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*Full Length Research Paper*

## Protective effect of 2,2,2-trichloroethanol on peptidoglycan-induced inflammation in murine macrophages

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The aim of this study was to investigate the effect of 2,2,2-trichloroethanol on peptidoglycan-induced murine macrophages inflammation. The effect of 2,2,2-trichloroethanol on the production of TNF- $\alpha$  and IL-6 by murine peritoneal macrophages with peptidoglycan-stimulation were investigated. Also, RAW264.7 macrophages transfected with a NF- $\kappa$ B luciferase reporter plasmid with the stimulation of peptidoglycan were used to test the effect of 2,2,2-trichloroethanol on NF- $\kappa$ B activity. Flow cytometry and Western blotting were used to check the expression levels of toll-like receptor 2 on treated RAW264.7 macrophages. 2,2,2-Trichloroethanol decreased the unregulated levels of IL-6 and TNF- $\alpha$  produced by the peritoneal macrophages stimulation with peptidoglycan. The NF- $\kappa$ B activities of the RAW264.7 macrophages stimulated by peptidoglycan were decreased. 2,2,2-Trichloroethanol decreased the peptidoglycan-induced murine macrophage inflammation response.

**Key words:** 2,2,2-Trichloroethanol, peptidoglycan, inflammation, macrophage.

### INTRODUCTION

Peptidoglycan is one of the major conserved components of Gram-positive bacteria walls and can be detected in the blood of 80% of the serious bacterial infected patients (Merdink et al., 2008). The mononuclear phagocyte represents an important cell type of the innate immune system and plays a key role against infection in the innate immune system, and murine macrophages are important models for studying response of immune system to infection. Many studies reported that after stimulation with peptidoglycan, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) which has long been considered a prototypical proinflammatory signaling pathway

factor becomes activated and then macrophages release many of the proinflammatory mediators, such as IL-6 and TNF- $\alpha$ . At the same time, levels of toll-like receptor 2 expression on macrophages become upregulated (Chen et al., 2009; Lin et al., 2011).

We have shown that chloral hydrate, a well-known sedative and anesthetic that is used in pediatric procedures, can attenuate the inflammation and improve the survival of lipopolysaccharide/D-galactosamine-induced acute lethal liver injury mice. These protective function of chloral hydrate on acute lethal liver injury mice was related to the inhibitory effects on NF- $\kappa$ B activity and serum levels of

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MCP-1, IL-6 and TNF- $\alpha$  (Pan et al., 2010).

Based on chloral hydrate being rapidly and extensively metabolized in the liver and erythrocytes by alcohol dehydrogenase to its major active metabolite, 2,2,2-trichloroethanol *in vivo* (Ann et al., 2001) is responsible for its physiological and psychological effects (Reinhard et al., 2007). The active metabolite of 2,2,2-trichloroethanol is 2,2,2-trichloroethanol (TCE). So, its necessary and very important to use 2,2,2-trichloroethanol instead of chloral hydrate to study its effect on inflammatory response of macrophages *in vitro*. So, here, we investigate the effect of 2,2,2-trichloroethanol on the production of the inflammatory cytokines and NF- $\kappa$ B activity in murine peritoneal macrophages and RAW264.7 macrophages stimulated with peptidoglycan. Also, the effects of 2,2,2-trichloroethanol treatment on toll-like receptor 2 expression in murine peritoneal macrophages and RAW264.7 macrophages stimulated with peptidoglycan were explored.

## MATERIALS AND METHODS

### Reagents

Peptidoglycan (*Staphylococcus aureus*, strain DSM346) and 2,2,2-trichloroethanol were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Fetal bovine serum and Iscove's Modified Dulbecco's Medium (IMDM) medium were obtained from Gibco (NY, U.S.A). IL-6 and TNF- $\alpha$  enzyme linked immunosorbent assay (ELISA) kits were from R&D Systems, Inc. (Minneapolis, USA). The fluorescein isothiocyanate (FITC)-anti-toll-like receptor 2 antibody (clone TL2.5), FITC-mouse IgG1, anti-mouse toll-like receptor 2 antibody (clone TL2.5), HRP-anti-mouse secondary antibody were obtained from Ebioscience (San Diego, CA, USA) and Biolegend (San Diego, CA, USA). The NF- $\kappa$ B-luciferase,  $\beta$ -galactosidase reporter vectors and the Dual-Luciferase reporter assay system were purchased from Promega Corporation (Madison, WI, USA). The immobilon membrane and the Electrochemiluminescence (ECL) were from Millipore (Watford, UK) and Amersham Life Science Ltd (Little Chalfont, UK), respectively.

### Effects of 2,2,2-trichloroethanol on IL-6 and TNF- $\alpha$ production by murine peritoneal macrophages with the stimulation of peptidoglycan (PGN)

After peritoneal macrophages separated from mice (Yin et al., 2006), the cells were seeded at the levels of  $10^5$  cells/well in IMDM media (5% fetal bovine serum) at 37°C in 5% CO<sub>2</sub>. At the indicated time, cells and culture supernatants were collected after stimulated with PGN (1.5  $\mu$ g/ml) or PGN (1.5  $\mu$ g/ml) plus 2,2,2-trichloroethanol (0.1 and 0.5 mg/ml) and measured with ELISA kits.

### Effects of 2,2,2-trichloroethanol on the activity of NF- $\kappa$ B of PGN-stimulated RAW264.7 macrophages

RAW264.7 macrophages were cultured in IMDM media (5% fetal bovine serum) and maintained at 37°C in 5% CO<sub>2</sub>. These cells were digested with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (2.5 g/L trypsin, 1 g/L EDTA) at 37°C for five minutes, and  $8.2 \times 10^6$  RAW 264.7 macrophages were transiently transfected with 8  $\mu$ g NF- $\kappa$ B-luciferase and 8  $\mu$ g  $\beta$ -galactosidase reporter

vectors which was used for normalization of the efficiency of the transfection in a volume of 640  $\mu$ l by electroporation at 250 V and 960  $\mu$ F of capacitance pulse<sup>21</sup>. Then, RAW264.7 macrophages were subsequently washed two times in IMDM media and divided into 40 wells (150  $\mu$ l/well) and cultured for 24 h in IMDM media (5% fetal bovine serum) before being stimulated with PGN (1.5  $\mu$ g/ml) for 12 or 24 h with or without 2,2,2-trichloroethanol (0.1 or 0.5 mg/ml). For the luciferase activity assays, the transfected RAW264.7 macrophages were stimulated for 6 or 12 h and subsequently harvested, and luciferase activity in extracts were analyzed with the dual-luciferase reporter assay system following manufacturer's instructions.

### Effects of 2,2,2-trichloroethanol on PGN-induced toll-like receptor 2 expression in RAW264.7 macrophages

Flow cytometry was performed to investigate levels of toll-like receptor 2 expression on RAW264.7 macrophages. RAW 264.7 macrophages were cultured in IMDM media (5% fetal bovine serum) for 12 h with PGN (1.5  $\mu$ g/ml) with or without 2,2,2-trichloroethanol (0.1 mg/ml). Then, RAW264.7 macrophages were incubated with FITC-anti-mouse-toll-like receptor 2 (1.0  $\mu$ g) for thirty minutes at room temperature after harvested, and FITC-mouse IgG1 was used as isotype control, the cells were washed two times with cell staining buffer. Finally, a six-parameter flow cytometer (FACScan; BD Biosciences, San Jose, CA) was used for data acquisition and analysis using CellQuest software (BD Bioscienc, San Jose, CA). Also, toll-like receptor 2 expression in the extracts of RAW264.7 macrophages stimulated with PGN (1.5  $\mu$ g/ml) for 12 h with or without 2,2,2-trichloroethanol (0.1 mg/ml) were semi-quantitatively analyzed by Western blotting using the anti-mouse-toll-like receptor 2 antibody by band scan software 5.0 (Glyko Inc). First, the extracts of RAW264.7 macrophages were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the immunoblotting was performed with a standard protocol onto the immobilon membrane. HRP-anti-mouse IgG as secondary antibody, followed by ECL chemiluminescence which was used to detect toll-like receptor 2 expression.

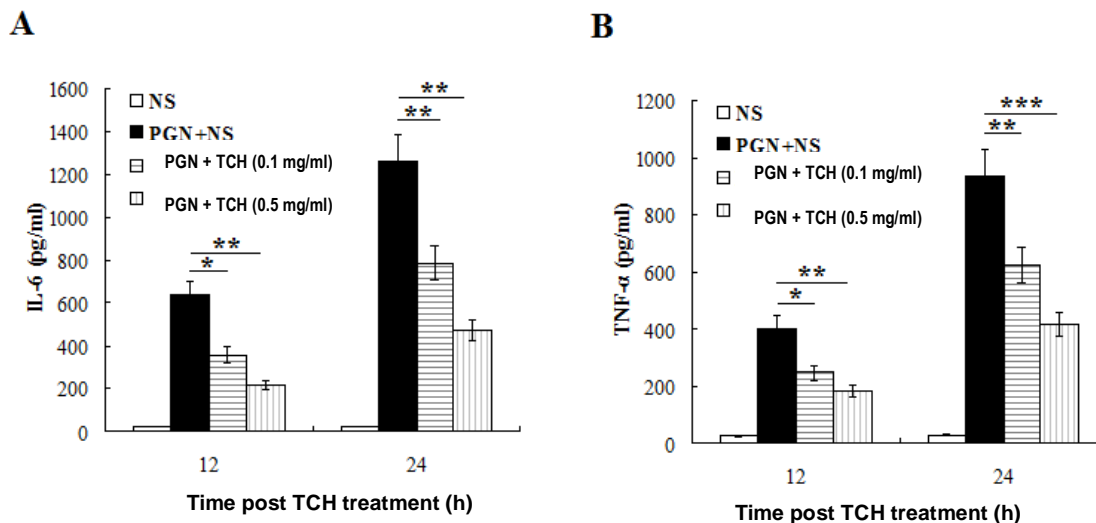
### Statistical analysis

Data were expressed as the means  $\pm$  standard deviation (SD) and statistical analysis with statistical package for social sciences (SPSS) version 15.0 statistical software. The statistical significance between two groups was determined by the unpaired Student's t-test. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### 2,2,2-Trichloroethanol-treatment decreased the production of IL-6 and TNF- $\alpha$ by peritoneal macrophages with the stimulation of peptidoglycan

The production of IL-6 (Figure 1A) and TNF- $\alpha$  (Figure 1B) increased significantly after stimulation with peptidoglycan (1.5  $\mu$ g/ml) for 12 and 24 h and decreased significantly post 2,2,2-trichloroethanol treatment (0.1 and 0.5 mg/ml) (all  $P < 0.01$ ). Also, the higher concentration of 2,2,2-trichloroethanol decreased the production of IL-6 significantly compared to the lower concentration (0.1 mg/ml) at 12 h and 24 h time points (all  $P < 0.05$ ) (Figure 1A), but not for TNF- $\alpha$ .



**Figure 1.** 2,2,2-trichloroethanol (TCH) decreased the production of IL-6 and TNF- $\alpha$  by peritoneal macrophages with stimulation of peptidoglycan (PGN). Effects of 2,2,2-trichloroethanol (0.1 and 0.5 mg/ml) on the levels of IL-6 (A) and TNF- $\alpha$  (B) produced by peritoneal macrophages ( $10^6$  cell/well) with PGN stimulation for 12 and 24 h were shown.

Data were expressed as the means  $\pm$  standard deviation (SD) of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

### 2,2,2-trichloroethanol-treatment decreased the NF- $\kappa$ B activity of peptidoglycan-stimulated RAW264.7 macrophages

Luciferase activities in the transfected RAW264.7 macrophages were measured after peptidoglycan stimulation for 6 and 12 h (Figure 2). The results showed about 3- and 5-fold increase of the luciferase activity post peptidoglycan stimulated for 6 and 12 h, respectively. But this increase of luciferase activity was significantly decreased by 2,2,2-trichloroethanol treatment (0.1 and 0.5 mg/ml) (all  $P < 0.01$ ). A higher concentration of 2,2,2-trichloroethanol (0.5 mg/ml) led to a significantly larger decrease in the activity of luciferase after 12 h when compared to the lower concentration of 2,2,2-trichloroethanol (0.1 mg/ml) ( $P < 0.01$ ).

