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Necroptosis mediates the antineoplastic effects of the soluble fraction of polysaccharide from red wine in Walker-256 tumor-bearing rats

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Chemicals:
TriZol Reagent
buffered 10% formalin
ethanol
xylol
paraffin
hematoxylin and eosin (HE)
phosphate buffer (pH 6.5)
Griess solution (0.1%
N-1-naphthyl-tilediamine, 1%
sulfanilamide in 5% H_3PO_4)
saline Triton X-100 0.1%, TMB 18.4 mM
dimethylformamide 8%
sodium acetate (NaOAc)
p-nitrophenyl-N-acetyl-β-D-glucosamine
N-acetyl-β-d-glucosamine
p-nitrofen
ketamine hydrochloride
xylazine hydrochloride;
metothrexate
phosphate buffered saline (PBS, 16.5 mM
phosphate, 137 mM NaCl, and 2.7 mM KCl)
at pH 7.4

ABSTRACT

Polysaccharides are substances that modify the biological response to several stressors. The present study investigated the antitumor activity of the soluble fraction of polysaccharides (SFP), extracted from cabernet franc red wine, in Walker-256 tumor-bearing rats. The monosaccharide composition had a complex mixture, suggesting the presence of arabinoglactans, mannans, and pectins. Treatment with SFP (30 and 60 mg/kg, oral) for 14 days significantly reduced the tumor weight and volume compared with controls. Treatment with 60 mg/kg SFP reduced blood monocytes and neutrophils, reduced the tumor activity of *N*-acetylglucosaminidase, myeloperoxidase, and nitric oxide, increased blood lymphocytes, and increased the levels of tumor necrosis factor α (TNF-α) in tumor tissue. Treatment with SFP also induced the expression of the cell necroptosis-related genes *Rip1* and *Rip3*. The antineoplastic effect of SFP appears to be attributable to its action on the immune system by controlling the tumor microenvironment and stimulating TNF-α production, which may trigger the necroptosis pathway.

Abbreviations: ALT, alanine aminotransferase; ANOVA, Statistical analysis of variance; AST, aspartate aminotransferase; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; DNA, Deoxyribonucleic acid; FADD, Fas -associated death domain; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; HE, Hematoxylin and eosin; Mlkl, mixed lineage kinase domain-like protein; MPO, myeloperoxidase; mRNA, Messenger ribonucleic acid; MTX, Metotrexato; NAG, N-acethyl-β-D-glucosaminidase; NaOAc, Sodium acetate; NF-κB, nuclear factor kappa B; NO, Oxide nitric; p53, Protein 53; SFP, Soluble fraction of polysaccharide; Rip-1, receptor-interacting protein kinase 1; Rip-3, receptor-interacting protein kinase 3; ROS, reactive oxygen species; TMB, tetramethylbenzidine; TNF-α, tumor necrosis factor-alpha; Vegf, vascular epidermal growth factor.

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ethylenediaminetetraacetic acid (EDTA)
(0.5 M, pH 8.0)
eTrypan blue, 2 M trifluoroacetic acid

Keywords:
Walker-256 tumor
polysaccharide
red wine
cabernet franc
necroptosis
immunomodulation

1. Introduction

Cancer is a group of diseases that are related to mutations in key genes that confer a selective growth advantage to cancer cells and regulate core cellular processes, such as cell survival and genome maintenance (Vogelstein, Papadopoulos, Velculescu, Zhou, & Kinzler, 2013). The most conventional treatment for cancer patients is chemotherapy. Drugs that are frequently used include vincristine, methotrexate, and alkylating agents, which induce cell death through different mechanisms of action (e.g., the inhibition of mitosis, metabolism, and angiogenesis). However, chemotherapy has severe side effects and is insufficient to induce complete tumor remission. This occurs mainly because of pharmacokinetics, resulting in lower intracellular drug concentrations, an increase in cell survival, and tumor cell resistance to chemotherapy (Merck, 2015). Therefore, the search for new substances that are able to circumvent the mechanisms of tumor resistance and have fewer side effects is important.

Recent studies have reported the antitumor activity and antimetastatic, immunomodulatory, and antioxidant properties of polysaccharides that are extracted from seaweed, fruits, fish, and mushrooms (Huang et al., 2015; Inngjerdingen, Thöle, Diallo, Paulsen, & Hensel, 2014; Mau, Chao, & Wu, 2001; Nascimento et al., 2013; Ooi & Liu, 2000; Park et al., 2013; Ren, Perera, & Hemar, 2012; Rout & Banerjee, 2007; Suo et al., 2014; Wasser, 2003; Zhou, Hu, Wu, Pan, & Sun, 2008). Polysaccharides are substances that modify biological responses. The effects of polysaccharides are not cell-specific and instead regulate major bodily systems, including the nervous, hormonal, and immune systems (Wasser, 2003).

