Effect of \textit{p}-cymene on chemotaxis, phagocytosis and leukocyte behaviors

Raquel Kummer\textsuperscript{1*}, Camila Fernanda Estevão-Silva\textsuperscript{1}, Rafael Lucena Bastos\textsuperscript{1}, Renata Grespan\textsuperscript{1}, Francielli Maria de Souza Silva-Comar\textsuperscript{1}, Ricardo Alexandre Spironello\textsuperscript{1}, Bruno Ambrosio Rocha\textsuperscript{1}, Expedito Leite Silva\textsuperscript{2}, Ciomar Aparecida Bersani-Amado\textsuperscript{1}, Roberto Kenji Nakamura Cuman\textsuperscript{1}

\textsuperscript{1}Department of Pharmacology and Therapeutic, State University of Maringá, Maringá, Paraná 870020-900, Brazil
\textsuperscript{2}Department of Chemistry, State University of Maringá, Maringá, Paraná 870020-900, Brazil

Summary. In this study, we investigated the effects of \textit{p}-cymene (CYM) \textit{in vitro} and \textit{in vivo} on leukocyte activity. In the cell viability assay, CYM (3, 10, 30, 90 \(\mu\)g/mL) had low cytotoxicity. \textit{In vitro} chemotaxis revealed that CYM (3, 10, 30, 60 \(\mu\)g/mL) promoted a significant reduction of neutrophil migration toward fMLP, but not toward LTB\textsubscript{4} stimulation. In carrageenan-induced peritonitis, CYM (100, 200, 400 mg/kg) decreased the infiltration of peritoneal exudate leukocytes and of polymorphonuclear leukocytes. CYM pretreatment resulted in a significant decrease in the number of rolling (100, 200 mg/kg) and in the number of adherent leukocytes (100, 200, 400 mg/kg) to the perivascular tissue. The macrophage phagocytic index was increased significantly in concentrations of CYM (3, 10, 30 \(\mu\)g/mL). Treatment with CYM (10, 90 \(\mu\)g/mL) also reduced TNF-\(\alpha\) levels but did not alter IL-10 levels in fMLP-stimulated neutrophils. In conclusion, CYM may be considered as a potential agent for treatment inflammatory injury, however, further studies are necessary to elucidate the anti-inflammatory mechanism.

Industrial relevance. The CYM is a secondary metabolite, belongs to the class of monoterpene and is found in essential oils from plants, food and in several spices. It is an important intermediate used in pharmaceutical, flavor and aroma industries and for the production of fungicides. The investigation of the anti-inflammatory activity of this compound may be useful to development of new drug anti-inflammatory, on a commercial scale.

Keywords. \textit{p}-cymene; chemotaxis; \textit{in vivo} microcirculation; phagocytosis; anti-inflammatory.

INTRODUCTION

Plants are one of the most important sources of substances with biological activities and their use for treatment, cure and prevention of diseases is one of the oldest forms of medical practice of humanity (Gu et al. 2014). In recent years have seen a growing interest in the use of natural compounds and, more importantly, their role as a basis for drug development due to their structural diversity and specific biological activities (Cragg and Newman 2013).

The \textit{p}-cymene (CYM), 4-isopropyltoluene or 1-isopropyl-4-methylbenzene, is a secondary metabolite, belongs to the class of monoterpene and is found in some foods, several spices and in essential oils from plants, including the \textit{Citrus latifolia} Tanaka essential oil (Siani et al. 1999; Xie et al. 2012; Kummer et al. 2013; Quintans et al. 2013). Furthermore, it is an important intermediate used in pharmaceutical, flavor and aroma industries and for the production of fungicides and pesticides (Selvaraj et al. 2002).

Recent works have demonstrated that CYM may present important biological activities including antifungal (Kordali et al. 2008), antiviral (Astani, Reichling, and Schnitzler 2009), antimicrobial (Rattanachaikunasopon and Phumkhachorn 2010), insecticide effect on the \textit{Aedes aegypti} (Lucia, Zerba, and Masuh 2013), antinociceptive (Santana et al. 2011; Quintans-júnior et al. 2013) and anti-inflammatory (Zhong et al. 2012; Xie et al. 2012).