### 2,2,2-Trichloroethanol-treatment decreased the increase of toll-like receptor 2 expression in peptidoglycan-treated RAW264.7 macrophages

Based on both concentrations (0.1 and 0.5 mg/ml) of 2,2,2-trichloroethanol treatment, it significantly decreased the inflammatory response of peritoneal macrophages and RAW264.7 macrophages after PGN-stimulation, so we tested the effect of 2,2,2-trichloroethanol treatment on the expression of toll-like receptor 2, the receptor for PGN, using the lowest effective concentration of 2,2,2-trichloroethanol (0.1 mg/ml). RAW264.7 macrophages were cultured in the IMDM media (5% fetal bovine serum) for 12 h with or without peptidoglycan (1.5  $\mu$ g/ml) in the

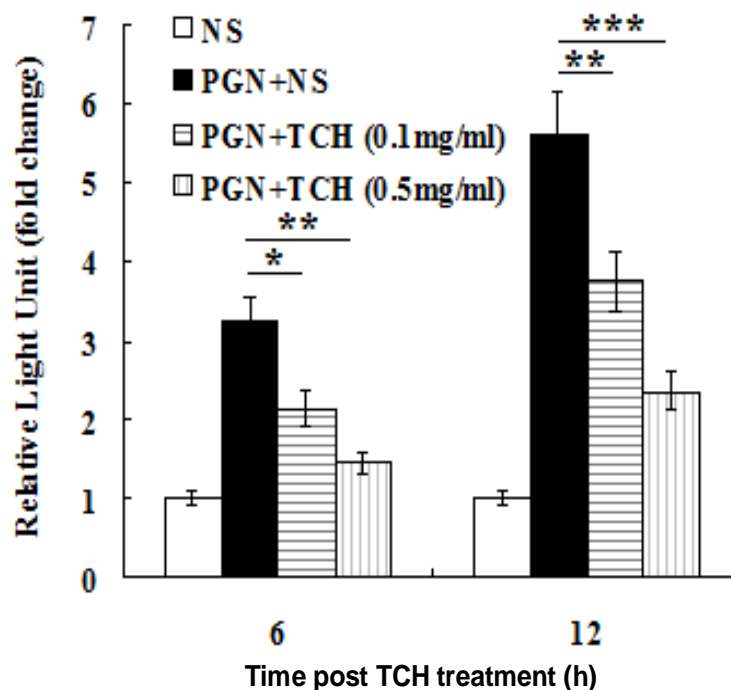
presence or absence of 2,2,2-trichloroethanol (0.1 mg/ml) (Figure 3A) and harvested, and the levels of toll-like receptor 2 were measured. In RAW264.7 macrophages stimulated with PGN (1.5  $\mu$ g/ml) for 12 h, toll-like receptor 2 expression was significantly increased (Figure 3B), and treatment of 2,2,2-trichloroethanol (0.1 mg/ml) significantly decreased the upregulation of toll-like receptor 2 expression stimulated with peptidoglycan ( $P < 0.05$ ). Also, the results of semi quantitative analysis of toll-like receptor 2 expression in the extracts of RAW264.7 macrophages by Western blotting showed a similar pattern (Figure 3C). It showed that if the related amounts of toll-like receptor 2 signal to the signal of a house-keeping proteins  $\beta$ -actin were similar, the increase and the reduction of the toll-like receptor 2 signal were not caused by cell death or variations in the number of cells in the samples.

## DISCUSSION

Over recent years, the mechanisms of bacteria-induced acute inflammation, anti-cytokines and anti-inflammatory therapies as therapeutic agents for the treatment of acute inflammation have been re-evaluated. Large quantities of reported cases of sepsis-related anti-cytokine therapeutic trials were unpromising or disappointing (Hall and Muszynski, 2009; Zeni et al., 1997; Ratsimandresy et al., 2009). So, recently, the developments of new strategies for modulating severe sepsis and acute inflammation have been investigated intensively.

As we all know, the new drug discovery, development





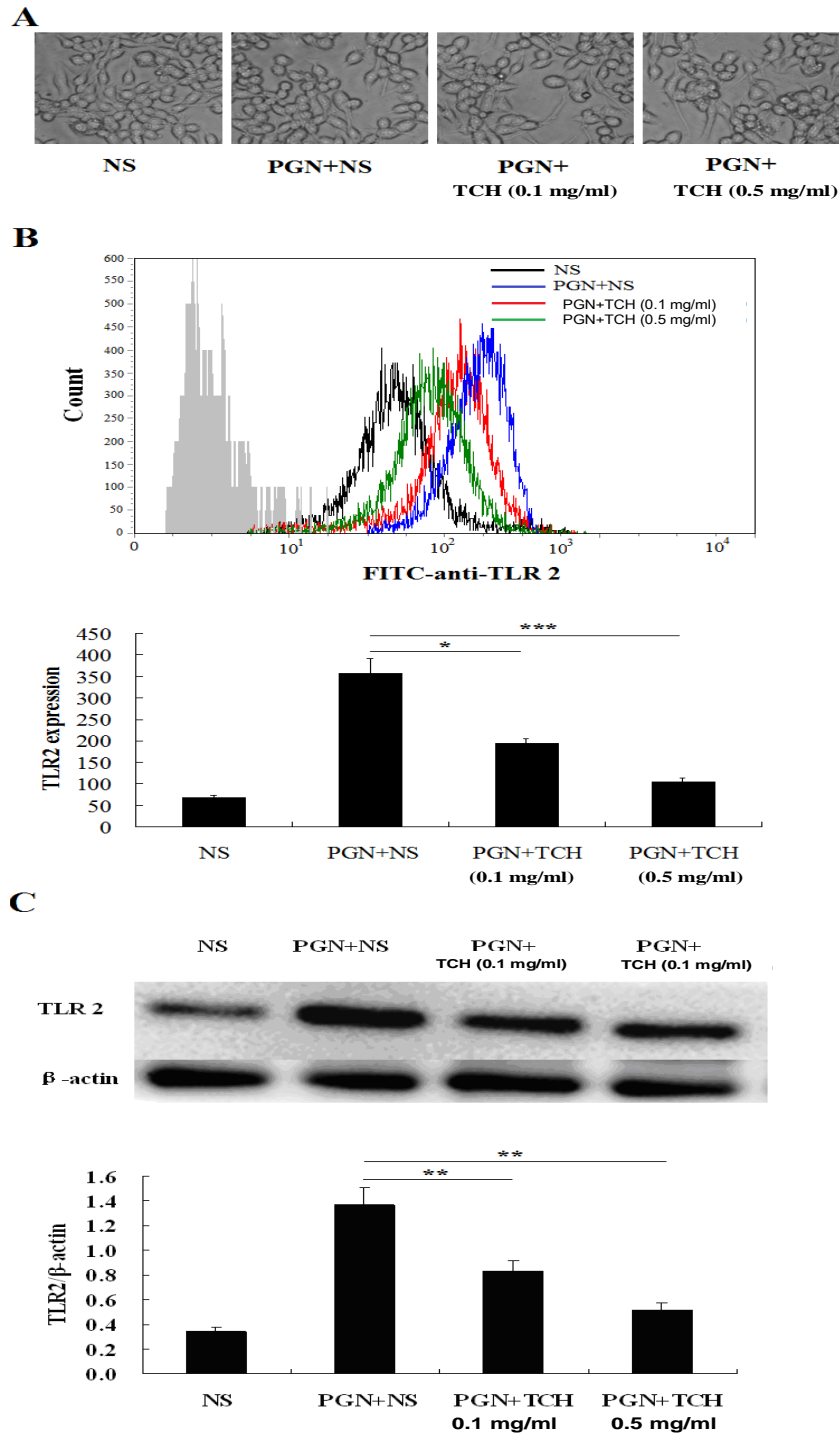
**Figure 2.** 2,2,2-trichloroethanol (TCH) treatment decreased the upregulation of NF- $\kappa$ B activity of RAW264.7 macrophages with peptidoglycan (PGN) stimulation. 24 h after the RAW264.7 macrophages were co-transfected with NF- $\kappa$ B-luciferase- and  $\beta$ -galactosidase- reporter vectors by electroporation, PGN (1.5  $\mu$ g/ml) or PGN (1.5  $\mu$ g/ml) plus 2,2,2-trichloroethanol (0.1 or 0.5 mg/ml) were added into the medium and incubated for 6 and 12 h. The luciferase activity of the cell extracts was expressed as the folds of luciferase-induction over saline-treated controls of three independent experiments. RLU stands for "Relative Light Unit", \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ .

and approval process of therapeutic programs requires investing significant amounts of time and effort. However, discovering new uses for the old drugs may accelerate the development and application of new therapies, take for example, the bisphosphonate zoledronic acid can decrease breast cancer metastasis beside treatment of osteoporosis and similar diseases (Coleman and Gnant, 2009) and thalidomide can treat multiple myeloma in addition to alleviate nausea and morning sickness (Cavo et al., 2009). Importantly, protective effect of some local and general anesthetics and sedatives have reported against infection, and inflammations (Plachinta et al., 2003; Gallos et al., 2004; Fuentes et al., 2006; de Klaver et al., 2002; Liu et al., 2009) are likely to yield new insights into anti-inflammatory therapies.

Chloral hydrate has been long used as a safe sedative and hypnotic drug in patients and animal models. Also, different from isoflurane, which is an inhalation agents and must be inhaled continuously, chloral hydrate can be used without control in many countries, but unlike other sedatives, for example, ketamine is under strictly regulated conditions in China and other countries, also, and is

not approved by US Food and Drug Administration (USFDA) for patients lower than 16 years old (Mellon et al., 2007).

In this study, we used 2,2,2-trichloroethanol, which is a major active metabolite *in vivo* after chloral hydrate rapidly and extensively metabolized in the liver and erythrocytes by alcohol dehydrogenase, to study its function on inflammatory response with macrophages *in vitro*. Here, our studies showed that 2,2,2-trichloroethanol treatment can decrease the rise of the inflammatory cytokine levels produced after peptidoglycan stimulation in murine peritoneal macrophages (Figure 1), indicating that the effect of 2,2,2-trichloroethanol on inflammation could be attributed to its inhibition of function of macrophages. The TNF- $\alpha$  and IL-6 levels sharply increased at 12 and 24 h after the peptidoglycan-challenge. Similar studies using peptidoglycan (10 or 25  $\mu$ g/ml) were performed by others (Shirasawa et al., 2004; Wang et al., 2004), but they only tested 24 h after one challenge. Also, the treatments with 2,2,2-trichloroethanol significantly decreased the rise of inflammatory cytokines production (Figure 1).



**Figure 3.** 2,2,2-trichloroethanol (TCH) treatment reduced the expression of toll-like receptor 2 (TLR 2) in peptidoglycan (PGN)-stimulated RAW264.7 macrophages. RAW 264.7 macrophages were cultured in IMDM media (5% FBS) for 12 h with PGN (1 µg/ml) with or without 2,2,2-trichloroethanol (0.1 mg/ml) (A). Flow cytometry was performed to investigate the expression levels of TLR 2 on the treated RAW264.7 macrophages represented as dot plots and represented as histograms. The mean channel fluorescence intensity (MFI) was calculated (B). The semi-quantitative analysis of the extracts from RAW264.7 macrophages by band scan and the ratio of TLR 2 to β-actin was shown, showed a western blot of β-actin and TLR 2 of Normal, PGN+NS and PGN+TCH group and showed a quantitation of them (C). The data were expressed as the means ± SD of three independent experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ .

Next, we examined whether peptidoglycan-induced NF- $\kappa$ B-dependent gene transcription is regulated by 2,2,2-trichloroethanol in RAW264.7 macrophages. The results of RAW264.7 macrophages transfected with a NF- $\kappa$ B luciferase reporter vector showed that 2,2,2-trichloroethanol treatment can significantly reduce NF- $\kappa$ B activity (Figure 2). Because NF- $\kappa$ B is a key mediator for inducible transcription of proinflammatory cytokine expression, these results suggested that 2,2,2-trichloroethanol could affect cytokine expression maybe by influencing the activity of NF- $\kappa$ B. After 6 and 12 h with the stimulation of peptidoglycan, the respective increase of NF- $\kappa$ B-induced luciferase activity in RAW264.7 macrophages was approximately 3- and 5-fold compared to control levels. A similar study using peptidoglycan (1.5  $\mu$ g/ml) was performed by others (Ito et al., 2005), and they observed the same trend.

Following this, we investigated whether peptidoglycan-induced upregulation of toll-like receptor 2 expression is regulated by 2,2,2-trichloroethanol. Flow cytometry was used to analyze the effect of 2,2,2-trichloroethanol on the expression of toll-like receptor 2 in RAW264.7 macrophages stimulated with peptidoglycan. The results showed that the expression of toll-like receptor 2 in RAW264.7 macrophages is significantly upregulated after PGN stimulation. Similar results have been found by others (Chen, 2009). After 2,2,2-trichloroethanol treatment, the highly upregulation of the toll-like receptor 2 expression in response to peptidoglycan exposure was remarkably decreased (Figure 3), which is consistent with the effect of 2,2,2-trichloroethanol on NF- $\kappa$ B activity and inflammatory cytokine production by RAW264.7 macrophages stimulated with peptidoglycan.

Taken together, this is the first time to report that 2,2,2-trichloroethanol can decrease the upregulation of peptidoglycan-induced inflammatory response by macrophages in a concentration and time-dependent manner. Also, this decrease was associated with a decreased upregulation of the peptidoglycan-induced-toll-like receptor 2 expression in macrophages. By knowing the effect and mechanisms of 2,2,2-trichloroethanol treatment in inflammatory response of macrophages may provide new opportunities to design new therapeutic strategies to reduce inflammation caused by Gram-positive bacteria.

### Conflict of interest

No competing interests were disclosed.