Several fruits, including grapes, are rich sources of polysaccharides. Red wine, such as carbenet franc, is an alcoholic beverage that is derived from the fermentation of grapes and has a soluble fraction of polysaccharides (SFP) that are mainly composed of arabinogalactans and rhamnogalacturonans. Some authors had described important immunomodulatory, antioxidant, antisepticemic, antineoplastic, and gastroprotective effects of the polysaccharides arabinogalactan and rhamnogalacturonan (Cipriani et al., 2006; Dartora et al., 2013; Inngjerdingen et al., 2014; Mellinger et al., 2008; Mueller & Anderer, 1990; Nascimento et al., 2013; Park et al., 2013). The "French paradox" phenomenon is associated with moderate wine drinking, which reduces the risk of cardiovascular, cerebrovascular, and peripheral vascular diseases and cancer (Pieszka, Szczurek, Ropka-Molik, Oczkowicz, & Pieszka, 2016). Some beneficial effects of wine on health have been attributed to resveratrol, a polyphenol that is present in the skin of grapes. Resveratrol has antioxidant activity, regulates plasma lipids and cardiac activity, and has protective effects against neurodegenerative diseases and several tumors (Jang et al., 1997; Singh, Liu, & Ahmad, 2015). Resveratrol has been extensively studied, but other components of wine that are present in higher concentrations, such as polysaccharides, require further investigation.

Thus, the aim of the present study was to evaluate the *in vivo* antitumor activity of SFP that was extracted from cabernet franc red wine in Walker-256 tumor-bearing rats, a model of solid carcinoma. This tumor is species-specific and characterized by fast growth. It is often used in studies of metabolism, oxidative stress, and inflammation that are related to cancer (Acco, Bastos-Pereira, & Dreifuss, 2012). Our hypothesis was that SFP modulates tumor development in Walker-256 rats.

2. Material and methods

2.1. Polysaccharide preparation

Cabernet franc polysaccharides were extracted from commercial wine bottles (Vinho Tinto Reserva Salton, Bento Gonçalves, RS, Brasil – production years: 2013 and 2015). The soluble liquid was initially reduced up to 25% of its volume under reduced pressure at 30 °C. The supernatants were combined, followed by the addition of 3 vols of cold ethanol and incubation for 24 h at −20 °C. The precipitated polysaccharides were washed twice with 70% cold ethanol and dialyzed against tap water in a membrane with a molecular mass cut-off (MMCO) of 8 kDa (Dartora et al., 2013; Bezerra, 2016). The retained fraction that contained polysaccharides was lyophilized and analyzed by gas chromatography-mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR).

2.1.1. Monosaccharide composition determined by NMR and GC–MS

Wine polysaccharides (5 mg) were hydrolyzed with 2 M trifluoroacetic acid (500 μl) at 100 °C for 8 h and evaporated to dryness under N_2 pressure. The residue material was dissolved in 0.5 ml of D_2O. One-dimensional ^1H NMR was performed at 600 MHz with the pulse program zgpr for HDO presaturation (relaxation delay = 5.0 s, number of time domain points = 65536) to obtain a spectrum width of 10 ppm. The monosaccharides were identified based on the chemical shifts of a standard mixture of 18 monosaccharides (Sassaki et al., 2014). After NMR analysis, the later was reduced with NaB^2H_4 for 12 h and evaporated to dryness. Boric acid was removed as trimethyl borate by co-distillation with MeOH. Acetylation was performed with Ac_2O-pyridine (1:1, v/v; 200 μl) at 100 °C for 1 h. Crushed ice-water was added to the solution, and the resulting 2-*O*-Me-Fuc, 2-*O*-Me-Xyl, and alditol acetate derivatives were extracted with $CHCl_3$ and analyzed by GC–MS (Varian-Saturn 4000-3800 mass spectrometer, 30 m × 0.25 mm VF-5MS column). The column temperature was set as the following: 50 °C for 1 min, increase to 220 °C at 40 °C/min, then held for 13.0 min. Partially *O*-methylated alditol acetates were identified based on the *m*/*z* of their positive ions, with comparisons to standards. The results are expressed as a relative percentage of each component (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).