to evaluate the anti-inflammatory activity in a pleurisy model in rats.

MATERIAL AND METHODS

Strain and medium

The microorganism *K. oxytoca* was isolated from the rhizosphere of *Aspidosperma polyneuron* and identified according to biochemical tests (Celloto *et al*., 2012). The strain was also phylogenetically identified by partial 16S rDNA sequence analysis and the GenBank data homology research search result was 99% according to the methodology described by Nogueira *et al.* (2004) and Procópio *et al.* (2009).

The *K. oxytoca* strain (1.5×10^8 CFU/mL standard bacterial suspension) was cultured in liquid medium (10 mL/L inoculum) as described by Sugihara *et al.* (2001). Cultures were grown in Sugihara medium (SM) (5.0 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 2.0 g/L polypeptone, and 20 g/L glucose) in 100 mL Erlenmeyer flasks at 28 °C with shaking at 200 rpm for 48 h. The bacterial culture was then transferred aseptically to 2000 mL Erlenmeyer flasks containing 900 mL of SM, incubated at 28 °C for 120 h then maintained at 4 °C prior to EPS isolation.

Isolation and purification of *K. oxytoca* EPS

Bacterial cells were separated by centrifugation at 12,000 × g for 20 min at 5 °C. The supernatant was collected and concentrated to 100 mL in a rotary evaporator at 50 °C. Following this, the concentrated solution containing EPS was precipitated from the clear supernatant by adding three volumes of cold acetone and maintained at 4 °C for 48 h. The crude exopolysaccharides from *K. oxytoca* (KEPSC) were obtained by filtration, lyophilized and weighed to calculate the yield (Kazy *et al.*, 2002; Sugihara *et al*., 2000; Sugihara *et al.*, 2001; Sugihara *et al.*, 2002). Once isolated, the KEPSC solution was deproteinized with trichloracetic acid (20%, w/v) at a ratio of 1:1, maintained at 4 °C for 2 h, and then centrifuged (12,000 × g for 20 min at 5 °C). Deproteination and precipitation were performed twice to remove remnant proteins. The polysaccharides were dissolved in bidistilled water and dialyzed (cellulose membrane, MWCO: 12,000, Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 4 days. Finally, the deproteinated exopolysaccharides (KEPS) were lyophilized, weighed to calculate the yield and stored at 4 °C until further use (Ruas-Adiedo, Creyes-Gavilan, 2005; Marcial *et al.*, 2013).

Chemical characterization

Colorimetric analyses

The total sugar content was determined using the phenol sulfuric acid method (Dubois *et al*., 1956); determination of reducing sugars was performed according to the spectrophotometric method of *p*-hydroxybenzoic acid hydrazide (Lever, 1972); uronic acid was assayed using the carbazol sulfuric acid method (Chaplin, Kennedy, 1994); pyruvic acid was analyzed using the colorimetric method of 2,4-dinitropheny-hydrazine reagent (Sloneker, Orentas, 1962); and total protein was quantified using the Hartree method (Hartree, 1972).

Analyses of the monosaccharide composition of KEPSC

Neutral monosaccharide components of the KEPSC (1 mg) were determined by gas chromatography–mass spectrometry. Their ratios were determined by hydrolysis with 1 mL of 2 M trifluoroacetic acid for 8 h at 100 °C, followed by conversion to alditol acetates by successive $NaBH_4$ (1 mg) reduction and acetylation with 1 mL acetic anhydride-pyridine (1:1, v/v) at room temperature for 14 h. The resulting alditol acetates were extracted with chloroform. The alditol acetate analysis was carried out using a Varian model 3300 gas chromatograph linked to

a Finnigan Ion-Trap model (ITD 800) mass spectrometer fitted with a DB-225 capillary column (30 m × 0.25 mm i.d.), held at 50 °C during injection for 1 min, then programmed at 40 °C/min to 220 °C and held at this constant temperature for 19.75 min. Helium was used as the carrier gas in the quantitative analysis. The alditol acetates were identified by their retention times and typical electron impact breakdown profiles compared with standards. The results were given as weight percentages, which take into account the coefficients from the detector response (York *et al*., 1985).

^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR)

^{1}H and ^{13}C NMR spectroscopy was performed using a Varian spectrometer, Mercury Plus model, operating at 300.06 MHz for ^{1}H and 75.45 MHz for ^{13}C. The ^{1}H and ^{13}C NMR spectra of the samples of polysaccharides were obtained using deuterated water as a solvent. The chemical shifts (δ) were expressed in ppm and compared with data obtained from the literature.

