Adjunctive role of *Calyptranthes tricona* extract with probiotic *Kluyveromyces marxianus* on colorectal adenocarcinoma Caco-2 cells

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ABSTRACT

The composition of microbiota may influence the development of colorectal cancer (CRC). In addition, probiotics can decrease the chance of developing cancer or its progress. For that reason, it is encouraging to assess the influence of plant extracts as adjuvants of the health-promoting effects of probiotics. Thus, this study aimed to investigate the *in vitro* beneficial properties of *Calyptranthes tricona* leaf ethanol extract in association or not with the lactic yeast *Kluyveromyces marxianus* on colon adenocarcinoma Caco-2 cells. *C. tricona* extract inhibited the *in vitro* p38α MAPK activity and exhibited immunomodulation on isolated human lymphocytes. Further, the extract did not induce cytotoxicity towards *K. marxianus* or Caco-2 cells; leading to an increased yeast adhesion to cells in a dose-dependent manner. In conclusion, this preliminary study demonstrates that *C. tricona* extract has effects on enzyme inhibition and immune function. In addition, when associated with *K. marxianus*, *C. tricona* may possess beneficial properties for application as enhancer of probiotic’s protective role on CRC cells. However, further studies are necessary in order to elucidate the mechanisms involved.

1. Introduction

Colorectal cancer (CRC) is the fourth most frequent cause of cancer deaths worldwide. It has a multifactorial etiology including inflammation, poor diet and gut microbiota dysbiosis (Marmol et al., 2017; McGuire, 2016). Recent reports have shown that probiotics decrease the chance of developing cancer or its progress (Chen et al., 2017; Cousin et al., 2016). Dietary supplementation (e.g. prebiotics and probiotics) represents an alternative and useful strategy to modulate the risk for CRC and/or to treat it. Besides, it can help to restore the normal flora (Rafer et al., 2007). In addition, the use of prebiotics and/or probiotics to manipulate gut microbiota has already been proposed since they have the ability to modulate the host’s immunologic response, stimulating anti-inflammatory cytokines, antioxidants compounds, and generating anti-carcinogenic compounds besides improving gut microbial balance (Satish Kumar et al., 2017). The microorganisms that satisfy the conditions for a good probiotic include strains of lactic acid producing bacteria such as *Lactobacillus* and *Bifidobacterium* (Jiang et al., 2016; Plaza-Diaz et al., 2017). In addition, some strains of *Propionibacterium freudenreichii* and yeast *Kluyveromyces marxianus* have presented important probiotic activities (Cousin et al., 2012, 2016; Maccaferri et al., 2012).

Plants of Myrtaceae family, including the genus *Calyptranthes*, are intensively studied and commercially exploited due to their innumerable biological properties and food components. Antiproliferative (Faleiro et al., 2017), neuromodulatory (Kich et al., 2016), antimicrobial and anti-inflammatory are some of their potentials (Borges et al., 2014; Figueiroa Ede et al., 2013). Moreover, we have previously demonstrated the antioxidant activity of *C. tricona* ethanol extract and the protective effect against H2O2-induced cell death in MCF-7 cells. These effects could be attributed to the identified phytoconstituents, such as steroids, triterpenoids, condensed tannins and flavones as the main...
compounds, highlighting the presence of the caffeic acid and ellagic acid (Kich et al., 2017). Phenolic compounds, such as caffeic acid and ellagic acid, have been associated to positive effects in terms of gut health protection. An important part of the ingested phenolic compounds reaches the large intestine where it undergoes a series of microbial transformations that leads to the generation of related metabolites. (Mosele et al., 2015) Phenolic compounds metabolites could present higher activity at a physiological level than the corresponding food precursors. These metabolites could also be absorbed, increasing polyphenol bioavailability (Dueñas et al., 2015).

Traditionally, probiotics are associated with dairy products (Ceugniez et al., 2017); however, considering the use of natural sources (i.e. plant extracts) in therapeutic regimens and the increase of yeasts consumption as human probiotics (Ceugniez et al., 2017; Newman and Cragg, 2016), it is encouraging to assess the influence of extracts as enhancers of the health-promoting effects of probiotics. Thus, the aim of this study was to investigate the in vitro beneficial properties of C. tricona leaf ethanol extract alone or in association with the lactic yeast K. marxianus on colon adenocarcinoma cells.