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### REFERENCES

- Ann Smith, Susan Budavari, Maryadele J. O'neil, Patricia E. Heckelman (2001). The Merck index: an encyclopedia of chemicals, drugs, and biologicals (13th Edition). John Wiley & Sons. ISBN-13: 978-0-911910-13-1, ISBN: 0-911910-13-1.
- Cavo M, Di Raimondo F, Zamagni E, Patriarca F, Tacchetti P, Casulli AF, Volpe S, Perrone G, Ledda A, Ceccolini M, Califano C, Bigazzi C, Offidani M, Stefani P, Ballerini F, Fiacchini M, de Vivo A, Brioli A, Tosi P, Baccarani M (2009). Short-Term Thalidomide Incorporated Into Double Autologous Stem-Cell Transplantation Improves Outcomes in Comparison With Double Autotransplantation for Multiple Myeloma. *J Clin. Oncol.* 27(30):5001-7.
- Chen BC, Kang JC, Lu YT, Hsu MJ, Liao CC, Chiu WT, Yeh FL, Lin CH (2009). Rac1 regulates peptidoglycan-induced nuclear factor-kappaB activation and cyclooxygenase-2 expression in RAW 264.7 macrophages by activating the phosphatidylinositol 3-kinase/Akt pathway. *Mol. Immunol.* 46(6):1179-1188.
- Coleman R, Gnant M (2009). New results from the use of bisphosphonates in cancer patients. *Curr. Opin. Support Palliat. Care* 3(3):213-8.
- de Klaver MJ, Manning L, Palmer LA, Rich GF (2002). Isoflurane pretreatment inhibits cytokine-induced cell death in cultured rat smooth muscle cells and human endothelial cells. *Anesthesiology* 97(1):24-32.
- Fuentes JM, Talamini MA, Fulton WB, Hanly EJ, Aurora AR, De Maio A (2006). General anesthesia delays the inflammatory response and increases survival for mice with endotoxic shock. *Clin. Vaccine. Immunol.* 13(2):281-288.
- Gallos G, Jones DR, Nasr SH, Emala CW, Lee HT (2004). Local anesthetics reduce mortality and protect against renal and hepatic dysfunction in murine septic peritonitis. *Anesthesiology* 101(4):902-911.
- Hall MW, Muszynski JA (2009). Immune modulation in sepsis. *J. Pediatr. Infect. Dis.* 4(2):127-136.
- Ito Y, Kawamura I, Kohda C, Tsuchiya K, Nomura T, Mitsuyama M (2005). Seeligeriolysin O, a protein toxin of *Listeria seeligeri*, stimulates macrophage cytokine production via Toll-like receptors in a profile different from that induced by other bacterial ligands. *Int. Immunol.* 17:1597-1606.
- Lin HY, Tang CH, Chen JH, Chuang JY, Huang SM, Tan TW, Lai CH, Lu DY (2011). Peptidoglycan induces interleukin-6 expression through the TLR2 receptor, JNK, c-Jun, and AP-1 pathways in microglia. *J. Cell. Physiol.* 226(6):1573-1582.
- Liu WL, Bian SZ, Gu ZL, Jiang XG, Qin ZH (2009). Advances in research of ketamine addiction mechanism. 25(3):200-203, 207.
- Mellon RD, Simone AF, Rappaport BA (2007). Use of anesthetic agents in neonates and young children. *Anesth. Anal.* 104(3):509-20.
- Merdink JL, Robison LM, Stevens DK, Hu M, Parker JC, Bull RJ (2008). Kinetics of chloral hydrate and its metabolites in male human volunteers. *Toxicology* 245(1-2):130-140.
- Pan QJ, Liu Y, Zheng J, Lu X, Wu S, Zhu P, Fu N (2010). Protective effect of chloral hydrate against lipopolysaccharide/D-galactosamine-induced acute lethal liver injury and zymosan-induced peritonitis in mice. *Int. Immunopharmacol.* 10(8):967-977.
- Plachinta RV, Hayes JK, Cerilli LA, Rich GF (2003). Isoflurane pretreatment inhibits lipopolysaccharide-induced inflammation in rats. *Anesthesiology* 98(1):89-95.
- Ratsimandresy RA, Rappaport J, Zagury JF (2009). Anti-cytokine therapeutics: history and update. *Curr. Pharm. Des.* 15(17):1998-2025.
- Jira R, Kopp E, McKusick BC, Röderer G, Bosch A, Fleischmann G (2009). "Chloroacetaldehydes". In: Ullmann's Encyclopedia of

- Industrial Chemistry. Wiley-VCH, Weinheim.
- Shirasawa S, Sugiyama S, Baba I, Inokuchi J, Sekine S, Ogino K, Kawamura Y, Dohi T, Fujimoto M, Sasazuki T (2004). Dermatitis due to epiregulin deficiency and a critical role of epiregulin in immune-related responses of keratinocyte and macrophage. *Proc. Natl. Acad. Sci. USA*, 101(38):13921-6.
- Wang N, Satoskar A, Faubion W, Howie D, Okamoto S, Feske S, Gullo C, Clarke K, Sosa MR, Sharpe AH, Terhorst C (2004). The cell surface receptor SLAM controls T cell and macrophage functions. *J. Exp. Med.* 199(9):1255-1264.
- Yin MZ, Li SQ, Yuan L, Dai HL (2006). Separation, Cultivation and Identification of Mouse Peritoneal Macrophages. *Med. J. Wuhan Univ.* 2:203-205.
- Zeni F, Freeman B, Natanson C (1997). Anti-inflammatory therapies to treat sepsis and septic shock: a reassessment. *Crit. Care. Med.* 25(7):1095-1100.

Full Length Research Paper

# Regulation of cartilage and inflammatory biomarkers in rheumatoid arthritis patients treated with green tea therapy

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Most of the conventional rheumatoid arthritis (RA) drugs used have severe adverse reactions. Therefore, natural plant products are continuously being sought for the management of RA. In the present study, *in vitro* antioxidant, joint protective and anti-inflammatory activity of aqueous green tea extract (AGTE) was evaluated in 50 patients with early RA, 30 patients with established RA and 50 healthy control subjects. All patients received 4 to 6 cups/day; 60 to 125 mg catechins of green tea for 24 weeks. The results obtained indicated that green tea possesses potent joint protective and anti-inflammatory action against RA by lowering disease activity parameters and improving COMP, HA, IL-6, and TNF- $\alpha$ . The biochemical observations were supplemented with radiographic analysis of RA patients. *In vitro* 1,1-diphenyl-2-picryldrazil (DPPH) radical scavenging and NBT assay tests of the green tea exhibited a moderate antioxidant activity (90.3 and 87.9%) in both tests used. The possible mechanism(s) of green tea extract (AGTE) activity may be due to free radical scavenging potential caused by the presence of antioxidant component(s) in AGTE. Consequently, green tea can be used as a therapeutic regime in treatment of some RA disorders.

**Key words:** Cartilage oligomeric matrix protein (COMP), interleukin-6 (IL-6), tumor necrosis factor (TNF- $\alpha$ ), rheumatoid arthritis, green tea, disease activity score (DAS)-28, pain numerical rating score (PNRS), European League Against Rheumatism Response (EULAR) criteria.

## INTRODUCTION

Rheumatoid arthritis (RA) is most prevalent in individuals aged 40 years or older with the risk of being up to 5 times higher in women (Brosseau et al., 2004). RA is the most common form of chronic joint inflammation which leads to varying degrees of functional impairment and disability. It

is characterized by chronic low-grade inflammation of multiple joints with periodic flare-ups of great intensity that lead to severe and irreversible cartilage, bone and joint destruction (Smith and Haynes, 2002; Lipsky, 2005). The severity of RA ranges from self-limited swelling in a

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limited number of joints to chronic progressive destruction of multiple joints with reduced muscle strength and impaired physical function (Ek Dahl and Broman, 1992). The cytokine network in RA is a complex and dynamic system in which cellular and humoral cytokines, chemokines, and growth factors regulate the initiation and perpetuation of inflammation (Choy and Panayi, 2001).

Previously, it has been shown that serum cytokine levels when screened are useful biomarkers for the diagnosis and prognosis of early inflammatory arthritis (Firestein, 2004; Hitchon et al., 2004), a dramatic increase in the proinflammatory cytokines such as tumour necrosis factor alpha (TNF- $\alpha$ ), interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ), and C-reactive protein (CRP) was reported in patients with RA (McInnes, 2003; Sattar et al., 2003).

Early confirmation of RA is important because aggressive therapy during the earliest stages of disease can lead to decreased disease activity and reduced joint damage (Kroot et al., 2002). Molecular-marker technology reported that cartilage oligomeric matrix protein (COMP) and hyaluronic acid may aid as promising candidates in evaluating the disease activity in early RA using immunoassay techniques (ELISAs) (Saxne and Månsson, 2000; Den Broeder et al., 2002; Majeed et al., 2004). It was reported that serum levels of COMP significantly correlates with low values of traditional prognostic markers including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (Rf) and disease activity score (DAS) (Skoumal et al., 2003).

Non-steroidal anti-inflammatory drugs have formed the mainstay of treatment of RA, but their prolonged use is associated with adverse reactions and discomfort (Kremers et al., 2004). Therefore, natural plant products that are beneficial against arthritis are continuously being sought for the management of RA (Berman, 2004). However, the mechanisms of action of such products are largely unexplored.

Multiple studies were conducted on the effect of green tea (GTPs) in arthritis using *in vitro* and *in vivo* animal models (Ahmed et al., 2002; Singh et al., 2002). These studies suggest that GTPs given in drinking water of mice prevented collagen-induced arthritis in the mice, and that this effect of GTPs was associated with the marked reduction of collagen-induced inflammatory mediators such as cyclooxygenase-2 and TNF- $\alpha$  in arthritic joints of GTP-fed mice (Haqqi et al., 1999). The polyphenolic compounds isolated from green tea extracts are rich in antioxidants that possess anti-inflammatory properties (McKenna et al., 2000; Arab and Il'yasova, 2003).

Recently, it was shown that epigallocatechin-3-gallate (EGCG), a major constituent of GTE, inhibits IL-1 $\beta$ -stimulation CC/CXC chemokine production, MMP-2 activation, IL-6 synthesis and trans-signalling in human RA synovial fibroblasts and rat adjuvant-induced arthritis (AIA) model (Ahmed et al., 2008).

Therefore, this study longitudinally analyzed levels of serum cartilage biomarkers and some proinflammatory cytokines during 24 weeks green tea therapy, taking the composition of aqueous green tea extract (AGTE) and its *in vitro* free radical scavenging activity into account to evaluate the feasibility of biomarkers for monitoring structural joint damage.

## PATIENTS AND METHODS

### Controls

In this study, a total of 80 patients were enrolled from the division of rheumatology and clinical immunology at Mansoura University, and all patients fulfilled the diagnostic criteria for RA according to American College of Rheumatology criteria (Arnett et al., 1988). Fifty patients with arthritis symptoms of  $1.6 \pm 2.21$  duration were classified as having early RA, and 30 patients with disease duration of  $4.17 \pm 2.21$  years were classified as showing established RA. Demographic and baseline characteristics of patients are shown in Table 1. All patients had clinically active disease, despite administration of conventional first-level disease-modifying anti-rheumatic drugs, and the mean 28-joint disease activity score (DAS28)-ESR at baseline was 5.4 for early RA and 6.6 for established RA. Only patients who were not receiving non-steroidal anti-inflammatory drugs and oral glucocorticoids (prednisolone-equivalents  $\leq 10$  mg/day) were included in the study. Furthermore, no intra-articular glucocorticoid injections were given during the study period or 3 months prior to enrolment. Patients are encouraged to follow a healthy, balanced diet that fosters a healthy weight. It is important to avoid elimination diets (Nutrition and Your Health, 1995). Dietary information was obtained from food diaries or by extensive dietary interviews. Patients were evaluated for therapeutic response at baseline and 12, and 24 weeks after starting green tea supplement (4 to 6 cups/day; 60 to 125 mg catechins). In addition to comparison with the manufacturer's reference samples (below) and upper limit of normal, 50 healthy age matched blood donor volunteers acted as controls. The control subjects completed standard blood donor health questionnaires, but were not assessed clinically and did not have acute phase or immunogenetic studies. At each evaluation, blood samples were obtained and sera were separated and stored at  $-80^{\circ}\text{C}$  until needed for biomarker analysis. All laboratory investigations were performed in serum samples regarding informed consent obtained from RA patients. The study protocol was approved by ethical committee of Rheumatology and Clinical Immunology department, Mansoura University, Mansoura, Egypt.

### Analysis of phenolic compounds in AGTE

Green tea, a commercial green tea drink produced by Kao Ltd. (Tokyo, Japan), was purchased from a convenience store. Total phenolic compounds of 100 mg of GTE water extract were analyzed at Bio-technology Lab., Plant Pathology Institute, Agricultural Research Center, Giza, Egypt. Analysis was performed with a liquid chromatography "HP1050" equipped with a  $4.6 \times 150$  mm ODS C<sub>18</sub> column with UV detector and the injection volume was 5  $\mu\text{l}$ . The mobile phase yielded results of 40% methanol:60% distilled water. The wave length in the UV detector was 230 nm, total run time for the separation was 15 min at a flow rate of 0.60 ml/min according to the proposed method of Waskmundzka et al. (2007). Green tea has minimum contents of 540 mg of catechins and 80 mg of caffeine/350 ml.

### Studies of the in vitro antioxidant activity of AGTE

The free radicals scavenging activity of green tea was determined using the 1,1-diphenyl-2-picrylhydrazil (DPPH) method (Ohinishi et al., 1994) and nitro blue tetrazolum (NBT) reduction method (Ravishankara et al., 2002) as the following.

#### DPPH free radical scavenging assay

One milliliter of DPPH solution (0.1 mM in ethanol) was mixed with 1 ml of aqueous green tea extract (from 0 to 248 µg/ml) and reacted for 30 min. After that, absorbance of this mixture was measured at 517 nm against 95% ethanol solution as the blank. Triplicate measurements were performed and the antioxidant activity was expressed as the percentage of scavenged DPPH:

$$\text{Scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A<sub>0</sub> and A<sub>1</sub> are the absorbance for the blank and green tea extract, respectively.

#### NBT superoxide scavenging assay

The reaction mixture contained: EDTA (6 M), riboflavin (2 µM), NBT (50 µM), aqueous green tea extract (from 10 to 248 µg/ml) and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent lamp for 15 min, and the optical density was measured at 530 nm before and after illumination.

### Clinical evaluation of therapeutic response

The following clinical and laboratory parameters were longitudinally examined in each patient: CRP, Rf IgG antibodies, modified health assessment questionnaire (HAQ) score, pain numerical rating score (PNRS, 0 to 10) and DAS28-ESR. Scores for DAS28-ESR are reported using the erythrocyte sedimentation rate (Inoue et al., 2007) and were defined as follows: ≥4.1, high activity; ≥2.7 to <4.1, moderate activity; ≥2.3 to <2.7, low activity; and <2.3, remission. DAS28-ESR scores were assessed using EULAR response criteria. By comparing a patient's DAS28 score over multiple time points improvement or response of RA patients to green tea therapy was substantiated. The EULAR response criteria are defined as good (>1.2), moderate (0.6 to 1.2), and no response (>0.6). In terms of radiographic analysis, radiographs of both hands and feet at baseline and 24 weeks were available for 43 patients in the early RA group and 26 patients in the established RA group. Two expert readers independently scored articular damage and progression in a blinded fashion according to the modified VDH-Sharp scoring method. Progression of TSS from baseline to week 24 (DTSS) was determined, and the proportion of patients with DTSS#0 was calculated.