Evaluation of the anti-inflammatory activity of EPS from *K. oxytoca* on carrageenan-induced pleurisy

The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the State University of Maringá (012/2013). Groups of Wistar rats (n = 6–7 per group), weighing 200–220 g, were kept under controlled temperature (22 ± 2 °C) and a light/dark cycle of 12 h with water and food *ad libitum*. The animals were anesthetized using a solution of 10% ketamine and 2% xylazine at a dose of 40 mg/kg and pleurisy was induced by injecting 0.25 mL of a carrageenan suspension (Cg; 200 μg) into the intra-pleural cavity (Vinegar, Truax, Selph, 1976; Goodman *et al*., 1993). In this experiment, the crude EPS obtained from the Sugihara culture (KEPSC) and KEPS were dissolved in water and saline, respectively. Groups of rats fasted for 12 h were treated orally (gavage) at a single dose of 400 mg/kg KEPSC and KEPS, or subcutaneously at a dose of 50, 100, 200, or 400 mg/kg KEPS, 1 h before the induction of pleurisy. Another group of rats were treated with indomethacin (Indo) at a dose of 5 mg/kg (positive control), or water or saline (negative controls), by the same routes of administration.

Statistical analyses

The results of the anti-inflammatory and chemical assays were expressed as the mean ± standard error of the mean. The data obtained in the anti-inflammatory and chemical assay were evaluated with GraphPad Prism 5.0 and Origin 7.0® software, respectively, and analyzed using analysis of variance followed by Tukey's test. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

EPS produced by *K. oxytoca* was isolated from the Sugihara culture medium at yields of 1.18 ± 0.20 and 0.96 ± 0.10 g/L (dry weight/volume) for KEPSC and KEPS, respectively. Table I shows the contents of total carbohydrate, reducing sugars, uronic acid, pyruvic acid, and protein.

TABLE I - Total carbohydrate, reducing sugars, uronic acid, pyruvic acid, and protein content of exopolysaccharides from *K. oxytoca*

Sample	Total carbohydrate (%, w/w)	Reducing sugars (%, w/w)	Uronic acid (%, w/w)	Pyruvic acid (%, w/w)	Protein (%, w/w)
KEPSC	65.57[a]	6.68[a]	7.90[a]	3.01[a]	16.83[b]
KEPS	62.82[b]	6.50[b,a]	6.21[a]	1.68[b]	8.92[c]

*Different letters indicate statistical differences by Tukey's test at 5% level of significance.
*Tukey's test was used to compare the means of triplicate observations in the different samples of exopolysaccharides.

The total carbohydrate contents in KEPSC and KEPS were 65.57% and 62.82%, respectively. Among the EPS tested, the relative contents of uronic acid in KEPSC and KEPS were 7.90% and 6.21%, respectively, while the contents of pyruvic acid in KEPSC and KEPS were 3.01% and 1.68%, respectively. The relative protein content of KEPSC (16.83%) and KEPS (8.92%) suggested they might be protein-bound polysaccharides (Qiao *et al*., 2009).

The low concentration of reducing sugars and the high content of total carbohydrate present in the KEPS indicated that the carbohydrates are present in the structural form of polysaccharides. Analyses of uronic acid and pyruvic acid confirmed that the EPS extracted from *K. oxytoca* are acidic. It is likely that these differences in composition are responsible for the different rheological behavior of the solutions, which provide strong gels (Lamb *et al*., 2004; Junhua *et al*., 2013). Gas chromatography revealed that the monosaccharide composition of the crude EPS consisted of rhamnose (Rha; 29.83%), glucose (Glc; 11.21%), galactose (Gal; 52.45%), and mannose (Man; 6.50%); thus, galactose and rhamnose are the major components. These data are in agreement with those found by Dlamini *et al*. (2007) and Feng, Li, Chen (2009), who used different media cultures for the production of EPS from *K. oxytoca*.

The ^{1}H NMR spectrum of KEPS contained seven signals in the anomeric region between 5.25 and 4.50 ppm (labeled **A–G** in order of decreasing chemical shift, Figure 1 A, B); these were assigned by monosaccharide composition through comparison with the structure and spectroscopic data of exopolysaccharide isolated from *K. oxytoca* BAS-10 described by Leone *et al.* (2007). The signals of anomeric hydrogens δ 5.23, 5.16, 5.14, and 5.04 were suggested to be α-Rha di-substituted (2 → 1, **A** and/or **B**), α-Rha di-substituted (3 → 1, **C**), and α-Rha tri-substituted (3 → 1,4, **D**), respectively. The signals at δ 4.79 and 4.69 were suggested to be β-GlcA terminal (**E**) and 4-β-GlcA (**F**), respectively. The signal at δ 4.55 was suggested to be 3-β-Gal (**G**). Moreover, between 1.11 and 1.31 ppm, the signals of four methyl groups were observed and each was suggested to be the 6-deoxy position of a Rha unit.

The ^{13}C NMR spectrum of KEPS showed anomeric carbon signals at 104.49, 103.20, 102.33, 101.43, 101.57, 101.19, and 98.13 ppm (Figure 1C). Designation of these signals was conducted by comparison with data obtained from the literature (Leone *et al*., 2007), giving the C-1 of β-galactose (**G**), β-glucuronic acid (**F**), β-glucuronic acid terminal (**E**), α-Rha 3-substituted (**C** and/or **B**), and α-Rha 2-substituted (**D** and/or **A**), respectively (Table II).