2. Material and methods

2.1. Reagents

All chemicals otherwise stated were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sabouraud Maltose Broth was purchased from Becton&Dickinson (Sparks, MD, USA).

2.2. Plant collection and extract preparation

C. tricona D. Legrand leaves were collected in September 2013 in Lajeado, Southern Brazil, geographical coordinates: 29º26’51”S and 51º57’54”W. A voucher specimen (#4996) was deposited at the Herbarium of Univates. The ethanol extract was prepared as previously described (Kich et al., 2016). For the experiments, the extract was diluted with DMSO resulting in a stock solution of 20 mg/mL.

2.3. In vitro enzyme activity assay

The extract was screened for p38α mitogen-activated protein kinase (MAPK) inhibition and the inhibitory potency was assessed by previously established direct ELISA assay measuring the inhibition of p38α mediated ATF-2 phosphorylation (Goettert et al., 2010). SB203580 was used as a p38-specific inhibitor. The half-maximal inhibitory concentration (IC50) was calculated.

2.4. Human primary cells isolation and cytotoxicity assay

The isolation of peripheral blood mononuclear cells (PBMCs) from human whole blood was performed as previously described (Haute et al., 2015). PBMCs were seeded in 96-well microtiter plates and cultured in RPMI 1640 medium supplemented with 20% autologous serum and 1% antibiotics at 37°C in a 5% CO2-humidified incubator. Cells (1.6 × 10^5/well) were treated with increasing concentrations of the extract (50, 100 and 200 μg/mL) for 96 h. The cellular viability was measured by trypan blue dye exclusion. The results were presented as percentage of control.

2.5. Lymphoproliferation assay

Phytohemagglutinin (PHA) was used for lymphocyte proliferation. Briefly, PBMCs (1.6 × 10^5 cells/well) were plated and cultured with different concentrations of the extract (50, 100 and 200 μg/mL) in the presence of the mitogen PHA (10 μg/mL) in 96-well microtiter plates for 96 h. Lymphocyte proliferation was determined by MTT assay as previously described (Mosmann, 1983). The plates were spectrophotometrically read using a wavelength of 540 nm and a reference wavelength of 620 nm.

2.6. Cell line and culture conditions

The human colon adenocarcinoma Caco-2 cell line was obtained from the Rio de Janeiro Cell Bank (BCRJ, # 0059), Brazil. Cells were cultured in DMEM-Low glucose medium supplemented with 1% non-essential amino acids, 10% foetal bovine serum and 1% antibiotics. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

2.7. Yeast culture conditions

Kluyveromyces marxianus (EC Hansen) van der Walt (ATCC, # 12,424) was routinely grown aerobically at 37°C in Sabouraud Maltose Broth (SMB). For all experiments, fresh overnight culture inoculated from a single colony was used. Yeast cells were diluted to 1 × 10^6 CFU/mL.

2.8. Cell viability assay

The assessment of Caco-2 cell viability was performed using Alamar Blue™ according to manufacturer’s instruction. Caco-2 cells (1 × 10^5 cells/mL) and K. marxianus (1 × 10^6 CFU/mL) were seeded in 96-well microplates and challenged with increasing concentrations of ethanol extract (25, 50, 100, 200 and 400 μg/mL). After 24 h, 48 h or 72 h, the absorbance was read at 540 nm and 630 nm using an ELISA microplate reader.

2.9. Adhesion of K. marxianus to Caco-2 cells

The adhesion of K. marxianus to Caco-2 cells treated with increasing concentrations of C. tricona extract (25, 50, 100, 200 and 400 μg/mL) was performed as previously described with minor modifications (Cravioto et al., 1979). Briefly, Caco-2 cells were seeded in 12-well plates containing 13-mm coverslips. After achieving monolayers, cells were washed with PBS and antibiotic-free medium with extract and K. marxianus were added and incubated for 3 h. After, cells were washed with PBS, fixed with methanol for 30 min and stained with May-Grunwald-Giemsa for 20 min. Adherent yeast cell number per Caco-2 cells was counted using an inverted light microscope.