However, there are few studies on this isolated constituent related to the anti-inflammatory activity, mainly on the behavior process involving leukocyte chemotaxis and phagocytosis. Given these considerations, the aim of the study was to evaluate the \textit{in vitro} and \textit{in vivo} effects of CYM on leukocyte activity.

MATERIALS AND METHODS

\textit{Plant material and constituent of essential oil}. The constituent CYM was isolated from \textit{Citrus latifolia} Tanaka essential oil as fractions of hydrodistillated oil, and was identified by GC-MS and NMR as previous described (Kummer et al. 2013).

\textit{Animals}. Male BALB/c mice (20-25 g) and adult male C57BL/6J mice (17-20 g), wild type, were used. The animals were obtained from the Central Animal House of the State University of Maringá. The animals were housed at 22 \(\pm\) 2\(^\circ\)C under a 12 h/12 h light/dark cycle. The experimental protocols were approved by the Ethical Committee in Animal Experimentation of the State University of Maringá (CEAE/UEM066/2010).
**Bioassays for cytotoxic activity.** The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide; Sigma) assay is based on the mitochondrial enzyme reduction of tetrazolium dye that detects and determines cell viability. Neutrophils were obtained from the peritoneal cavity of BALB/c mice 4 h after zymosan injection (1 mg/cavity, i.p.). Briefly, the cells (5 x 10^5 cells/well) were exposed to CYM (3, 10, 30 or 90 µg/ml) for 90 min at 37°C in 5% CO₂. CYM emulsions were prepared with RPMI-1640 medium and emulsified by sonication. A volume of 10 µl MTT (5 mg/ml; Sigma) was added to each well. After 2 h, 150 µl of the supernatant was removed, and 100 µl of dimethyl sulfoxide was added to each well. The cells were incubated at 25°C for an additional 10 min, and absorbance was measured using a Biochrom Asys Expert plus microplate reader (Asys) at a wavelength of 540 nm. The values of the blank wells were subtracted from each well of treated and control cells. The percentage of viability was determined by the following formula:

\[
\% \text{ Viable cells} = \frac{\text{Absorbance of the treated cells} - \text{Absorbance of the blank}}{\text{Absorbance of the control} - \text{Absorbance of the blank}} \times 100
\]

In vitro chemotaxis assay. To evaluate the effects of CYM on chemotaxis, neutrophils were obtained from the peritoneal cavity of BALB/c mice 4 h after the zymosan injection (1 mg/cavity, i.p). The cell number was adjusted to 1 x 10^6 cells/ml in RPMI-1640 medium that contained 0.1% bovine serum albumin (BSA). The chemotaxis assay was performed using a 48-well microchemotaxis plate (Neuro Probe), in which the chambers were separated by a polycarbonate membrane (5 µm pore size). The chemoattractants N-formyl methionyl leucyl phenylalanine (fMLP; 10^-6 M) and leukotriene B₄ (LTB₄; 10^-7 M) and a negative control (RPMI-1640) were placed in the lower chamber. A neutrophil suspension (1 x 10^6 cells/ml) pretreated with CYM (1, 3, 10 or 60 µg/ml) for 30 min was then placed in the upper chamber. CYM emulsions were prepared with RPMI-1640 medium and emulsified by sonication. The cells were allowed to migrate into the membrane for 1 h at 37°C in 5% CO₂. Following incubation, the membrane was washed and stained using Instant Prov (Newprove). The membrane area of each well was scored using light microscopy to count the intact cells present in five random fields. The results are expressed as the mean number of neutrophils per field and representative of three separate experiments.

**Determination of leukocytes migration into peritoneal cavity.** In vivo leucocytes migration was performed in C57BL/6 mice. Mice were orally pretreated with CYM (100, 200 or 400 mg/kg), indomethacin (5 mg/kg) or vehicle (0.2% of an aqueous Tween 80 solution, 0.1 ml) as the control. Thirty minutes later, all of the animals received an intraperitoneal carrageenan injection (500 µg/cavity) or an equivalent volume of vehicle (saline). Four hours after the animals were anesthetized and euthanized, the cells present in the peritoneal cavity were harvested by introducing 2.0 ml of phosphate-buffered saline (PBS) that contained ethylenediaminetetraacetic acid (EDTA). Counts were then performed in total and differential cells. The results are expressed as the number of leucocytes per cavity.