Colorimetric determination of glucuronic acid present in KEPS was 6.20% and its presence was confirmed by the carboxyl group signal at 172.95 ppm. The presence of C-6 (CH_3) of rhamnose units was evidenced by signals at δ 16.83 and 16.69. The signal at 61.18 ppm (C-6) represents galactose present in the biopolymer.

This partial chemical characterization of EPS showed that their structures could be related to the EPS isolated from *K. oxytoca* BAS-10 by Leone *et al.* (2007).

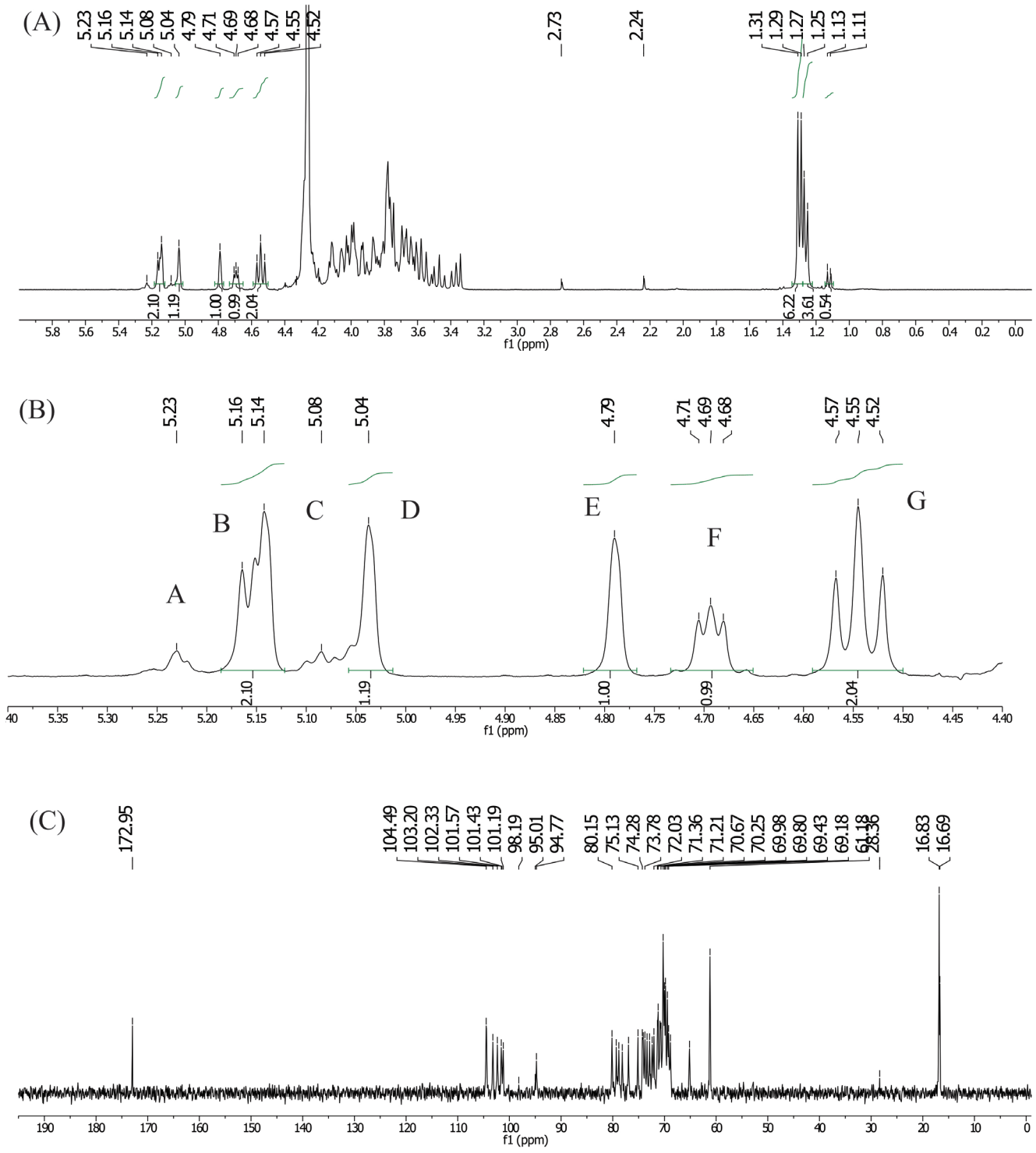

FIGURE 1 - NMR spectra of the KEPS from *K. oxytoca* in D_2O: (A) ^1H NMR spectrum (300.06 MHz); (B) Zoom into anomeric region of the ^1H NMR spectrum; (C) ^{13}C NMR spectrum (75.45 MHz).