### Cartilage biomarkers analyses

Serum samples obtained at baseline and after 12 and 24 weeks of green tea therapy were analysed for COMP by a sandwich ELISA utilizing two monoclonal antibodies directed against separate antigenic determinants on the human COMP molecule (AnaMar Medical, Lund, Sweden). The detection limit is <0.1 µg/L and the intra-assay and inter-assay coefficient of variation is <5%. Serum HA levels were determined using an HA Assay Kit (IBA method; Seikagaku, Tokyo, Japan) utilizing HA-binding protein.

### Statistical analyses

Statistical analysis was carried out with Statistical Package for Social Science (SPSS) program version 10 for Windows (spss Inc, Chicago, IL, USA). Comparisons were done by Wilcoxon's matched-pairs test, the Mann-Whitney U-test, or the chi-square test where appropriate and correlations were calculated using Spearman's rank correlation coefficient. A P value of <0.05 was considered significant.

## RESULTS

Eighty patients with rheumatoid arthritis (RA) and 50 healthy control subjects were included in this study. Patients were classified according to the diagnostic criteria of rheumatology, DAS28-ESR, radiographic analysis, and RA biomarkers into two groups. Fifty patients with arthritis symptoms of 1.6 ± 2.21 duration and DAS28-ESR of 5.4 were classified as having early RA, and 30 patients with disease duration, 4.17 ± 2.21 years and DAS28-ESR of 6.6 were classified as showing established RA. The biochemical profile of rheumatoid and immunological markers along with HA and COMP cartilage biomarkers showed a significant (P = 0.01) correlation with the degree of RA as shown in Table 1.

The varieties of phenolic compounds in green tea extracts (AGTEs) were estimated using liquid chromatography. Catechins were the highest amount (540 mg/g) followed by caffeine (80 mg/g), chlorogenic acid (22.47 mg/g), and pyrogallol (13.41 mg/g). This was in addition to small amounts of other phenolic compounds such as vanillic, synergic, salicylic, benzoic and ferulic acids as shown in Table 2.

Also, the results of DPPH/NBT scavenging colorimetric assays confirm a high scavenging activity of AGTE, in which anti-radical activity is 90.3 and 87.9% at 64 µg/ml of AGTE against DPPH and NBT radicals, respectively. The potential decrease of the concentration of DPPH and NBT free radicals are due to AGTE scavenging activity as shown in Table 2.

As expected, laboratory indices for RA disease activity, such as CRP, ESR, Rf, DAS28-ESR, and body mass index (BMI) had decreased significantly by week 24 green tea supplement in both groups (Table 3). The decrease in DAS28-ESR was prominent in patients with early RA, with mean score at week 24 below the level of clinical remission as compared to baseline data. Also, mean PNRS score was significantly decreased at week 24 in the early and established RA groups as compared to baseline group. Similarly, a significant (P=0.0001) decrease in BMI as demographic parameter was reported in both early and established RA groups during 24 week green tea supplement as compared to the data of baseline group (Table 3).

When DAS28-ESR scores were assessed using EULAR response criteria, 96 and 83% of patients were categorized as showing good or moderate response to

**Table 1.** Demographics and baseline characteristics of the patients with early and established rheumatoid arthritis (RA) enrolled in this study.

Parameter	Control	Rheumatoid arthritis (RA)	
		Early RA (<9 months)	Established RA (>10 years)
No. of patients	50	50	30
Gender (M/F)	(30/20)	(15/35)	(12/18)
Mean age (Year)	45.4 ± 4.7	45.6 ± 5.32*	55.6 ± 12.41**
BMI (kg/m <sup>2</sup> )	23.2 ± 3.2	25.6 ± 2.18*	26.9 ± 3.20**
Duration of RA (years)	-	8.6 ± 2.21*	14.17 ± 2.21**
RF IgG (U/ml <sup>2</sup> ) ELISA	12.4 ± 2.5	92.8 ± 28.5*	110.8 ± 31.4**
ESR (mm/hr)	16.9 ± 7.5	45.26 ± 4.7*	56.4 ± 5.2**
CRP (mg/L)	1.85 ± 0.72	6.3 ± 8.3*	8.5 ± 5.6**
COMP (ng/ml) ELISA	21.6 ± 4.6	34.86 ± 8.85*	45.9 ± 11.2*
HA (ng/ml)	23 ± 10.2	42 ± 9.23*	35 ± 4.1*
TNF- $\alpha$ (pg/ml)	7.4 ± 5.5	35.35 ± 12.56*	42.3 ± 6.7**
IL-6 (pg/ml)	10.4 ± 2.3	40.33 ± 13.98*	56.36 ± 17.6**
DAS28-ESR	-	5.4 ± 0.43	6.6 ± 0.65**
Swollen joint counts	-	9.8 (0-28)	11.6 (0-28)
Tender joint counts	-	7.9 (0-28)	8.5 (0-28)
HAQ score	-	2.68 (0.8-2.9)	3.6 (0.8-4.0)
PNRS	-	5.3 ± 1.96	6.2 ± 2.85*

Except where indicated otherwise, values are expressed as the mean. \*P < 0.01 versus baseline levels. \*\*P < 0.001 versus baseline levels DAS28= Disease activity score-28; HAQ =Health Assessment Questionnaire score; PNRS= (Pain Numerical Rating Score;0-10); COMP= Cartilage Oligomeric Matrix Protein; CRP = C-reactive protein; ESR= erythrocyte sedimentation rate; TNF- $\alpha$  = Tumor necrosis factor; Rf= Rheumatoid Factor; IL-6= interleukin-6.

**Table 2.** Phenolic compounds content (mg/g) and free-radical scavenging activities of 350 ml aqueous green tea extract (AGTE) using liquid chromatography and spectrophotometry analysis.

Parameter	Phenolic content (mg/g)
<b>Phenolic component</b>	
Catechins (EGCG, ECG )	540
Caffeine	80
Chlorogenic acid	22.47
Pyrogallol	13.41
Vanillic	4.69
Synergic	1.94
Salicylic	1.58
POH benzoic	0.54
Ferulic	0.53
P-Coumatic acid	0.11
Coumarin	0.09
Naringinin	0.1
<b>AGTE scavenging activity</b>	
% Inhibition of DPPH reagent	90.3%
% Inhibition of NBT/Riboflavin reagent	87.9%

green tea supplement in the early and established RA groups, respectively, with significant difference apparent between groups. Also, mean HAQ score was significantly

decreased at week 24 in both the early and the established RA group (Table 3). Also, a significant decrease in inflammatory cytokines, such as TNF- $\alpha$  and



**Table 3.** Time-course changes in biochemical, clinical, radiographic, and functional measures during 24th week green tea therapy.

Parameter	Time after starting green tea supplement (4 to 6 cups/day)					
	0 W (Baseline)		12 W		24 W	
	Early RA	Established RA	Early RA	Established RA	Early RA	Established RA
BMI (kg/m <sup>2</sup> )	25.6 ± 1.8	26.9 ± 2.8	24.9 ± 1.4**	25.6 ± 1.7**	23.8 ± 1.2**	24.5 ± 1.9**
RF IgG (U/ml <sup>2</sup> ) ELISA	92.8 ± 28.5	110.8 ± 31.4	85.8 ± 4.6**	98.8 ± 17.4*	72.5 ± 22.6**	85.3 ± 18.3**
ESR (mm)	45.26 ± 4.7	56.4 ± 5.2	36.2 ± 6.7**	48.6 ± 5.2**	25.8 ± 2.5*	32.2 ± 2.8**
CRP (mg/L)	6.3 ± 8.3	8.5 ± 5.6	2.8 ± 2.6**	4.8 ± 3.2**	1.8 ± 1.6*	2.7 ± 2.3**
DAS28-ESR	5.4 ± 0.43	6.6 ± 0.65	4.3 ± 0.16**	5.3 ± 0.18**	2.4 ± 1.6**	2.6 ± 2.13**
HAQ score	2.38	3.2	1.96**	2.9*	1.75**	2.88*
TSS (SD)	12.5 (21.7)	217.2 (93.2)	n.d.	n.d.	16.4 (25.8)	221.5 (98.4)
JNS (SD)	5.7 (8.6)	87.5 (46.3)	n.d.	n.d.	9.6 (12.7)	91.3 (48.3)
DTSS (mean/median)					4.2/0	5.3/0
Rate of DTSS#0 [% (cases)]					72 (31/43)	77 (20/26)
PNRS	5.3 ± 1.96	6.2 ± 2.85	3.36 ± 0.73**	4.9 ± 0.53**	2.32 ± 0.83**	3.8 ± 0.61**
<b>EULAR category of response</b>						
Good (>1.2%):	-	-	-	-	40 (50); 80%	18 (30); 60%
Moderate (0.6 to -1.2%):	-	-	-	-	8 (45); 16%	7 (30); 23%
No response (> 0.6%):	-	-	-	-	2 (45); 4%	5 (30); 17%

Except where indicated otherwise, values are expressed as the mean. \*P < 0.01 versus baseline levels. \*\*P < 0.001 versus baseline levels; DAS28= Disease Activity Score-28; HAQ =Health Assessment Questionnaire score; PNRS= (Pain Numerical Rating Score;0-10); COMP= Cartilage Oligomeric Matrix Protein; CRP = C-reactive protein; ESR= erythrocyte sedimentation rate; TNF- $\alpha$  = Tumor necrosis factor; Rf= Rheumatoid Factor; IL-6= interleukin-6;EULAR= European League against Rheumatism.

IL-6 were reported in both the early and established RA groups as compared to baseline data, suggesting that our clinical study using green tea yielded successful clinical results (Table 4).

Gradually, there were significant changes in serum levels of HA and COMP cartilage biomarkers during 24-week green tea therapy were reported in the early and established RA groups. The levels of HA and COMP cartilage biomarkers at weeks 12 and 24 were significantly lower than each base-line level (P = 0.001). The change in cartilage biomarkers appeared to synchronize with

decreasing CRP, Rf, ESR levels along with DAS-ESR and PNRS over the 24 weeks of green tea therapy (Table 4).

Radiographic structural assessment using the TSS revealed that mean DTSS per 6 month (6 month progression) was 4.2 in the early RA group and 5.3 in the established RA group, while the proportion with DTSS#0 exceeded 70% in both groups, suggesting that our clinical study using green tea yielded successful clinical results (Table 3).

For both early and established RA groups, correlations between levels of cartilage biomarkers,

biomarkers, inflammatory cytokines, and degree of RA disease activity (ESR, CRP, RF, and DAS-28) were investigated according to EULAR category response at 24 W of green tea therapy. Several marker pairs with significant correlations are summarized in Table 5. A significant positive (P = 0.001) correlation for cartilage HA and COMP biomarkers towards the decrement of RA disease activity markers in good and moderate EULAR response grades was reported; however, negative significant (P = 0.01) correlation was reported in non-responder EULAR group which has no change in RA disease activity markers. Similarly, A

**Table 4.** Time-course changes in the levels of cartilage and inflammatory biomarkers during 24th week green tea therapy.

Parameter	Time after starting Green Tea supplement (4-6 cups/day)					
	0W (Baseline)		12W		24 W	
	Early RA	Established RA	Early RA	Established RA	Early RA	Established RA
COMP (ng/ml) ELISA	34.86 ± 8.85	45.9 ± 11.2	28.15 ± 2.6**	32.15 ± 3.4**	18.5 ± 3.22**	23.5 ± 2.7**
HA (ng/ml)	42 ± 9.23	35 ± 4.1	38.5 ± 6.23	29.4 ± 4.2	28.5 ± 3.5	22.3 ± 2.7
TNF- $\alpha$ (pg/ml)	35.35 ± 12.56	42.3 ± 6.7	26.2 ± 3.2*	27.15 ± 1.7*	20.4 ± 3.65*	16.2 ± 2.4**
IL-6 (pg/ml)	40.33 ± 13.98	56.36 ± 17.6	24.3 ± 2.5**	30.27 ± 3.5**	20.21 ± 4.0**	26.4 ± 2.0**

Except where indicated otherwise, values are expressed as the mean. \*P < 0.01 versus baseline levels. \*\*P < 0.001 versus baseline levels; COMP= cartilage oligomeric matrix protein; TNF- $\alpha$  = tumor necrosis factor; IL-6= interleukin-6.

significant positive (P = 0.01) correlation for inflammatory cytokines TNF- $\alpha$  and IL-6 towards the decrement of RA disease activity markers in good and moderate EULAR response grades was reported; however, negative significant (P = 0.01) correlation was reported in non-responder EULAR group which has no change in RA disease activity markers (Table 5).

## DISCUSSION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the activation of synovial tissue lining the joint capsule, which results in severe morbidity and subsequent physical disability (Pope, 2002).

The traditional treatments for arthritis improve the inflammatory component of the disease and lead to reduced pain and swelling. However, it lacks activity against degenerative process of cartilage (Dingle, 1991). Therefore, the identification of common dietary substances of natural plant origin capable of affording beneficial protection or modulating the onset and severity of arthritis may have important health implications (Berman, 2004).

Green tea (*Camellia sinensis*) has been extensively evaluated by its potential anti-rheumatic activity due to its higher content of polyphenols known as catechins (30 to 36% of dry weight) including epigallocatechin-3-gallate (EGCG), which constitutes up to 63% of total catechins (Manning and Roberts, 2003).