2.10. Statistical analysis

All experiments were performed at least in triplicate and results were presented as mean ± SEM. Data were analysed and graphed using GraphPad Prism 6.01 software. Statistical significance was determined using one-way ANOVA with Dunnett’s or Tukey’s correction for multiple comparisons. A P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Inhibitory potential of C. tricona extract on p38α MAPK

Considering that the composition of microbiota may influence the development of CRC, there is an increase in drug discovery programs using natural products and probiotics as adjuvant therapies to reduce dosage and frequency of chemotherapeutic agents (Marmol et al., 2017). In addition, due to the occurrence of chemical drugs-related adverse effects, inhibitors from natural source are the potential agents to replace them in therapeutic regimens. The quantification of enzyme activity after inhibitor treatment is a key step and a prerequisite for efficient drug discovery programs (Laufer et al., 2002). Thus, we started our investigation screening C. tricona extract for its ability to inhibit the...
activity of the protein kinase p38α, since this MAPK is directly involved in many human disorders, including chronic inflammation and CRC (Del Reino et al., 2014). Here, it was demonstrated that *C. tricona* extract presented a great inhibitory activity with an IC$_{50}$ of 1.4 μg/mL, while the p38-specific inhibitor SB203580 showed an IC$_{50}$ of 0.06 μM (Fig. 1).

3.2. *C. tricona* extract exhibits immunomodulatory activity on PBMC

After the cell-free screening, we decided to explore the possible cytotoxic potential of *C. tricona* extract on immune cells, once these cells enhance premalignant cell growth and survival by activating signalling pathways such as MAPKs (Maccaferri et al., 2012). Previously, we have reported that *C. tricona* ethanol extract has genotoxic potential in a dose dependent-manner on lymphocytes in whole blood culture. Even though the intensity of DNA damage was proportional to the concentration of the extract, it was lower than the one promoted by the positive control ethyl methanesulfonate. In addition, it was an early DNA response demonstrated upon a short period of 3 h (Rich et al., 2017). As known, genotoxic effects may be transient and prone to repair (Avishai et al., 2003). Hence, in the present study we evaluated the cytotoxicity of the extract after a long-term stimulation. PBMCs were isolated from healthy volunteers and challenged with increasing concentrations of *C. tricona* extract for 96 h and the survival rate was evaluated by trypan blue assay. As depicted in Fig. 2a, the extract did not present any cytotoxicity after 96 h.

Immune cells are associated with the intestinal mucosa and regulate intestinal immune response against the adhesion and invasion of pathogens. Thus, it would be interesting to have a natural immunomodulator to interact with probiotics and improve their ability to bind to the intestinal epithelium (Maccaferri et al., 2012). We evaluated the effect of *C. tricona* extract on lymphoproliferation. PHA-stimulated cells were cultured for 96 h in the presence or absence of the previous tested concentrations of the extract. As shown in Fig. 2b, cells treated with the mitogen PHA exhibited intense proliferation compared with control cells. However, the ethanol extract hampered the mitogenic effect of PHA as observed in the cultures treated with 200 μg/mL extract, suggesting a possible immunomodulatory activity of this extract.

3.3. Cytotoxicity of *C. tricona* ethanol extract on *K. marxianus* and Caco-2 cells

Probiotics are described as non-pathogenic microorganisms that exert health-promoting effects in addition to the usual nutritional benefits (Nagpal et al., 2012). Most probiotics are lactic acid bacteria strains (Plaza-Díaz et al., 2017); however, yeasts might offer more advantages compared to bacteria, as they are insensitive to antibiotics. Thus, the research on consumption of yeasts as human probiotics has increased in the last decade (Saxelin, 2008). Considering that, we have selected a strain of the lactic yeast *K. marxianus*, of which other strains have been previously reported as probiotics (Ceunegiez et al., 2017; Maccaferri et al., 2012). Initially, we evaluated the potential toxicity of *C. tricona* extract on *K. marxianus* cell viability. The yeast was challenged with increasing concentrations of the extract for 24 h and cell viability was assessed by Alamar blue colorimetric assay. As shown in Fig. 3a, the positive control amphotericin B significantly decreased the yeast cell viability. However, none of the extract’s concentrations affected *K. marxianus* viability. Later, we verified a possible cytotoxicity of *C. tricona* extract on human colon adenocarcinoma Caco-2 cell line. Fig. 3b shows that ethanol extract did not affect cell viability after 48 h of exposure. However, a minor decrease on cell viability (P < 0.05) was seen in cells cultured with 400 μg/mL ethanol extract after 72 h (Fig. 3c). However, according to Ioset et al. (2009), an extract is considered cytotoxic when it has an IC$_{50}$ < 90 μg/mL. Consequently, the slight reduction in Caco-2 viability does not classify the 400 μg/mL extract as potentially cytotoxic.