TABLE II - ^{13}C (75.45 MHz, D_2O) NMR chemical shifts of KEPS from *K. oxytoca*

	^{13}C Chemical shifts (ppm)					
Position	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**
2-α-Rha A	101.19 (100.3)	78.16 (78.0)	69.98 (69.7)	72.36 (71.9)	69.18 (69.1)	16.69 (16.5)
2-α-Rha B	98.19 (99.5)	79.32 (79.0)	69.80 (69.8)	-	-	-
3,4-α-Rha D	101.57 (101.9)	70.25 (69.6)	80.15 (80.6)	78.82 (78.2)	-	16.83 (16.9)
3-α-Rha C	101.43 (101.9)	69.43 (69.7)	-	71.21 (71.1)	69.18 (69.2)	-
t-β-GlcA E	102.33 (102.4)	72.88 (73.4)	75.13 (75.7)	72.03 (72.0)	76.96 (76.5)	-
4-β-GlcA F	103.20 (103.3)	73.78 (73.6)	74.28 (74.4)	-	-	-
3-β-Gal G	104.49 (104.0)	71.36 (71.1)	-	68.83 (68.6)	-	61.18 (61.10)

Signs in parentheses have been cited by Leone *et al*., 2007, for exopolysaccharides from *K. oxytoca* BAS-10.

Anti-inflammatory activity of EPS from *K. oxytoca*

Cg-induced pleurisy in animals is an effective model that has often been used to investigate the pathophysiology of acute inflammatory response and to evaluate the anti-inflammatory activity of numerous compounds. The intrapleural injection of Cg in rats induces an acute inflammatory response characterized by a significant increase in the volume of pleural inflammatory exudate and the cells that migrate into the cavity when compared to normal animals (without injection of Cg). Polymorphonuclear leukocytes are the dominant cell type, which are recruited to the site up to 12 h after injection, after which they are replaced by mononuclear leukocytes (Amdekar *et al*., 2012; Adebayo *et al*., 2012).

Oral administration of 400 mg/kg EPS (KEPSC, KEPS) prior to Cg-induced pleurisy did not significantly change the volume of inflammatory exudate or the number of recruited leukocytes into the pleural cavity (Figure 2; Table III). Possible explanations for this include: 1) when administered orally, EPS (KEPSC, KEPS) isolated from *K. oxytoca* may be degraded in the acidic environment of the stomach, or 2) that the high molecular weight polysaccharides may not be absorbed in the systemic circulation via oral administration (Wang *et al*., 2017).

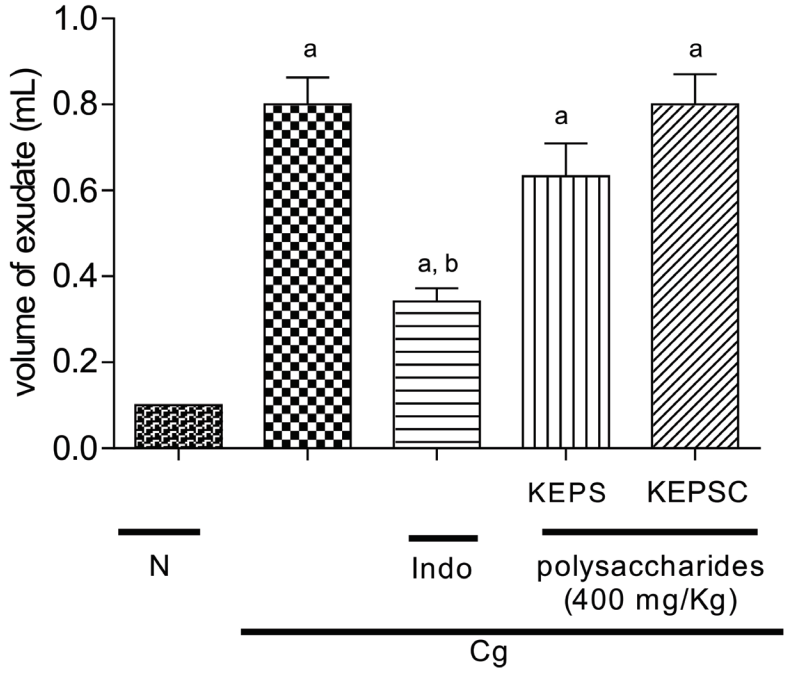

FIGURE 2 - KEPS and KEPSC effects on the volume of pleural inflammatory exudate. The pleurisy was induced by intrapleural carrageenan injection (Cg - 200 µg/cavity) in rats (n = 6-7), 1 h following oral administration of the EPS at 400 mg/kg or saline (Cg). Indomethacin (Indo, 5 mg/kg) was orally administered as an anti-inflammatory reference drug (positive control). N = normal animals that received no pleural injection of carrageenan. Each point represents the mean ± standard error of the mean of volume of exudate 4 h after injection of carrageenan. [a] $p < 0.05$ when compared with normal rats; [b] $p < 0.05$ when compared with group of rats injected with carrageenan and untreated (Cg) (ANOVA, Tukey's test).