Real time in situ microscopic analysis for rolling and adhesion events of leucocytes in the mesenteric microcirculation.

Rolling and adhesion of leucocytes to the endothelium were evaluated in the mesenteric microcirculation of C57BL/6J mice two hours after intraperitoneal carrageenan injection (500 µg/cavity). CYM (100, 200 or 400 mg/kg), indomethacin (5 mg/kg), celecoxib (5 mg/kg) or vehicle (0.2% of an aqueous Tween 80 solution) were administered orally one hour before carrageenan injection to different groups of mice. In brief, animals were anesthetized (ketamine/xilasine 1:1) and the mesenteric tissue was exposed for microscopic examination in situ. The animals were maintained on a special board thermostatically controlled at 37°C keeping the tissue moist and warm by irrigating with Ringer Locke’s solution (pH 7.2–7.4) containing 1% gelatin. The postcapillary venules, with a diameter of 18–25 µm were chosen and the interaction of leucocytes with the luminal surface of the venular endothelium was evaluated counting the number of rolling leucocytes at 5 min intervals. Adherent leucocytes were considered to be adherent to the venular endothelium if remained stationary for >30 s. Cells were counted in the recorded image using three different fields for each animal to avoid variability due to sampling. Data were then averaged for each animal.

Peritoneal macrophage preparation and determination of phagocytosis activity. To evaluate the effects of CYM on phagocytosis activity, macrophages were obtained from the peritoneal cavity of BALB/c mice (Silva-comar et al. 2014). To collect the macrophages, the animals were euthanized, and 5 ml of phosphate-buffered saline (PBS) was injected intraperitoneally. Phosphate-buffered saline that contained peritoneal exudate cells was recollected and kept on ice. The suspended cells were centrifuged at 1500 rotations per minute for 10 min and resuspended with complete RPMI-1640. Peritoneal exudate cells were then isolated on 24-well culture plates and allowed to adhere for 1 h at 37°C in 5% CO₂. After nonadherent cells were removed, the remaining adherent cells were designated as peritoneal macrophages. Cell viability, evaluated by the exclusion test with Trypan blue, was ≥90% in all of the experiments. The cells were incubated for 24 h at 37°C in 5% CO₂ with different concentrations of CYM (3, 10 or 30 µg/ml) in complete RPMI-1640 medium. Lipopolysaccharide (LPS; 20 µg/ml) was used as a positive control, and RPMI-1640 medium was used as a negative control. After incubation, 100 µl of a 3% suspension of chicken red blood cells (CRBCs) was added to each well. CRBCs, to be nucleated, were used to assess macrophage phagocytosis. After 1 h, the macrophages were fixed and stained. The phagocytosis index was measured by counting the number of phagocytosed CRBCs per 100 macrophages.

Measurements of cytokine levels by enzyme-linked immunosorbent assay. The levels of TNF-α and IL-10 were determined neutrophils obtained from the peritoneal cavity of BALB/c mice 6 h after the zymosan injection (1 mg/cavity, i.p). The cell number was adjusted to 5 x 10⁶ cells/ml in complete RPMI-1640, plated and exposed to CYM (10 or 90 µg/ml) or RPMI-1640 medium as the control for 30 min at 37°C in 5% CO₂. Subsequently, neutrophils were stimulated with fMLP (except the negative control) at 0.1 µM for 60 min under previously challenging conditions, then the supernatant was separated for dosing and rapidly frozen and stored at -70°C for later analysis. We used commercial kits for the enzyme-linked immunosorbent assay according to the manufacturer's recommendations (R&D Systems, Cayman Chemical). Cytokine levels in the supernatant are expressed as picograms and per milliliter.
Statistical analysis. Data were evaluated using GraphPad Prism version 5.0 (GraphPad Prism Software Inc., San Diego, CA, USA). The data are expressed as the mean ± SEM for each group. The data were statistically analyzed by using one-way variance analysis (ANOVA) followed by Tukey’s test. Differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Recently, some works have demonstrated antinociceptive and anti-inflammatory properties of monoterpenes and these components are the main chemical constituents found in the essential oils (Yamada et al. 2013; Estevão-Silva et al. 2014; Ritter et al. 2013). Our previous studies have shown that monoterpenoid CYM is present in the *Citrus latifolia* Tanaka essential oil (Kummer et al. 2013).