In this study before studying the anti-rheumatic activity of green tea, the polyphenol content and free radical scavenging activity of aqueous green tea leaf extracts (AGTE) were estimated using liquid chromatography and DPPH/NBT scavenging colorimetric assays.

Analysis of AGTE showed higher content of polyphenols. Catechins amount were the highest between all phenolic compounds, where it represented 32.6 mg/g followed by caffeine (25.84 mg/g), chlorogenic acid (22.47 mg/g) and pyrogallol (13.41 mg/g). This is in addition to small amounts of other phenolic compounds such as vanillic, synergic, salicylic, benzoic and ferulic. Similar levels were shown by Hoff and Singleton (1997). However, different results of total polyphenol content (80.5 to 134.9 mg/g and 65.8 to 106.2 mg/g) in black and green tea leaves were obtained by Khokhar and Magnusdottir (2002), this may be related to brewing time, commercial

brand, and producing country.

Also, this study shows higher activity of AGTE, in which anti-radical activity was about 90.3 and 87.9% at 64  $\mu$ g/ml of AGTE against DPPH and NBT radicals, respectively. The potential decrease in the concentration of DPPH and NBT radicals are due to the scavenging ability of AGTE. These results were in accordance with other studies (Yen and Chen, 1997; Yokozawa et al., 1998), which reported higher radicals scavenging efficiency of green tea extract. Also, green tea polyphenols were widely reported as significant antioxidant, anti-carcinogenic, anti-inflammatory, thermogenic, probiotic, and antimicrobial properties (Graham, 1992; Alschuler, 1998).

To study anti-rheumatic activity of green tea, 80 patients with early and established rheumatoid arthritis were subjected to 12, 24 week green tea supplement (4 to 6 cups/day), whereas a cup of green tea typically provides 60 to 125 mg catechins, including EGCG (Cooper et al., 2005).

In this study, patients with early and established RA showed an increase in BMI which correlates significantly with disease activity and pro-inflammatory state of RA patients. The association of BMI with disease activity could be due to physical inactivity leading to an increase in BMI (Dessein et

**Table 5.** Spearman's correlation coefficients of cartilage and inflammatory biomarkers versus RA disease markers (ESR, Rf, CRP, and DAS28-ESR) according to EULAR category of response at 24 week of green tea therapy.

Item	EULAR category response at 24 W of green tea therapy					
	Early RA (Spearman's r)			Established RA (Spearman's r)		
	Good (n=40)	Moderate (n=8)	No Response (n=2)	Good (n=18)	Moderate (n=7)	No Response (n=5)
<b>Cartilage biomarker</b>						
COMP vs ESR	0.32**	0.21 **	- 0.35*	0.5**	0.63**	- 0.51*
vs Rf	0.12**	0.41 **	- 0.65*	0.25**	0.73**	- 0.31*
vs CRP	0.42**	0.81 **	- 0.55*	0.45**	0.83**	- 0.91*
vs DAS28	0.49**	0.78 **	- 0.95*	0.48**	0.33**	- 0.53*
HA vs ESR	0.24**	0.35**	- 0.42*	0.34**	0.71**	- 0.34*
vs Rf	0.64**	0.55**	- 0.62*	0.44**	0.51**	- 0.37*
vs CRP	0.56**	0.25**	- 0.48*	0.53**	0.68**	- 0.41*
vs DAS28	0.49**	0.75**	- 0.32*	0.64**	0.70**	- 0.35*
<b>Inflammatory biomarker</b>						
TNF- $\alpha$ vs ESR	0.37*	0.91*	- 0.25**	0.35*	0.75*	- 0.59**
vs Rf	0.29*	0.85*	- 0.27**	0.45*	0.80*	- 0.61**
vs CRP	0.43*	0.75*	- 0.23**	0.55*	0.82*	- 0.70**
vs DAS28	0.47*	0.68*	- 0.31**	0.25*	0.67*	- 0.73**
IL-6 vs ESR	0.45*	0.81*	- 0.43**	0.58*	0.25*	- 0.68**
vs Rf	0.49*	0.87*	- 0.38**	0.52*	0.35*	- 0.71**
vs CRP	0.65*	0.79*	- 0.36**	0.56*	0.31*	- 0.75**
vs DAS28	0.58*	0.72*	- 0.40**	0.61*	0.30*	- 0.78**

Values are correlation coefficients calculated using Spearman's rank correlation. P values are expressed in parentheses. P < 0.05 is considered as statistically significant. \* P < 0.01 and \*\*P < 0.001.

(Dessein et al., 2007; Sokka et al., 2008). Therefore, BMI may consider as a predictor of RA disease activity.

When patients with early and established RA treated with green tea therapy for 12 and 24 weeks, BMI was greatly reduced towards normal values compared to baseline data. The control of higher BMI towards normal values may be related to green tea's thermogenic effect properties as a result of a synergistic interaction between caffeine and catechin polyphenols that appears to prolong sympathetic stimulation of thermogenesis (Dulloo et al., 2000; Juhel et al., 2000). This matched with Maki et al. (2009) who reported that, the consumption of 625 mg EGCG-containing catechins daily for 24 weeks caused a greater loss of body weight and a decrease in the fasting serum triglyceride levels in the catechin-administered group. Also data showed that the decrease in BMI was significantly correlated with reduction in the parameters of RA disease activity DAS28 and PNRS during 24 week green tea therapy indicating the anti-rheumatic activity of green tea and the importance of BMI as a predictor of RA disease activity (Sokka et al., 2008).

In the present study, patients with early and established RA treated with green tea therapy for 24 week showed a significant decrease in clinical measures of disease activity

such as CRP, Rf and ESR as compared to baseline data. The decrease in disease activity measures exhibited significant correlation with enhancement of DAS28-ESR and PNRS criteria. The established pharmacological properties of green tea are attributed to its high content of EGCG (Doss et al., 2005; Cooper et al., 2005). The anti-rheumatic activity of green tea supplement may be related to certain pathways such as the modulation of energy balance, endocrine system, food intake, lipid and carbohydrate metabolism, antiobesity, anti-inflammatory, and redox status (Yang et al., 2001; Sabu et al., 2010).

Recent understanding of the RA pathogenesis has clarified the role of cytokines and other inflammatory mediators in this process and has provided a scientific rationale in developing targeted therapies (Feldmann and Maini, 2008), whereas cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 promote imbalance between excessive cartilage destruction and cartilage repair processes via induction of reactive oxygen species (ROS) and inflammatory mediators (Malemud, 2004).

Our results showed elevated levels of TNF- $\alpha$  and IL-6 in early and established RA groups which coincided with previous studies (Dube et al., 2008; Patrick et al., 2010). These results were reversed by supplementation of green

tea therapy for 24 week. The reported beneficial effects of green tea may be related to their antioxidant, anti-inflammatory and antiproteinase properties of its phenolic contents (Frei and Higdon, 2003), the anti-inflammatory effects of green tea may partially be due to its ability to regulate TNF- $\alpha$  gene expression, encoding anti-inflammatory/pro-inflammatory factors (Wahyudi and Sargowo, 2007). The decrease in the level of TNF- $\alpha$  and IL-6 was supported with a significant correlation between disease activity parameters and the improvement of DAS28-ESR and PNRs criteria, whereas the level of cytokines and RA disease parameters especially CRP are interrelated (Hye Soon Parka et al., 2005; Patrick et al., 2010). The obtained results may be related to the ROS-scavenging activity of green tea catechins (Chen et al., 2004; Erba et al., 2005; Riegsecker et al., 2013).

Soon after a discovery that GT polyphenols may inhibit arthritis in murine model, majority of the studies were focused on its beneficial effect on progressive cartilage degradation, a hallmark of OA and RA (Malemud, 2006).

In this study, a significant increase in both serum HA and COMP levels was reported at baseline stage of patients with early and established RA. However, patients with established RA showed a higher HA and COMP values as compared to early group. This may be related to the severity of joint damage due to physical activity (Mundermann et al., 2005). The elevation of serum COMP and HA showed a significant correlation with the parameters of RA disease activity, especially DAS28-ESR and PNRs. The correlation of these parameters in patients demonstrating low values of traditional prognostic markers including ESR, CRP, RF and DAS was greatly reported (Månsson et al., 1999; Skoumal et al., 2004). Additionally, COMP may be of prognostic value for radiological outcome and proposed its feasibility in monitoring articular cartilage damage in RA (Skoumal et al., 2003; Young-Min et al., 2007; Morozzi et al., 2007).

Also, in the present study, there was a significant decrease in the level of COMP and HA in both groups with RA during the initial 6 months of green tea therapy. This suggests that green tea modifies the release of COMP and HA from the tissue and supports the interpretation that this treatment modality retards the development of joint destruction. Interestingly, some recent pharmacological studies using EGCG or green tea to suppress arthritis have focused equally on bone resorption observed in RA (Lin et al., 2009; Natsume et al., 2009; Takai et al., 2008).

The EULAR response criteria classify patients as good, moderate or non-responders, using the individual amount of change in the DAS28 according to patients responding for novel treatments or clinical trials (Smolen et al., 2003; Fransen et al., 2003).

In the present study, the response of RA patients to green tea treatment at 24 week was reported using DAS28-ESR and EULAR response criteria. When

DAS28-ESR scores were assessed using EULAR response criteria, 96 and 83% of patients were categorized as showing good or moderate response in the early and established RA groups, respectively with no significant difference apparent between groups. However, 4 and 17% of patients were non-responders at 24 week green tea therapy. The data obtained showed a significant correlation between the decrease in the levels of CRP, ESR, Rf, IL-6 and TNF- $\alpha$  in good and moderate green tea RA responders as compared to non-responders, whereas the disease activity remained unchanged. These results demonstrated the anti-rheumatic activity of green tea which may be related to the antioxidant and anti-inflammatory of its phenolic compounds, especially catechins (Morinobu et al., 2008; Lin et al., 2008). Also, many studies reported that treatment of green tea reduced oxidative stress free radicals induced production of proinflammatory cytokines TNF- $\alpha$ , IL-6, CRP and NF- $\kappa$ B activation in a dose dependent manner (Kim et al., 2000; Wahyudi and Sargowo, 2007; Ramesh et al., 2009).

Similarly, in the present study radiographic structural assessment using the TSS revealed that mean DTSS per 6 month (6 month progression) was 4.2 in the early RA group and 5.3 in the established RA group, while the proportion with DTSS#0 exceeded 70% in both groups, suggesting that our clinical study using green tea yielded successful clinical results comparable to those in a previous study in Japan (Takeuchi et al., 2008), which reported the notable efficacy of infliximab therapy in a rheumatoid arthritis management.

Finally, according to EULAR response criteria, elevated serum levels of HA and COMP were significantly decreased in all groups of good, moderate and non-responders to green tea therapy at 24 week. Our results showed that green tea treatment inhibited the degradation of human cartilage through enhancement of the serum levels of HA and COMP cartilage biomarkers as previously reported (Adcocks et al., 2002; Vankemmelbeke et al., 2003; Ahmed et al., 2004; Niki et al., 2012). Also, it could be hypothesized that the effect might be due to the joint protective effect suggested for green tea polyphenols (Lambert et al., 2007). Our results further confirm the conclusion that COMP levels are highly specific markers for the cartilage degradation process in RA (Di Cesare et al., 1999), and could be used as biomarker for clinical treatments.

## Conclusion

The pattern of changes of serum cartilage biomarkers (HA and COMP) and inflammatory cytokines (IL-6, TNF- $\alpha$ ) in patients with early and established RA, supports the interpretation that green tea has a joint protective and anti-inflammatory effect. Serum cartilage biomarkers could

be useful for evaluating tissue effects of novel treatment modalities in rheumatoid arthritis. Our results suggest that this promising anti-arthritic activity of green tea should be further explored as a dietary therapy for the management of RA in conjunction with conventionally used drugs.

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## ABBREVIATIONS

**RA**, Rheumatoid arthritis; **ECM**, extracellular matrix; **DAS28**, disease activity score-28; **PNRS**, pain numerical rating score 0-10; **COMP**, cartilage oligomeric matrix protein; **CRP**, C-reactive protein; **ESR**, erythrocyte sedimentation rate; **TNF- $\alpha$** , tumor necrosis factor; **Rf**, rheumatoid factor; **IL-6**, interleukin-6; **AGTE**, aqueous green tea extract; **EGCG**, epigallocatechin-3-gallate; **DPPH**, 1,1-diphenyl-2-picryldrazil; **NBT**, nitro blue tetrazolum; **EULAR**, European league against rheumatism response criteria.

## Authors' contributions

All the authors contributed equally to this study, read and approved final version of manuscript.

## Conflict of Interest

The authors declare that they have no competing interests.