TABLE III - Effect of oral administration of KEPS and KEPSC on the number of total and differential leukocytes in pleural inflammatory exudate of rats

Groups of animals	TL	Cells/mm^3 MN	PMN
N	5200 ± 230	3640 ± 159	1560 ± 121
Cg	73470 ± 2530[a]	10238 ± 990[a]	63232 ± 2406[a]
Indo $_{5\ mg/kg}$	66727 ± 2938[a]	11909 ± 340.2[a]	54818 ± 3654[a]
KEPS $_{400\ mg/kg}$	62625± 3647[a]	11305 ± 815.0[a]	51320 ± 4241[a]
KEPSC $_{400\ mg/kg}$	65851 ± 4437[a]	12880 ± 1734[a]	52971 ± 4016[a]

Pleurisy was induced by intrapleural carrageenan injection (Cg - 200 µg/cavity) in rats (n = 6 – 7) 1 h following oral administration of polysaccharides at 400 mg/kg or saline (Cg). Indomethacin (Indo; 5 mg/kg) was administered subcutaneously as an anti-inflammatory reference drug (positive control). N = normal animals that received no pleural injection of carrageenan; TL = total leukocytes; MN = mononuclear leukocytes; PMN = polymorphonuclear leukocytes. Each point represents the mean ± standard error of the mean of the number of leukocytes recruited into the pleural cavity 4 h following injection of carrageenan. [a] $p < 0.05$ compared with normal rats (ANOVA, Tukey's test).

Literature data report that *K. oxytoca* EPS isolated from the soil, and produced under the same cultivation conditions as that employed in this work, have an average molecular weight of approximately 200 kDa (Sugihara *et al*., 2000; Sugihara *et al*., 2001) and KEPSC and KEPS are probably high molecular weight macromolecules.

The treatment of rats, by subcutaneous administration, with KEPS significantly reduced both the volume of the pleural inflammatory exudate (edema) and the number of recruited leukocytes into the pleural cavity (polymorphonuclear and mononuclear) (Figure 3, Table IV). The inhibitory effect on edema was similar for all four doses tested (Figure 3). However, KEPS treatment at doses of 50, 100, 200 and 400 mg/kg reduced the number of recruited leukocytes by 68%, 84%, 83% and 90%, respectively. No significant difference was found between the 100 and 200 mg/kg doses of KEPS (Table IV).

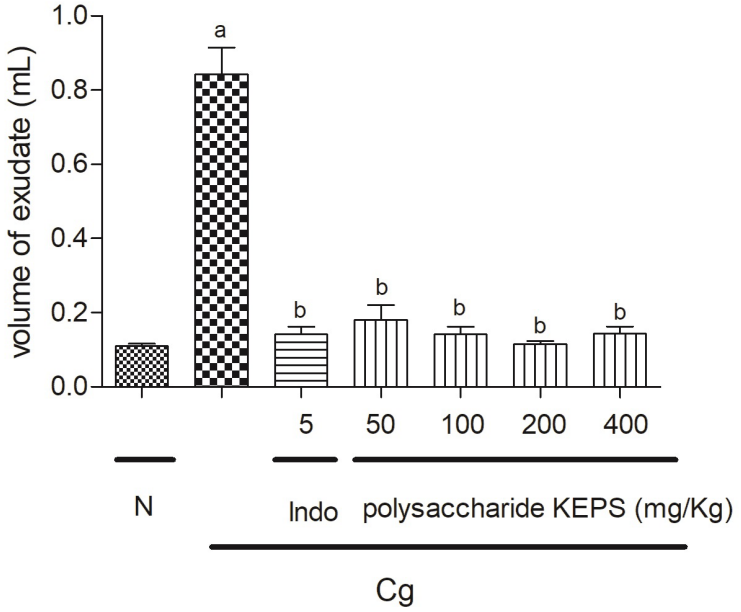

FIGURE 3 - KEPS effect on the volume of pleural inflammatory exudate. The pleurisy was induced by intrapleural carrageenan injection (Cg - 200 µg/cavity) in rats (n = 6–7), 1 h following subcutaneous administration of the EPS at 50, 100, 200, 400 mg/kg or saline (Cg). Indomethacin (Indo, 5 mg/kg) was subcutaneously administered as an anti-inflammatory reference drug (positive control). N = normal animals that received no pleural injection of carrageenan. Each point represents the mean ± standard error of the mean of the volume of exudate 4 h after injection of carrageenan. [a] $p < 0.05$ when compared with normal rats; [b] $p < 0.05$ when compared with group of rats injected with carrageenan and untreated (Cg) (ANOVA, Tukey's test).