In the cell viability assay, CYM was tested at different concentrations. CYM at concentrations of 3, 10, 30 or 90 µg/ml presented cell viability of 93%, 89%, 85%, and 82%, respectively. Our data indicated that CYM treatments did not present in vitro cytotoxicity, as claimed by similar studies, which showed that CYM did not display any cellular toxicity against some inflammatory cells (Zhong et al. 2012).

In inflammatory conditions, the neutrophils represent the body’s primary line of defense against invading pathogens and is known that a large number of signaling molecules most common in this process are the phosphatidylinositol 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK) (Kim and Haynes 2013; Kolaczkowska and Kubes 2013). To evaluate the direct effect of CYM on in vitro neutrophil chemotaxis, different concentrations were applied. The chemoattractants fMLP ($10^{-6}$ M) and LTB₄ ($10^{-8}$ M) were tested. CYM at concentrations of 3, 10, 30 or 60 µg/ml significantly reduced ($P < 0.05$) neutrophil migration in response to fMLP stimulation (60.2%, 64.1%, 69.1% and 58.1%, respectively), whereas CYM at the same concentrations tested did not significantly reduce ($P < 0.05$) neutrophil migration in response to LTB₄ stimulation (Fig. 1A and 1B) suggesting that the mechanism activated by this chemoattractant cannot be inhibited by this compound in vitro. Since CYM treatment did not affect the viability of neutrophils in the concentrations tested, our data suggest that CYM has a direct effect on inhibition of in vitro neutrophils migration.

Figure 1. Effect of CYM on neutrophils chemotaxis in vitro. Neutrophils were obtained from zymosan-induced peritonitis (1 mg/cavity) and stimulated with fMLP ($10^{-6}$ M) (A) or LTB₄ ($10^{-8}$ M) (B) after 30 min of treatment with CYM at doses of 1, 3, 10, 30 or 60 µg/ml. Values are mean ± S.E.M. (n = 5) and are representative of three independent experiments. *$P < 0.05$ versus medium (RPMI-1640). **$P < 0.05$ versus group of neutrophils stimulated with fMLP or LTB4 (One-way ANOVA, Tukey’s test).

A few studies suggest that p38 MAPK enzyme is very important in neutrophil chemotaxis signal and in cell migration (Kim and Haynes 2013; Kolaczkowska and Kubes 2013). Recent research demonstrated that CYM significantly blocked MAPK
activities in acute lung injury, which may partially explain the inhibitory effect of CYM on leukocyte chemotaxis toward the fMLP (Xie et al. 2012; Zhong et al. 2012).

Many essential oils have showed an expressive anti-inflammatory activity, by reducing the migration of leukocytes to the inflammatory focus (Nogueira de Melo et al. 2011; Yamada et al. 2013). To evaluate the effects of CYM pretreatment on the in vivo migration of inflammatory cells, peritonitis was induced by carrageenan. After 4 h of inflammation induced, there was an increase in the number of peritoneal exudate leukocytes (11.53 ± 1.18×10⁶ cells/cavity) compared with the control group (5.37 ± 0.20×10⁶ cells/cavity). The animals pretreated with CYM (100, 200 or 400 mg/kg) presented a significant reduction of leukocyte infiltration into peritoneal cavity (35.2 %, 39.8 % and 22.9 %, respectively) compared with untreated animals, and this response was mainly attributable to a reduction of the number of polymorphonuclear leukocytes infiltration (Fig. 2). Indeed, in the acute lung injury CYM demonstrated effectiveness in inhibiting cell recruitment to the bronchoalveolar fluid (L. Chen et al. 2013).