## REFERENCES

- Adcocks C, Collin P, Buttle DJ (2002). Catechins from green tea (*Camellia sinensis*) inhibit bovine and human cartilage proteoglycan and type II collagen degradation in vitro. *J. Nutr.* 132:341-346.
- Ahmed S, Marotte H, Kwan K, Ruth JH, Campbell PL, Rabquer BJ, Pakozdi A, Koch AE (2008). Epigallocatechin-3-gallate inhibits IL-6 synthesis and suppresses trans signaling by enhancing soluble gp130 production. *Proc. Natl. Acad. Sci. USA*105:14692-7.
- Ahmed S, Rahman A, Hasnain A, Lalonde M, Goldberg VM, Haqqi TM (2002). Green tea polyphenol epigallocatechin-3-gallate inhibits the IL-1 $\beta$ -induced activity and expression of cyclooxygenase-2 and nitric oxide synthase-2 in human chondrocytes. *Free Radic. Biol. Med.* 33:1097-1105.
- Ahmed S, Wang N, Lalonde M, Goldberg VM, Haqqi TM (2004). Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1 beta-induced expression of matrix metalloproteinase-1 and-13 in human chondrocytes. *J. Pharmacol. Exp. Ther.* 308:767-773.
- Alschuler L (1998). Green Tea: Healing tonic. *Am. J. Nat. Med.* 5:28-31.
- Arab L, Il'yasova D (2003).The epidemiology of tea consumption and colorectal cancer incidence. *J. Nutr.* 133:S3310-8.
- Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA Jr., Mitchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JT, Wilder RL, Hunder GG (1988). The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31:315-324.
- Berman B (2004). Complementary and alternative medicine: is it just a case of more tools for the medical bag? *Clin. J. Pain* 20:1-2.
- Brosseau L, Wells GA, Tugwell P, Egan M, Wilson KG, Dubouloz CJ, Casimiro L, Robinson VA, McGowan J, Busch A, Poitras S, Moldofsky H, Harth M, Finestone HM, Nielson W, Haines-Wangda A, Russell-Doreleyers M, Lambert K, Marshall AD, Veilleux L; Ottawa Panel Members (2004). Ottawa panel evidence-based clinical practice guidelines for therapeutic exercises in the management of rheumatoid arthritis in adults. *Phys. Ther.* 84(10):934-972.
- Chen JH, Tipoe GL, Liang EC, So HS, Leung KM, Tom WM, Fung PC, Nanji AA (2004). Green tea polyphenols prevent toxin-induced hepatotoxicity in mice by down-regulating inducible nitric oxide-derived prooxidants. *Am. J. Clin. Nutr.* 80:742-751.
- Choy EH, Panayi GS (2001). Cytokine pathways and joint inflammation in rheumatoid arthritis. *N. Engl. J. Med.* 344: 907-16.
- Cooper R, Morre DJ, Morre DM (2005). Medicinal benefits of green tea: part I. Review of non-cancer health benefits. *J. Altern. Compl. Med.* 11:521-528.
- den Broeder AA, Joosten L, Saxne T, Heinegard D, Fenner H, Miltenburg A, Frasa W, van Tits LJ, Buurman W, van Riel PLCM, van de Putte LBA, Barrera P (2002). Long term anti-tumour necrosis factor alpha monotherapy in rheumatoid arthritis: effect on radiological course and prognostic value of markers of cartilage turnover and endothelial activation. *Ann Rheum Dis.*61:311-8.
- Dessein PH, Norton GR, Woodiwiss AJ, Joffe BI, Solomon A (2007). Independent role of conventional cardiovascular risk factors as predictors of C-reactive protein concentrations in rheumatoid arthritis. *J. Rheumatol.* 34:681-8.
- Di Cesare PE, Fang C, Leslie MP, Della Valle CJ, Gold JM, Tulli H, Perris R, Carlson CS (1999). Localization and expression of cartilage oligomeric matrix protein by human rheumatoid and osteoarthritic synovium and cartilage. *J. Orthop. Res.* 17:437-445.
- Dingle JT (1991). Cartilage maintenance in osteoarthritis: interaction of cytokines, NSAID and prostaglandins in articular cartilage damage and repair. *J. Rheumatol.* 18: 30-37.
- Doss MX, Potta SP, Hescheler J, Sachinidis A (2005). Trapping of growth factors by catechins: a possible therapeutical target for prevention of proliferative diseases. *J. Nutr. Biochem.* 16:259-266.
- Dube B, Rita T B, Satya P, Kalra A, Michael G (2008). Increased leptin expression selectively in the hypothalamus suppresses inflammatory markers CRP and IL-6 in leptin-deficient diabetic obese mice. *Peptides* 29: 593-598.
- Dulloo AG, Seydoux J, Girardier L, Chantre P, Vandermander J (2000). Green tea and thermogenesis: interactions between catechin polyphenols, caffeine, and sympathetic activity. *Int. J. Obes. Relat. Metab. Disord.* 24:252-258.
- Ekdahl C, Broman G (1992). Muscle strength, endurance, and aerobic capacity in rheumatoid arthritis: a comparative study with healthy subjects. *Ann. Rheum. Dis.* 51(1):35-40.
- Erba D, Riso P, Bordonni A, Foti P, Biagi PL, Testolin G (2005). Effectiveness of moderate green tea consumption on antioxidative status and plasma lipid profile in humans. *J. Nutr. Biochem.* 16:144 - 9.
- Feldmann M, Maini SR (2008).Role of cytokines in rheumatoid arthritis: an education in pathophysiology and therapeutics. *Immunol. Rev.* 223:7-19.
- Firesten GS (2004). The T cell cometh: Interplay between adaptive immunity and cytokine networks in rheumatoid arthritis. *J. Clin. Invest.* 114: 471-4.

- Fransen J, Stucki G, Van Rielplc M (2003). Rheumatoid arthritis measures. *Arthritis Rheum.* 49 (Suppl.5):S214-24.
- Frei B, Higdon JV (2003). Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. *J. Nutr.* 133:3275S-84S.
- Graham HN (1992). Green tea composition, consumption, and polyphenol chemistry. *Prev. Med.* 21:334-350.
- Haqqi TM, Anthony DD, Gupta S, Ahmad N, Lee MS, Kumar GK, Mukhtar H (1999). Prevention of collagen-induced arthritis in mice by a polyphenolic fraction from green tea. *Proc. Natl. Acad. Sci. USA* 96:4524-4529.
- Hitchon CA, Alex P, Erdile LB, Frank MB, Dozmorov I, Tang Y, Wong K, Centola M, El-Gabalawy HS (2004). A distinct multi-cytokine profile is associated with anti-cyclical citrullinated peptide antibodies in patients with early untreated inflammatory arthritis. *J. Rheumatol.* 31:2336-46.
- Hoff JF, Singlengleton KIA (1997). A method for determination of tannin in foods by means of immobilized enzymes. *J. Food Sci.* 42:1566.
- Hye SP, Jung YP, Rina Y (2005). Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF- $\alpha$  and IL-6. *Diabetes Res. Clin. Pract.* 69:29-35.
- Inoue E, Yamanaka H, Hara M, Tomatsu T, Kamatani N (2007). Comparison of Disease Activity Score (DAS)-28- erythrocyte sedimentation rate and DAS28- C-reactive protein threshold values. *Ann. Rheum. Dis.* 66:407-409.
- Juhel C, Armand M, Pafumi Y, Rosier C, Vandermander J, Lairon D (2000). Green tea extract (AR25) inhibits lipolysis of triglycerides in gastric and duodenal medium in vitro. *J. Nutr. Biochem.* 11:45-51.
- Khokhar S, Magnusdottir SGM (2002). Total phenol, catechin and caffeine contents of teas commonly consumed in the United Kingdom. *J. Agric. Food Chem.* 50:565.
- Kim S, Lee MJ, Hong J, Li C, Smith TJ, Yang GY, Seril DN, Yang CS (2000). Plasma and tissue levels of tea catechins in rats and mice during chronic consumption of green tea polyphenols. *Nutr. Cancer* 37:41-48.
- Kremers HM, Nicola P, Crowson CS, Michael O' fallon W, Gabriel SE (2004). Therapeutic strategies in rheumatoid arthritis over a 40-year period. *J. Rheumatol.* 31:2366-2373.
- Kroot EJ1, de Jong BA, van Leeuwen MA, Swinkels H, van den Hoogen FH, van't Hof M, van de Putte LB, van Rijswijk MH, van Venrooij WJ, van Riel PL (2000). The prognostic value of anti-cyclic citrullinated peptide antibody in patients with recent-onset rheumatoid arthritis. *Arthritis Rheum.* 43:1831-1835.
- Lambert JD, Sang S, Yang CS (2007). Biotransformation of green tea polyphenols and the biological activities of those metabolites. *Mol. Pharm.* 4:819-25.
- Lin RW, Chen CH, Wang YH, Ho ML, Hung SH, Chen IS, Wang GJ (2009). (-)-Epigallocatechin gallate inhibition of osteoclastic differentiation via NF- $\kappa$ B. *Biochem. Biophys. Res. Commun.* 379:1033-1037.
- Lin SK, Chang HH, Chen YJ, Wang CC, Galson DL, Hong CY, Kok SH (2008). Epigallocatechin-3-gallate diminishes CCL2 expression in human osteoblastic cells via up-regulation of phosphatidylinositol 3-Kinase/Akt/ Raf-1 interaction: a potential therapeutic benefit for arthritis. *Arthritis Rheum.* 58:3145-3156.
- Lipsky PE (2005). Rheumatoid arthritis. In: Kasper D, Braunwald E, Fauci A, Hauser S, Longo D, Jameson J (eds.), *Harrison's principles of internal medicine*, 16th ed. New York: McGraw-Hill.
- Majeed M, McQueen F, Yeoman S, McLean L (2004). Relationship between serum hyaluronic acid level and disease activity in early rheumatoid arthritis. *Ann. Rheum. Dis.* 63:1166-1168 doi:10.1136/ard.2003.010942
- Maki KC, Reeves MS, Farmer M, Yasunaga K, Matsuo N, Katsuragi Y, Komikado M, Tokimitsu I, Wilder D, Jones F, Blumberg JB, Cartwright Y (2009). Green tea catechin consumption enhances exercise-induced abdominal fat loss in overweight and obese adults. *J. Nutr.* 139:264-270.
- Malemud CJ (2004). Cytokines as therapeutic targets for osteoarthritis. *Biol. Drugs* 18:23-35.
- Malemud CJ (2006). Matrix metalloproteinases: role in skeletal development and growth plate disorders. *Front. Biosci.* 11:1702-15.
- Manning J, Roberts JC (2003). Analysis of catechin content of commercial green tea products. *J. Herbal Pharmacother.* 3:19-32.
- Månsson B, Carey D, Alini M, Ionescu M, Rosenberg LC, Poole AR, Heinegård D, Saxne T (1995). Cartilage and bone metabolism in rheumatoid arthritis. Differences between rapid and slow progression of disease identified by serum markers of cartilage metabolism. *J. Clin. Invest.* 95:1071-7.
- McInnes IB (2001). Rheumatoid arthritis: From bench to bedside. *Rheum. Dis. Clinics North Am.* 27(2):373-387.
- McKenna DJ, Hughes K, Jones K (2000). Green tea monograph. *Altern. Ther. Health Med.* 6:61-8, 70-2, 4.
- Morinobu A, Biao W, Tanaka S, Horiuchi M, Jun L, Tsuji G, Sakai Y, Kurosaka M, Kumagai S (2008). (-)-Epigallocatechin-3-gallate suppresses osteoclast differentiation and ameliorates experimental arthritis in mice. *Arthritis Rheum.* 58:2012-2018.
- Morozzi G, Fabbro M, Bellisai F, Cucini S, Simpatico A, Galeazzi M (2007). Low serum level of COMP, a cartilage turnover marker, predicts rapid and high ACR70 response to adalimumab therapy in rheumatoid arthritis. *Clin. Rheumatol.* 26:1335-1338.
- Mundermann A, Dyrby CO, Andriacchi TP, King KB (2005). Serum concentration of cartilage oligomeric matrix protein (COMP) is sensitive to physiological cyclic loading in healthy adults. *Osteoarthritis Cartilage* 13:34-38.
- Natsume H, Adachi S, Takai S, Tokuda H, Matsushima-Nishiwaki R, Minamitani C, Yamauchi J, Kato K, Mizutani J, Kozawa O, Otsuka T (2009). (-)-Epigallocatechin gallate attenuates the induction of HSP27 stimulated by sphingosine 1-phosphate via suppression of phosphatidylinositol 3-kinase/Akt pathway in osteoblasts. *Int. J. Mol. Med.* 24:197-203.
- Niki Y, Takeuchi T, Nakayama M, Nagasawa H, Kurasawa T, Yamada H, Toyama Y, Miyamoto T (2012). Clinical Significance of Cartilage Biomarkers for Monitoring Structural Joint Damage in Rheumatoid Arthritis Patients Treated with Anti-TNF Therapy. *PLoS ONE* 7(5):e37447.
- Nutrition and Your Health: Dietary Guidelines for Americans (1995). U.S. Department of Agriculture, U.S. Department of Health and Human Services. 4th edition.
- Ohinishi M, Morishita H, Iwahashi H, Shizuo T, Yoshiaki S, Kimura M, Kido R (1994). Inhibitory effects of chologenic acids on linoleic acid peroxidation and haemolysis. *Phytochemistry* 36:579-583.
- Patrick B, Tobias B, Rüdiger H, Georg B, Roland J, Felix B, Michael S, Heike N (2010). Diet-induced obesity, exogenous leptin-, and MADB106 tumor cell challenge affect tissue leukocyte distribution and serum levels of cytokines in F344 rats. *Endocrinology* 38:104-112.
- Pope RM (2002). Apoptosis as a therapeutic tool in rheumatoid arthritis. *Nat. Rev. Immunol.* 2:527-535.
- Ramesh E, Geraldine P, Thomas PA (2009). Regulatory effect of epigallocatechin gallate on the expression of C-reactive protein and other inflammatory markers in an experimental model of atherosclerosis. *Chem. Biol. Interact.* [Epub ahead of print].
- Ravishankara M N, Shrivastava N, Padh H, Rajani M (2002). Evaluation of antioxidant properties of root bark of *Hemidesmus indicus* R. Br. (Anantmul). *Phytomedicine* 9:153-160.
- Riegsecker S, Wiczynski D, Kaplan MJ, Ahmed S (2013). Potential benefits of green tea polyphenol EGCG in the prevention and treatment of vascular inflammation in rheumatoid arthritis. *Life Sci.* 3:93(8):307-12.
- Sabu MC, Priya TT, Ramadnan K, Ikuo Nishigaki I (2010). Beneficial effect of green tea; A literature review. *Chin. Med* 5:13.
- Sattar N, McCarey DW, Capell H, McInnes IB (2003). Explaining how "high-grade" systemic inflammation accelerates vascular risk in rheumatoid arthritis. *Circulation* 108(24):2957-2963.
- Saxne T, Månsson B (2000). Molecular markers for assessment of cartilage damage in rheumatoid arthritis. In: Firestein G, Panayi GS, Wollheim FA (eds.), *Rheumatoid Arthritis: New Frontiers in Pathogenesis and Treatment*. Oxford: Oxford University Press. pp. 291-304.
- Singh R, Ahmed S, Islam N, Victor M. Goldberg VM, Haqqi TM (2002). Epigallocatechin-3-gallate inhibits interleukin-1 $\beta$ -induced expression