TABLE IV - KEPS effect on the number of total and differential leukocytes in pleural inflammatory exudate of rats

Groups of animals	TL	Cells/mm^3 MN	PMN
N	6767 ± 129.5	4897 ± 493.1	1870 ± 103.9
Cg	73167 ± 471[8]a	9347 ± 1158[a]	63820 ± 584.5[a]
Indo $_{5\ mg/kg}$	71800 ± 5530[a]	12078 ± 1606[a]	59722 ± 6316[a]
KEPS$_{50\ mg/kg}$	23501 ± 1159[a,b]	3361 ± 34.6[a,b]	20140 ± 325.2[a,b]
KEPS$_{100\ mg/kg}$	11211 ± 896.5[a,b,c]	2119 ± 502.8[a,b]	9092 ± 37.91[a,b,c]

TABLE IV - KEPS effect on the number of total and differential leukocytes in pleural inflammatory exudate of rats

Groups of animals	TL	Cells/mm^3 MN	PMN
$KEPS_{200\ mg/kg}$	$12083 \pm 840.9^{a,b,c}$	$1235 \pm 294.3^{a,b}$	$10848 \pm 356.5^{a,b,c}$
$KEPS_{400\ mg/kg}$	$7072 \pm 897.6^{a,b,c,d}$	$2125 \pm 446.7^{a,b}$	$4947 \pm 328.9^{a,b,c,d}$

The pleurisy was induced by intrapleural carrageenan injection (Cg; 200 µg/cavity) in rats (n = 6–7) 1 h after the respective treatments with polysaccharide concentrations of 50, 100, 200, or 400, mg/kg or saline (Cg). Indomethacin (Indo; 5 mg/kg) was administered subcutaneously as an anti-inflammatory reference drug (positive control). N = normal animals that received no pleural injection of carrageenan; TL = total leukocytes; MN = mononuclear leukocytes; PMN = polymorphonuclear leukocytes. Each point represents the mean ± standard error of the mean of the number of leukocytes recruited into the pleural cavity 4 h following injection of carrageenan. $^a p < 0.05$ compared with normal rats; $^b p < 0.05$ compared with rats injected with carrageenan and untreated (Cg); $^c p < 0.05$ compared with rats injected with carrageenan and treated with KEPS 50 mg/kg; $^d p < 0.05$ compared with rats injected with carrageenan and treated with KEPS 100 and 200 mg/kg (ANOVA, Tukey's test).

Although cell migration is important for the body's defense in inflammatory processes, heightened mobilization to the site of injury can damage tissue depending on the activity of metalloproteinases and the generation of reactive oxygen/nitrogen species (Paula-Neto *et al*., 2011). Thus, a decrease in the number of recruited leukocytes into the pleural cavity following treatment with KEPS indicates potential clinical benefits of this compound by reducing the extent of the injury.

Corroborating our data, previous studies utilizing other experimental models (*in vitro* and *in vivo*) have shown that exopolysaccharides isolated from a variety of microorganisms (bacteria and fungus) have anti-inflammatory activity (Du *et al*., 2016; Du *et al*., 2017; Gangalla *et al*., 2018). These studies have also shown that the anti-inflammatory response can be associated with an inhibitory action on enzymes and mediators involved in the inflammatory response (COX-2, 5-LOX, NO, cytokines) (Zha *et al*., 2015).

In both experimental assays, indomethacin treatment (oral and subcutaneous administration), which was used as an anti-inflammatory drug reference, caused a reduction in the exudate volume but did not change the total number of recruited leukocytes. This can be explained by the fact that indomethacin was given at a low dose (5 mg/kg). Previous reports have shown that the effect of indomethacin on leukocyte migration depends on the administered dose and the experimental model used (Higgs *et al*., 1980; Jain, Parmar, 2011).

It is important to highlight that in the present study, in the evaluation of anti-inflammatory activity administered subcutaneously, only KEPS was used, as it is a more purified compound than KEPSC, and therefore less likely to interfere with the inflammatory response and confound interpretation of the results (Hamuro *et al*., 2017).

Overall, our data showed that the production of EPS by *K. oxytoca* is viable using the method described in this study, easy to perform, and results in a good yield. In addition, we showed that EPS exhibit important anti-inflammatory activity when administered subcutaneously in rats, and that the choice of drug administration route is a crucial factor in the experimental model used (Hamuro *et al*., 2017).

CONCLUSION

Overall, the deproteinated exopolysaccharides from *K. oxytoca* exhibited anti-inflammatory activity. Subcutaneous administration of KEPS decreased both the volume of inflammatory exudate and the number of leukocytes recruited to the pleural cavity in an experimental rat pleurisy model.

It could be suggested that EPS from *K. oxytoca* may be a candidate for the development of new therapeutic

anti-inflammatory agents, nevertheless, further studies are warranted to isolate and to identify their mechanisms in modulating the inflammatory process.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest. All authors have contributed equally.

REFERENCES

Adebayo EA, Oloke JK, Majolagbe ON, Ajane RA, Bora TC. Antimicrobial and anti-inflammatory potential of polysaccharide from *Pleurotus pulmonarius* LAU 09. Afr J Microbiol Res. 2012;6(13):3315-23.