Figure 2. Effect of CYM treatments on leukocyte (LT) and polymorphonuclear number (PMN) 4 hours after carrageenan-induced peritonitis in C57BL/6 mice. Mice were orally pretreated with CYM (100, 200 or 400 mg/kg), indomethacin (5 mg/kg) or vehicle. Results are expressed as mean ± S.E.M. of 5 animals per group. a

In response to inflammatory signals, leukocytes migrate from circulating blood and accumulate in the injured tissue and this migration is predominantly in postcapillary venules (Napimoga et al. 2012; Longhi-Balbinot et al. 2012). This is a key step in the inflammatory response, which involves rolling, adhesion and transmigration of leukocytes in endothelial cells (Dal Secco et al. 2006; Wright et al. 2010). These events result mainly from the release inflammatory mediators by resident and infiltrating cells in the inflammatory site, such as cytokines and chemokines, components of the complement system, the nitric oxide (NO), prostanoids and leukotrienes (Verri et al. 2010; Jin 2013). The proinflammatory effects induced by carrageenan in the peritoneal cavities include activation of macrophages, which are source cells of many inflammatory mediators, such as cytokines (TNFα, IL-6, IL-1β), arachidonic acid metabolites and NO (Alencar et al. 2010). Since in vitro and in vivo leukocytes migration was reduced by CYM, we also investigated its effect on leukocyte behavior (rolling and adhesion) in mesenteric postcapillary venules.

The in situ carrageenan injection (500 μg/cavity, i.p.) significantly increased rolling (Fig. 3A) and adhesion (Fig. 3B) of leukocytes to the endothelium when compared to only saline administration group. Our result showed that CYM pretreatment (100 and 200 mg/kg) promoted a significant decrease in the number of rolling leukocytes (41.1 % and 62.6 %, respectively) whereas CYM pretreatment (100, 200 and 400 mg/kg) resulted in a significant decrease in the number of adherent (55.8 %, 72.6 % and 56.5 %, respectively) cells when compared with the positive control group, as showed in Figure 3A and 3B. Indomethacin and celecoxib control drugs significantly decreased rolling leukocytes (58.6 % and 46.4 %, respectively) and adhesion (34.7 % and 23.2 %, respectively) whereas CYM pretreatment (100, 200 and 400 mg/kg) of that observed for the reference drugs. Indeed, studies have reported that CYM inactivated Nuclear Factor-Kappa B (NF-κB) (Xie et al. 2012), a nuclear transcription factor able to regulate the
expression of many immune and inflammatory genes, including adhesion molecules and cytokines (Peng et al. 2007; Zhong et al. 2012). Taken together our data suggest that the antichemotactic activity of CYM by reducing rolling and adherent leukocytes could be NF-κB involved, as previous observed by others authors (Xie et al. 2012).

Figure 3. Effect of CYM on leukocyte rolling (A) and adhesion (B) induced by carrageenan. Mice were orally pretreated with CYM (100, 200 or 400 mg/kg), indomethacin (5 mg/kg), celecoxib (5 mg/kg) or vehicle. After 60 min, saline or carrageenan was injected i.p. Leukocyte rolling and adhesion were evaluated by intravital microscopy in the mesentery 2 h later. Results are expressed as mean ± S.E.M. of 5 animals per group. *P < 0.05 versus saline (negative control). #P < 0.05 versus carrageenan-injected group (positive control). INDO, indomethacin; CEL, celecoxib (One-way ANOVA, Tukey’s test).

Macrophages are located in all body tissues, where they have roles as ingest and process foreign materials, tissue debris and dead cells; recruit additional macrophages during an inflammatory injury and infectious diseases; and secretion of proinflammatory mediators (Shapiro, Lutaty, and Ariel 2011; Murray and Wynn 2011). Lipopolysaccharide (LPS) is found in the cell walls of Gram-negative bacteria and is a potent initiator of inflammatory response. It activates macrophages by binding the CD14/TLR4 receptor complex, signaling pathways activates NF-κB and release of mediators and proinflammatory cytokines (Zhou et al. 2009; Cheng et al. 2010; Shoeb and Ramana 2012).