- of nitric oxide synthase and production of nitric oxide in human chondrocytes: suppression of nuclear factor  $\kappa$ B activation by degradation of the inhibitor of nuclear factor  $\kappa$ B. *Arthritis Rheum.* 46:2079-2086.
- Skoumal M, Haberhauer G, Feyertag J, Kittl EM, Bauer K, Dunky A (2004). Serum levels of cartilage oligomeric matrix protein are elevated in rheumatoid arthritis, but not in inflammatory rheumatic diseases such as psoriatic arthritis, reactive arthritis, Raynaud's syndrome, scleroderma, systemic lupus erythematosus, vasculitis and Sjögren's syndrome. *Arthritis Res. Ther.* 6:73-74.
- Skoumal M, Kolarz G, Klingler A (2003). Serum levels of cartilage oligomeric matrix protein. A predicting factor and a valuable parameter for disease management in rheumatoid arthritis. *Scand. J. Rheumatol.* 32:156-61.
- Smith JB, Haynes MK (2002). Rheumatoid arthritis; a molecular understanding. *Ann. Intern. Med.* 136:908-22.
- Smolen JS, Breedveld FC, Schiff MH, Kalden JR, Emery P, Eberl G, van Riel PL, Tugwell P (2003). A simplified disease activity index for rheumatoid arthritis for use in clinical practice. *Rheumatology* 42:244-57.
- Sokka T, Häkkinen A, Kautiainen H, Maillefert JF, Toloza S, Mørk Hansen T, Calvo-Alen J, Oding R, Liveborn M, Huisman M, Alten R, Pohl C, Cutolo M, Immonen K, Woolf A, Murphy E, Sheehy C, Quirke E, Celik S, Yazici Y, Tlustochowicz W, Kapolka D, Skacic V, Rojkovich B, Müller R, Stropuviene S, Anderson D, Drosos AA, Lazovskis J, Pincus T; QUEST-RA Group (2008). Physical inactivity in patients with rheumatoid arthritis: data from twenty-one countries in a cross-sectional, international study. *Arthritis Rheum.* 59:42-50.
- Takai S, Matsushima-N R, Adachi S, Natsume IH, Minamitani C, Mizutani J, Otsuka T, Tokuda H, Kozawa O (2008). (-)-Epigallocatechin gallate reduces platelet-derived growth factor-BB-stimulated interleukin-6 synthesis in osteoblasts: suppression of SAPK/JNK. *Mediators Inflamm.* 2008: 291808.
- Takeuchi T, Yamanaka H, Inoue E, Nagasawa H, Nawata M, Ikari K, Saito K, Sekiguchi N, Sato E, Kameda H, Iwata S, Mochizuki T, Amano K, Tanaka Y (2008). Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan: one year outcome of joint destruction (RECONFIRM-2J). *Mod Rheumatol.* 18:447-454.
- Vankemmelbeke MN, Jones GC, Fowles C, Ilic MZ, Handley CJ, Day AJ, Knight CG, Mort JS, Buttle DJ (2003). Selective inhibition of ADAMTS-1, -4 and -5 by catechin gallate esters. *Eur. J. Biochem.* 270:2394-2403.
- Wahyudi S, Sargowo D (2007). Green tea polyphenols inhibits oxidized LDL- induced NKKB activation in human umbilical vein endothelial cell. *Acta Med. Indones.* 39(2): 66-70.
- Waskmundzka M, Wianowska D, Szewczyk K, Oniszczyk A (2007). Effect of sample-preparation methods on the HPLC quantitation of some phenolic acids in plant materials. *Acta Chromatographica* 19:227-237.
- Yang MH, Wang CH, Chen HL (2001). Green tea, Oolong and black tea extract modulate lipid metabolism in hyper lipidemic rats fed high sucrose diet. *J. Nutr. Biochem.* 12:14-20.
- Yen GC, Chen HY (1997). Antioxidant activity of various tea extracts in relation to their mutagenicity. *J. Agric. Food Chem.* 43:27.
- Yokozawa T, Dong E, Nakagawa T, Kashiwagi H, Nakagawa H, Takeuchi S, Chung HY (1998). In vitro and in vivo studies on the radical-scavenging activity of tea. *J. Agric. Food Chem.* 46:2143.
- Young-Min S, Cawston T, Marshall N, CoadyD, Christgau S, Saxne T, Robins S, Griffiths I (2007). Biomarkers predict radiographic progression in early rheumatoid arthritis and perform well compared with traditional markers. *Arthritis Rheum.* 56:3236-3247.

## Full Length Research Paper

# Effect of Korean arbor vitae (*Thuja koraiensis*) extract on antimicrobial and antiviral activity

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The present study was carried out to develop a new natural product reagent which has antimicrobial and antiviral effect, so we assayed the extract from Korean Arbor vitae (*Thuja koraiensis*) on antimicrobial and antiviral *in vitro*. The antimicrobial activity was assayed at two gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*); two gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*) and the results were measured by the paper disc diffusion assay and minimum inhibitory concentration (MIC). The antiviral activity of *T. koraiensis* extract was assayed at the Bovine viral diarrhoea (BVD) virus which is a RNA virus replication in Madin-Darby bovine kidney (MDBK) cells and the results were measured by maximum non cytotoxic concentration (MNCC) and maximum non-toxic dose (MNTD). The result of paper disk diffusion assay showed that extract had the high antimicrobial effect at *S. aureus* strain. The MNCC of extract on MDBK cells was 0.031% and the MNTD of extract was 0.0195% on BVD virus. These results suggested that *T. koraiensis* extract had antimicrobial and antiviral effect, especially at low concentrations which had a strong antiviral effect at BVD virus. The *T. koraiensis* extract could also be useful as disinfectant for bacterial. The study of *T. koraiensis* function perhaps would be the first and more research is needed in the future.

**Key words:** *Thuja koraiensis* extract, antimicrobial activity, minimum inhibitory concentration, maximum non cytotoxic concentration, maximum non-toxic dose, antiviral activity.

## INTRODUCTION

Arbor vitae is the common name for any of the coniferous evergreen trees or shrubs comprising the genus *Thuja* in the cypress family (William and Jackson, 1967). The foliage of *Thuja* is rich in Vitamin C, and was used by Native Americans and early European explorers as a cure for scurvy. The leaves have been used as a treatment for rheumatism. The oil of arbor vitae is often quoted as a herbal remedy topically used to aid in the

treatment of human papillomavirus (HPV), genital or common warts (Aljos, 2005). Korean Arbor vitae (*Thuja koraiensis*) is a species of *Thuja*, an evergreen shrub or small tree growing up to 3 to 10 m tall. The foliage forms flat sprays with scale-like leaves of about 2 to 4 mm long (up to 15 mm long on strong-growing shoots), matt dark green above and with broad and vivid white stomata wax bands below. The cones are oval, yellow-green ripening

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to red-brown, 7 to 11 mm long and 4 to 5 mm broad (opening to 6 to 9 mm broad), with 8 to 12 overlapping scales ("Flora of China" editorial board, 1978).

*Thuja* is very resistant to most pests (Aljos, 2005). A lot of research has shown that *Arbor vitae* exhibits anti-inflammatory (Kim et al., 2013), antidiuretic (Dubey and Batra, 2008), antimicrobial (Jain and Garg, 1997), fungi toxic (Guleria et al., 2008) and antiviral (Loizzo et al., 2008) activities. However, there are no studies for *T. koraiensis*. In this study, we investigate the antimicrobial and antiviral activities of the extract of *T. koraiensis* in order to develop a new natural product reagent. For the antimicrobial activity, two strains each of Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) and Gram-negative bacteria (*Salmonella typhimurium*, *Escherichia coli*) were selected and the experiment conducted. For antiviral activity, the Bovine viral diarrhoea (BVD) virus which resulted in large losses of domestic animals was selected for the experiment. BVD virus is an enveloped positive-strand RNA virus that holds generic status in the family Flaviviridae (Collett et al., 1988). BVD virus causes major economic losses in domestic ruminants worldwide (Houe, 1999). It can lead to a variety of clinical outcomes that range from subclinical infections to the more severe presentations including abortion, infertility and the fatal mucosal disease (Baker, 1995).

## MATERIALS AND METHODS

### Plant

*T. koraiensis* was purchased from the Natural Space and dissolved in 100% dimethyl sulfoxide (DMSO).

### Solvent extraction

*T. koraiensis* was extracted by steam distillation method (Cassel et al., 2009).

### Micro-organisms

The bacteria used in this study were two kinds of Gram-positive bacteria *B. subtilis* (ATCC11774), *S. aureus* (KCCM1335) and two kinds of Gram-negative bacteria *E. coli* (KCCM12181), *S. typhimurium* (KCCM11862). The strains were purchased at the Korean Culture Center of Microorganisms (KCCM). The medium used was Muller Hinton Broth (Difco, USA).

### Paper disc diffusion assay

Antimicrobial activity of *T. koraiensis* extract was determined by the disc diffusion method (Bauer et al., 1966). The bacteria were cultured in Mueller–Hinton broth. The concentrations of the cultures were  $10^6$  colony forming units (CFU) per ml. The extract to be tested was dissolved in 100% DMSO. For the purpose of screening, 10  $\mu$ l of each extract solution was loaded on paper disc (Whatman no. 6 mm). The disc was placed on the surface of the Mueller–Hinton agar plate previously inoculated with bacteria. The agar plates were then inverted and incubated for 24 h at 37°C. The

antimicrobial activity was recorded by measuring the zone of inhibition (in mm) around each disc and the diameter zone of inhibition was measured using a transparent plastic ruler. Each test was carried out in triplicate. Amoxicillin (10  $\mu$ g/ml) and colistin sulfate (10  $\mu$ g/ml) were used as a positive control and 100% DMSO as a negative control in the assays.

### Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration (MIC) assay was determined by following the micro broth dilution method (Shin and Kim, 2005) performed with the 96-well plate. A range of two-fold dilution (50 to 0.3%) of the *T. koraiensis* extract in Muller Hinton Broth (MHB) containing 100% DMSO was prepared. The extract suspensions (100  $\mu$ l) were added to each well. The test bacterial strains were adjusted with MHB to match the  $10^6$  colony forming units (CFU) per ml. Subsequently, 100  $\mu$ l volume of the strain medium was added to each well and the plate were incubated at 37°C for 24 h under micro plate spectrophotometer (Biotech, Eon, USA) using the absorbance of 600 nm measured once every 4 h. MHB was used as a negative control and each strain medium were used as a positive control. The MIC was defined as the lowest concentration of a test sample that completely inhibited visible bacterial growth.

### Cell and virus cultures

The Madin-Darby bovine kidney (MDBK) cell and Bovine viral diarrhoea (BVD) virus were purchased at the American Type Culture Collection (ATCC). MDBK cells were grown in 75T-Flask by using minimum essential medium (MEMA, Thermo, USA) supplemented with 5% (v/v) fetal bovine serum (FBS, GIBCO/ BRL, USA) and 1% (v/v) anti-anti (Antibiotic-Anti mycotic, GIBCO/ USA). The BVD virus was propagated using MDBK cells and cytopathic effect (CPE) was observed. Repeated freezing and thawing was done twice and then centrifuged for 5 min at 2000 rpm. The aqueous layer was removed and put into cryogenic vials, stored at -70°C until use. Titer of virus infection was done using the Spearman-Karrer method (Jung, 2001), 50% tissue culture infective dose (TCID<sub>50</sub>) was calculated and the result of  $1.5 \times 10^6$ /ml was obtained.

### Cell cytotoxicity test

The cytotoxicity assay for dimethyl sulfoxide (DMSO) was used to obtain the cell attachment test. MDBK cells were seeded into 96-well plate at a density of  $3.5 \times 10^3$  cell/ml. DMSO was diluted with MDBK cell growth medium by two fold serial dilutions in 96-well plate and incubated for 72 h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After incubation, cytopathic effect (CPE) was observed. The plate was examined under binocular microscope and the dead cells in each well were counted. Monolayers of MDBK cells incubated only with growth medium were used as a control. The result for DMSO concentration is 0.7% than that of non-cytotoxic at MDBK cells. For extract cytotoxicity assay, 1% *T. koraiensis* extract was diluted in 0.7% DMSO and in the same way as DMSO cytotoxicity assay to be experimented. CPE was observed and the maximum non-cytotoxic concentration of extract was determined.

### Antiviral activity test

The antiviral activity of *T. koraiensis* extract was also assayed in 96-well plates, since the initial dilution of the extract was done in 0.7% DMSO and the MNCC of extract in MDBK cells were used. The

**Table 1.** Antimicrobial activity of the Korean Arbor vitae (*Thuja koraiensis*) extract on several micro-organisms.

	Strain	Zone of inhibitory (mm)	
		*Control	Extract 100%
Gram(+)	<i>Bacillus subtilis</i> (ATCC11774)	10	13
	<i>Staphylococcus aureus</i> (KCCM1335)	30	17
Gram(-)	<i>Escherichia coli</i> (KCCM12181)	15	15
	<i>Salmonella typhimurium</i> (KCCM11862)	15	12

\*Control: Gram (+), Amoxicillin; Gram (-), Collistin Sulfate.