Amdekar S, Roy P, Singh V, Kumar A, Singh R, Sharma P. Anti-inflammatory activity of lactobacillus on carrageenan-induced paw edema in male wistar rats. Int J Inflam. 2012;2012:1-6.

Baldi F, Minacci A, Pepi M, Scozzafava A. Gel sequestration of heavy metals by *Klebsiella oxytoca* isolated from iron mat. FEMS Microbiol Ecol. 2001;36(2-3):169-74.

Barbara UV, Chen M, Russell J, Crawford I, Ivanova EP. Bacterial extracellular polysaccharides involved in biofilm formation. Molecules. 2009;14(7):2535-54.

Celloto VR, Oliveira AJB, Gonçalves JE, Watanabe CSF, Matioli G, Gonçalves RAC. Biosynthesis of indole-3-acetic acid by new *Klebsiella oxytoca* free and immobilized cells on inorganic matrices. Sci World J. 2012; Article ID 495970:1-7.

Chaplin MF, Kennedy JF. Carbohydrate analysis: A practical approach, second ed.IRL Press; Oxford, 1994.

Dlamini AM, Peiris PS, Bavor JH, Kailasapathy K. Rheological characteristics of an exopolysaccharide produced by a strain of *Klebsiella oxytoca*. J Biosci Bioeng. 2009;107(3):272-74.

Dlamini AM, Peiris PS, Bavor JH, Kailasapathy K. Characterization of the exopolysaccharide produced by a whey utilizing strain of *Klebsiella oxytoca*. Afr J Biotechnol. 2007;6(22):2603-11. DOI: 10.5897/AJB2007.000-2416

Donot F, Fontana A, Baccou JC, Schorr-Galindo S. Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction. Carbohyd Polym. 2012;87(2):951-62.

Du B, Zeng H, Yang Y, Bian Z, Xu, B. Anti-inflammatory activity of polysaccharide from *Schizophyllum commune* as affected by ultrasonication. Int J Biol Macromol. 2016;91:100-5.

Du B, Yang Y, Bian Z, Xu B. Characterization and Anti-Inflammatory Potential of an Exopolysaccharide from Submerged Mycelial Culture of *Schizophyllum Commune*. Front Pharmacol. 2017;8(252):1-11.

Dubois MK, Gilles A, Hamilton JK, Reberes PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956;28(3):350-56.

Feng L, Li D, Chen J. Characterization and fouling properties of exopolysaccharide produced by *Klebsiella oxytoca*. Bioresour Technol. 2009;100(13):3387-94.

Gangalla R, Macha B, Kasarla S, Ee R, Thampu R K. Anti-inflammatory activity of the Exopolysaccharides (EPS) produced from polluted soil. Int J Pharm Biol Sci. 2018;8(1):623-31.

Goodman R, Mantegna LR, Mcallister CL, Bruin E, Dowling RL, George H, et al. IL-1 and its rolein rat carrageenin pleurisy. Mediators Inflamm. 1993;2(1):33–39.

Hamuro L, Kijanka G, Kinderman F, Kropshofer H, Bu D-X, Zepeda M, et al. Perspectives on Subcutaneous Route of Administration as an Immunogenicity Risk Factor for Therapeutic Proteins. J Pharm Sci. 2017;106(10):2946-54.

Hartree EF. A modification of the Lowry method that gives a linear photometric responser. Anal Biochem. 1972;48(2):422-27.

Higgs GA, Eakins KE, Mugridge KG, Moncada S. The effects of non-steroid anti-inflammatory drugs on leukocyte migration in carrageenin-induced inflammation. Eur J Pharmacol. 1980;66(1):81-86.

Hirota Y, Fujii T, Sano Y, Iyana S. Nitrogen fixation in the rhizo-sphere of rice. Nature. 1978;276:416-17.

Jain M, Parmar HS. Evaluation of antioxidative and anti-inflammatory potential of hesperidin and naringin on the rat air pouch model of inflammation. Inflamm Res. 2011;60(5):483-91.

Junhua W, Mengxian L, Ling L, Qi A, Jinlu Z, Jingkai Z, et al. Nitric oxide and interleukins are involved in cell proliferation of RAW264.7 macrophages activated by viili exopolysaccharides. Inflammation. 2013;36(4):954-61.

Kazy SK, Pinaki SAR, Singh SP, Asishand KS, D'Souza SF. Extracellular polysaccharides of a copper-sensitive and a copper-resistant *Pseudomonas aeruginosa* strain: synthesis, chemical nature and copper binding. World J Microbiol Biotechnol. 2002;18(6):583-88.

Lamb DH, Lei QP, Hakim N, Rizzo S, Cash P. Determination of meningococcal polysaccharides by capillary zone electrophoresis. Anal Biochem. 2005;338(2):263-9.

Leone S, Castro C, Parrilli M, Baldi F. Lanzetta R. Structure of the iron-binding exopolysaccharide produced anaerobically by the Gram-negative bacterium *Klebsiella oxytoca* BAS-10. Eur J Org Chem. 2007;(31):5183-89.