The effect of CYM on phagocytic activity and the phagocytosis ability of the macrophages stimulated by different treatment is shown in Figure 4. The phagocytic index was increased significantly by different concentrations of CYM (3, 10 and 30 µg/ml) similarly to that observed for LPS (20 µg/ml - positive control). Literature data showed that essential oil from Eucalyptus globulus, that contains CYM, also was able to stimulate phagocytic response of macrophages (Serafino et al. 2008). Beyond of TLR4 receptor the macrophages present several other receptors, such as integrins, Fc receptors, lectins and scavenger receptors that are involved in the recognition and binding of pathogens on the cell surface (Dai et al. 2013). However, it is known that TLR activation and phagocytosis are functionally linked (Y.-J. Chen et al. 2012; Murray and Wynn 2011). Indeed, the expression of TLR is crucial in macrophages phagocytosis and it has been shown that there is a TLR4 increased expression in macrophages following LPS exposure (Y.-J. Chen et al. 2012). Thus, CYM can also have stimulated the phagocytosis via binding with TLR4 receptor, but your mechanism on phagocytosis is not well established, future studies should be conducted.
Effect of *p*-cymene on chemotaxis, phagocytosis and leukocyte behaviors

**Figure 4.** Effect of CYM on phagocytosis of macrophages. Macrophages were obtained from the peritoneal cavity of mice and treated with different doses of CYM (3, 10 or 30 µg/ml). LPS at 20 µg/ml was used as positive control. *P < 0.05 versus NC (negative control). #P < 0.05, compared to the NC (ANOVA, Tukey’s test).

Activated neutrophils are related cytokine synthesis (eg. IL-1, IL-6, TGFβ and TNF-α), mediators present in the inflammatory response (Wright et al. 2010). Knowing the mechanisms of neutrophil-derived cytokines modulation is an important step to understand how these cells may influence the pathophysiological processes and contribute to the development of new drugs. Treatment with CYM significantly inhibited the release of TNF-α at concentrations of 10 and 90 µg/ml (Fig. 5A), but not showed significant inhibitory effects on the release of IL-10 (Figure 5B) in fMLP-stimulated neutrophils. Indeed, fMLP is able to activate all the functional activities of leukocytes (Pan 2000).

**Figure 5.** Effect of CYM on TNF-α (A) and IL-10 (B) levels in fMLP-stimulated neutrophils. Cells were exposed to CYM (10 or 90 µg/ml) or RPMI 1640 (controls) for 30 min and stimulated with 0.1 µM fMLP for 60 min (except the negative control). The concentrations of TNF-α and IL-10 were measured by ELISA. *P < 0.05 versus negative control. #P < 0.05 versus fMLP-stimulated neutrophils (positive control) (One-way ANOVA, Tukey’s test).

The fact of the CYM blocks NF-κB and MAPK could be related to the inhibitory effect of this compound on the production of proinflammatory cytokines (Xie et al. 2012). TNF-α is a proinflammatory cytokine, and IL-10 is a regulatory cytokine. TNF-α up-regulates the expression of adhesion molecules, can cause damage to the vascular endothelial cells and at high local concentrations can stimulate reactive oxygen species production in adherent neutrophils (Wright et al. 2010), while IL-10 promotes an inhibitory effect on few functional responses of neutrophils, including cytokine and chemokine production (Futosi, Fodor, and Mócsai 2013). Our data are in agreement with other studies, that showing that CYM significantly decreased the IL-1β, IL-6, TNF-α levels after stimulation with LPS, but not significantly alter the levels of IL-10, an anti-inflammatory cytokine (Zhong et al. 2012). Thus, our results suggest that CYM could modulate the anti-inflammatory response through synergic activity between IL-10 and other cytokine that conducted the inhibition of proinflammatory cytokines.
CONCLUSION

Our results have demonstrated that CYM showed an effect on leukocyte chemotactic behavior, inhibiting rolling and adherent leukocytes modulated by cytokines, responsible for regulating these effects. Furthermore CYM could be considered a promising drug as an anti-inflammatory agent in the processes that take place in leukocyte infiltration, such as rheumatoid arthritis. However, further studies are necessary to fully elucidate its mechanism of action.

ACKNOWLEDGMENTS

This study was supported by grants from CAPES (Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and Fundação Araucária, Brazil. We thanks Mr. Jailson Araujo Dantas and Mrs. Célia Regina Miranda for technical assistance.

REFERENCES


Kummer et al.