3.4. Combined effect of *C. tricona* extract and *K. marxianus* on Caco-2 cells

Based on the hypothesis that the origin of CRC might be dysbiosis, probiotics have an important protective role (Marmo et al., 2017). It is noteworthy that the health-promoting effects of probiotic strains might be partly dependent on their persistence in the intestine and their adhesion to mucosal surfaces (Van Raay and Allen-Vercoe, 2017). In addition, the adhesion ability is a strain-specific characteristic. Adhesion to gut epithelium is an important requisite for allowing probiotics to modulate the immune system. Probiotics that present this capacity play a crucial role in the immune response (Jungersen et al., 2014). A previous report demonstrated that *K. marxianus* B0399 strain was extremely adherent to Caco-2 cells and had the potential to modulate the immune response (Maccaferri et al., 2012). In the current study, *K. marxianus* was evaluated for its adhesion ability to Caco-2 cells treated with *C. tricona* ethanol extract. As depicted in Fig. 4, the yeast adhesion to Caco-2 cells was increased by the extract within 3 h of treatment. The adhesion of the microorganism *K. marxianus* to the cells seems to depend on the concentration of the *C. tricona* extract or some phytoconstituent, suggesting a dose-dependent activity. However, the effects were significant (P < 0.01) only at two concentrations (200 and 400 μg / mL) of the extract.

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**Fig. 1.** Inhibitory activity of *C. tricona* leaf ethanol extract on p38 MAPK. Mean values ± SEM are shown (n = 3). Results are showed as IC$_{50}$ for *C. tricona* (μg/mL) and SB203580 (μM).

**Fig. 2.** Effects of *C. tricona* leaf ethanol extract on peripheral blood mononuclear cells (PBMCs). (a) PBMCs were challenged with the extract for 96 h and cytotoxicity was assessed using trypan blue exclusion assay. (b) Immunomodulatory effect of the extract was assessed by MTT assay. Cells were treated with DMSO vehicle control, challenged with the extract with or without phytohemagglutinin (PHA) for 96 h. Vehicle Mean values ± SEM are shown (n = 3). *** P < 0.001 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Freitas who identified the plant, and Fabiola Dresch who provided technical assistance.

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4. Conclusion

This preliminary study improves the knowledge on C. trcona properties. It presents data to argue that the leaf ethanol extract of C. trcona has effects on enzyme inhibition and immune function. In addition, when associated with K. marxianus, it possesses beneficial properties that may be applied to enhance probiotic’s protective role on CRC cells. Evidently, further studies are necessary to elucidate the possible mechanisms involved.

Conflict of interest

None.

Author contributions

CS and MG conceived and designed the experiments. DK, SB, DF, SI, DM, TB, GH performed the experiments. DK, SB, and MG analyzed the data. DK, SB and MG wrote the paper. JO, SI and RP critically revised the manuscript and contributed reagents/materials/analysis tools.

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Fig. 3. Toxicity of ethanol extract of C. tricona on Klyveromyces marxianus and Caco-2 cells. (a) K. marxianus was challenged with increasing concentrations of the extract for 24 h. The antifungal amphotericin B (AFB; 100μg/mL) was used as positive control. Caco-2 cells were challenged with different concentrations of the extract for (b) 48 h and (c) 72 h. Cell viability was assessed using Alamar blue assay. Mean values ± SEM are shown (n = 3). ** P < 0.01; *** P < 0.001 compared with C (control) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
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