Lever MA. New reaction for calorimetric determination of carbohydrates. Anal Biochem.1972;47(1):273-79.

Manivasagana P, Kanga K, Kimb D, Kima S. Production of polysaccharide-based bioflocculant for the synthesis of silver nanoparticles by *Streptomyces* sp. Int J Biol Macromol. 2015;77:159-67.

Marcial G, Messinga J, Menchicchi B, Goycoolea FM, Faller G, Graciela FV, et al. Effects of polysaccharide isolated from *Streptococcus thermophiles* CRL1190 on human gastric epithelial cells. Int J Biol Macromol. 2013;62:217-224.

Matsumoto Y, Ohmori H, Production of immunosuppressive polysaccharide, AZ9, in the culture of *Klesiella oxytoca* strain TNM3. *J Biosci Bioeng*. 2001;92:485-87.

Nogueira CAM, Momesso CAS, Machado RLD, Almeida MED, Rossita ARP. Performance of commercial kits and laboratory protocols for bacterial genomic DNA extraction. Panam Infectology. 2004;6:35-38.

Paula-Neto HA, Alves-Filho JC, Souto FO, Spiller F, Amêndoa RS, Freitas A, et al. Inhibition of guanylyl cyclase restores neutrophil migration and maintains bactericidal activity increasing survival in sepsis. Shock. 2011;35(1):17-27.

Procópio RL, Araújo WL, Maccheroni W, Azevedo JL. Characterization of an endophytic bacterial community associated with *Eucalyptus* spp. Genet Mol Res. 2009;8(4):1408-22.

Qiao D, Ke C, Hu B, Luo J, Ye H, Sun Y, et al. Antioxidant activities of polysaccharides from *Hyriopsis cumingii*. Carbohydr Polym. 2009;78(2):199-204.

Ruas-Adiedo P, Creyes-Gavilan CG. Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. J Dairy Sci. 2005;88(3):843-56.

Shi L. Bioactivities, isolation and purification methods of polysaccharides from natural products: A review. Int J Biol Macromol. 2016;92:37-48.

Sloneker JH, Orentas DG. Pyruvic acid, a unique component of an exocellular bacterial polysaccharide. Nature. 1962;194:478-79.

Snapper CM. Differential regulation of polysaccharide-specific antibody responses to isolated polysaccharides, conjugate vaccines, and intact Gram-positive *versus* Gram-negative extracellular bacteria. Vaccine. 2016;34(30):3542-48.

Sugihara R, Yoshimura M, Mori M, Kanayama N, Hikida M, Ohmori H. Prevention of collagen-induced arthritis in DBA/1 mice by oral administration of AZ-9, bacterial polysaccharide from *Klebsiella oxytoca*. Immunopharmacology. 2000;49(3):325-33.

Sugihara R, Oiso Y, Matsumoto Y, Ohmori H. Production of immunosuppressive polysaccharide, AZ9, in the culture of *Klesiella oxytoca* strain TNM3. J Biosci Bioeng. 2001;92(5):485-87.

Sugihara R, Matsumoto Y, Ohmori H. Suppression of IgE antibody response in mice by a polysaccharide, AZ9, produced by *Klebsiella oxytoca* strain TNM3. Immunopharmacol Immunotoxicol. 2002;24(2):245-54.

Sun L, Chu J, Sun Z, Chen L. Physicochemical properties, immunomodulation and antitumor activities of polysaccharide from *Pavlova viridis*. Life Sci. 2016;144:156–61.

Tian H, Yin X, Zeng Q, Zhu L, Chen J. Isolation, structure, and surfactant properties of polysaccharides from *Ulva lactuca* L. from South China Sea. Int J Biol Macromol. 2015;79:577-582.

Vinegar R, Truax JF, Selph JL. Quantitative comparison of the analgesic and anti-inflammatory activities of aspirin, phenacetin and acetaminophen in rodents. Eur J Pharmacol. 1976;37(1):23-30.

Wang K, Cheng F, Pan X, Zhou T, Liu X, Zheng Z, et al. Investigation of the transport and absorption of *Angelica sinensis* polysaccharide through gastrointestinal tract both *in vitro* and *in vivo*. Drug Deliv. 2017;24(1):1360-1371.

Zha X, Lu C, Cui S, Pan L, Zhang H, Wang J, et al. Structural identification and immunostimulating activity of a *Laminaria japonica* polysaccharide. Int J Biol Macromol. 2015;78:429-38.

Wang Y, Li Y, Liu Y, Chen X, Wei X. Extraction, characterization and antioxidant activities of Se-enriched tea polysaccharides. Int J Biol Macromol. 2015;77:76-84.

York WS, Darvill AG, McNeil M, Stevenson TT, Albersheim P. Isolation and characterization of plant cell walls and cell wall components. Methods in Enzymol. 1985;118:3-40.

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