**Table 2.** Minimum inhibitory concentration (MIC) of the Korean Arbor vitae (*Thuja koraiensis*) extracts several micro-organisms.

	Microorganism	MIC (mg/ml) extract (%)
Gram (+)	<i>Staphylococcus aureus</i> (KCCM1335)	12.5
	<i>Bacillus subtilis</i> (ATCC11774)	0.6
Gram (-)	<i>Salmonella typhimurium</i> (KCCM11862)	12.5
	<i>Escherichia coli</i> (KCCM12181)	12.5

virus were serially diluted in MEMA containing 1% (v/v) anti-anti and the virus titer was determined at 10<sup>4</sup> TCID<sub>50</sub>/ml. 50 ul of BVD virus growth medium was placed in each well and 50 ul of extract at serial dilutions corresponding to MNCC were added. BVD virus suspension (10<sup>4</sup> TCID<sub>50</sub>/ml) of 50 ul was added in each well, incubated at 37°C and 5% CO<sub>2</sub> adsorbed in 90 min. MDBK cells (3.5 × 10<sup>3</sup> cells/well) of 100 ul were added in each well and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, and viral plaques were counted after 72 h. The BVD virus growth medium containing the MDBK cells was used as a positive control. The BVD virus growth medium contained the MDBK cells and virus was used as a negative control.

## RESULTS AND DISCUSSION

The antimicrobial activity of the *T. koraiensis* extract by paper disk diffusion assay against the test organisms are shown in Table 1. The extract inhibited Gram-positive bacteria *S. aureus* and *B. subtilis*, with diameters of inhibition zone 17 and 13 mm, Gram-negative bacteria *E. coli* and *S. typhimurium*, with diameters of inhibition zone 15 and 12 mm. Particularly, the antimicrobial effect of extract at the *B. subtilis* strain was higher than in the control. Table 2 shows the MIC values of the *T. koraiensis* extract against the test organisms. The MIC values ranged from 0.6 to 12.5% of the extract. The extract exhibited the highest activity against *B. subtilis*, with MIC of 0.6%.

The results showed that *T. koraiensis* extract has antimicrobial activity both at gram-positive and gram-

negative bacteria. At other studies, the growth of gram-positive bacteria was inhibited very effectively than gram-negative bacteria in a study using quercetin and naringenin which are single compounds in a phenolic compound (Rauha et al., 2000), while it had similar analysis results for the *T. koraiensis* extract single compound. But accordingly, the results of the *T. koraiensis* extract had a good antimicrobial effect at gram-positive bacteria in this study which may contain phenolic compound in the *T. koraiensis* extract. Further study was done analysing *T. koraiensis* antimicrobial effective single compound using high performance liquid chromatography (HPLC) profiling.

The antiviral activity of 1% *T. koraiensis* extract against BVD virus is shown in Table 3. The result showed that the MNCC of *T. koraiensis* extract on MDBK cells was 0.031% and the MNTD of extract was 0.0195% on BVD virus replication in MDBK cells. Especially, the extract at low concentrations had a strong antiviral effect at BVD virus; BVD virus is a RNA virus. *T. koraiensis* extract had antiviral effect at RNA virus. *T. koraiensis* extract, which anti-virus affects, will appear at DNA virus and needs to be more experimental in the future.

In summary, *T. koraiensis* extract had good antibacterial and antiviral effect. And we had first identified that Korean Arbor vitae (*Thuja koraiensis*) extract has both effect about antibacterial and antiviral. We suggest that the Korean Arbor vitae (*Thuja koraiensis*) extract will be useful as disinfectant for gram positive bacterial. Future

**Table 3.** Antiviral activity of Korean Arbor vitae (*Thuja koraiensis*) extracts 1% against BVD virus.

Solvent	*MNCC (%)	†MNTD (%)
Korean Arbor vitae ( <i>Thuja koraiensis</i> ) extract	0.031	0.0195
Control (P.C)	nag	nag
Control (N.C)	-	-

\*MNCC: maximum non cytotoxic concentration, †MNTD: maximum non-toxic dose, PC: Positive control, N.C: Negative control, 1% extract dissolved 0.7% DMSO

study, Korean Arbor vitae identify effective single compound about antibacterial and antiviral, also another single compound will confirm pharmacology function.

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## Conflict of Interests

The author(s) have not declared any conflict of interests.

## REFERENCES

- Aljos F (2005). A monograph of Cupressaceae and Sciadopitys. Surrey: Royal Botanic Gardens. "Flora of China" editorial board (1978). Flora of China. Science 7:318.
- Baker JC (1995). The clinical manifestations of bovine viral diarrhoea infection. Vet. Clin. North Am. Food Anim. Pract. 11:425-445.
- Bauer AW, Kirby WM, Sherris JC, Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45:493-496.
- Cassel E, Vargas R, Martinez N, Lorenzo D, Dellacassa E (2009). Steam distillation modeling for essential oil extraction process. Ind. Crops Prod. 29:171-176.
- Collett MS, Larson R, Belzer SK, Retzel E (1988). Proteins encoded by bovine viral diarrhoea virus: the genomic organization of a pestivirus. Virology 165:200-208.
- Dubey S, Batra A (2008). Antidiabetic activity of *Thuja occidentalis* Linn. Res. J. Pharm. Technol. 1:362.
- Guleria S, Kumar A, Tiku AK (2008). Chemical composition and fungitoxic activity of essential oil of *Thuja orientalis* L. grown in the North-western Himalaya. Z. Naturforsch. C. 63:211-214.
- Houe H (1999). Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet. Microb. 64:89-107.
- Jain RK, Garg SC (1997). Antimicrobial activity of the essential oil of *Thuja orientalis* L. Anim. Sci. Life 16:186-189.
- Jung O (2001). Quantitation of Virus. Korean J. Clin. Microbiol 4:1-4.
- Kim TH, Li H, Wu Q, Lee HJ, Ryu JH (2013). A new labdane diterpenoid with anti-inflammatory activity from *Thuja orientalis*. J. Ethnopharmacol. 146:760-767.
- Loizzo MR, Saab AM, Tundis R, Statti GA, Menichini F, Lampronti I, Gambari R, Cinatl J, Doerr HW (2008). Phytochemical analysis and in vitro antiviral activities of the essential oils of seven Lebanon species. Chem. Biodivers. 5:461-470.
- Rauha JP, Remes S, Heineken M, Hopia A, Kujala T, Pihlaja K, Vuorela H, Vuorela P (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. Int. J. Food Microbiol. 56(1):3-12.
- Shin S, Kim JH (2005). In vitro inhibitory activities of essential oils from two Korean Thymus species against antibiotic-resistant pathogens. Arch. Pharm. Res. 28:897-901.
- William D, Jackson A (1967). A handbook of *Coniferae and Ginkgoaceae*. New York: St. Martin's Press.

*Full Length Research Paper*

## Awareness of epilepsy in pharmacy students in different universities of Karachi, Pakistan

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The aim of this study was to determine knowledge and awareness regarding epilepsy among under graduate pharmacy students in Karachi, Pakistan. The questionnaire was distributed to the pharmacy students of 4th and 5th year in three different universities of Karachi, Pakistan from August to September, 2013 and the data was assessed using descriptive analysis. Five hundred questionnaires were distributed among the students and 467 were returned (93.4% response rate) after filling. Majority of the students were female (n = 380, 81.4%). It was found that all the students were aware about epilepsy (100%) while (71.9%) were aware of its types. It was also found that printed information was the main information source regarding epilepsy apart from class lectures. Overall responsiveness for most of the questions was in affirmative. The result of this study indicates a satisfactory level of awareness and knowledge of epilepsy among undergraduate pharmacy students in Karachi but it is necessary to advance understanding of the disease and its treatment among all the pharmacy students as they represent a well-informed fraction of society and healthcare system regarding the drug use.

**Key words:** Epilepsy, awareness, pharmacy students.

### INTRODUCTION

Epilepsy or recurring seizures is one of the most common and non-communicable brain disease world-wide (Scott et al., 2001). Patients with epilepsy also face stigma placed upon them by the people in addition to their disease which deprives patients of disclosing their condition and seeking proper treatment (World Health Organization (WHO), 1999). Knowledge and approach worldwide regarding epilepsy differs broadly but has improved very much than before ensuing more favorable social environment (Mirnics et al., 2001; Jacoby, 2002). Surveys are also conducted to raise the awareness of the extent of social recognition of epilepsy (Jacoby et al., 2004).

In the urban areas, civic stance towards epilepsy has very much enhanced over the years (Jacoby, 2002). Studies have reported that more educated people present more positive attitude and (Jensen et al., 1992; Chung et al., 1995) proper perception of epilepsy but fallacy still continues in some areas (Nyame et al., 1997). In countries like United States and Denmark, enhanced public knowledge and approach about epilepsy have been seen due to sturdy public education and understanding (Caveness and Gallup, 1980; Canger and Cornagsia, 1985; Jensen and Dam Me, 1992) whereas inadequate studies are offered in developing countries (Radhakrishnan et al., 2000; Gambhir et al., 1995). On

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the other hand, the awareness about epilepsy is revealed to be enhanced in a number of studies from developing as well as developed countries (Jensen and Dam M, 1992; Radhakrishnan et al., 2000; Cuong et al., 1995; Choi et al., 2004; Rwiza et al., 1993).

The pharmacist is a reliable and easy to get to health care professional. Community pharmacists present a significant role in educating patients about their drugs and diseases hence providing medication therapy management (Bluml, 2005). There is an optimistic impact of pharmaceutical care services provided in various clinical settings in developed countries; findings regarding developing countries are still deficient (Awad et al., 2006). The pharmacists are more seen as provider of pharmaceutical care service now (Lawrence et al., 2004) as they take direct responsibility for patients' drug related needs (Dugan, 2006; Pearson, 2007). The provision of pharmaceutical care in community pharmacy is still emerging in different parts of world like in Europe (Germany, Sweden), Canada and USA (Christensen and Farris, 2006; Hughes et al., 2010; Jones et al., 2005; Eickhoff, 2006; Westerlund and Bjork, 2006).

Awareness and stance among the general population regarding epilepsy differ extensively worldwide. In the urbanized areas, civic attitude towards epilepsy has significantly been enhanced, leading to more constructive social milieu (Mirnics et al., 2001; Jacoby, 2002). Studies have shown that primary care physicians desire to have community pharmacists to manage a whole medication report and screening for drug interactions for patients with epilepsy (McAuley et al., 1999; 2009). It was observed in England (Tinelli et al., 2007) after experiencing a pharmacy-led medication management service that patients had an attitudinal swing towards the pharmacists. In turn, this would enhance pharmacist job satisfaction as they dedicate more time in consultation and drug use management (Schommer et al., 2006).

Pharmacists should continue to be involved in the care of patients with epilepsy because it was observed that specialized sessions given by pharmacists increased caregiver's knowledge about epilepsy and medication adherence (Chen et al., 2013). Studies have shown that patient education is essential to develop compliance and to raise contentment levels in patients during critical therapies (Rafique et al., 2006; Saeed and Ibrahim, 2005) but unfortunately, this is hardly ever acknowledged by even major health care institutions (Rafique et al., 2006) in Pakistan. The burden of epilepsy on the whole is not fully calculated and valid information regarding this is stumpy (Khatri et al., 2003). There are about 50 million epilepsy patients known worldwide among which approximately 85% are existing in developing countries.

The healthcare professionals have found it alarming that Pakistan is having high prevalence of epilepsy (Aziz et al., 2005). According to WHO, approximately 50% of epilepsy cases start at youth and 70 to 80% could have normal lives after appropriate treatment (Aziz et al., 2005).

This necessitates the education and practice of pharmacists since it was found that treatment condition for epilepsy was pitiable with short rate of improvement in rural as compared with optimum therapeutic effect in urban persons receiving the treatment at same time (Patsalos et al., 1993). Hence, pharmacists have an important role for the patients with epilepsy providing best possible care to them (McAuley et al., 1999). Since pharmacists are involved in counseling and communication with the patients therefore the patients also preferred that their pharmacist correspond to their epileptologist about drug interactions and adverse effects (James et al., 2009). Carole Brown reported that there are complexities with diagnosis, counseling and prescribing of antiepileptic drugs and the participation of a pharmacist practitioner with a particular interest in epilepsy (PwSI epilepsy) within the neurology team can maximize the skill blend with general practitioners (Carole, 2012).

This study was conducted to assess the level of knowledge of pharmacy students of 4th and 5th year about epilepsy and its management as they are eminent part of the healthcare system. Their contribution as healthcare professional in the future regarding epilepsy can play a distinguished role in improving the quality of life for such patients.

## METHODOLOGY

The questionnaires were distributed to the pharmacy students of 4th and 5th year in three different universities of Karachi, Pakistan, conducting Pharm.D courses, from August to September, 2013. The students of higher classes were recruited for this study as they have been taught about the basics of the disease and its management in pharmacy curriculum up to satisfactory extent. The questionnaire comprised of a series of questions to assess the level of information and understanding of under graduate pharmacy students regarding epilepsy. Most of the questions were adapted from previous studies (Mielke et al., 1997; Kankirawatana, 1999; Rahman, 2005). It took about 15 minutes for each student to complete a single questionnaire and the questionnaires were returned to the researchers on the same day after filling. The data obtained was analyzed via descriptive statistics.

## RESULTS AND DISCUSSION

The analysis of students' awareness regarding disease and their management is essential as it permits the deterrence of inadequate information of future health care providers. Consequently, this may result in improved health support and quality of life of one suffering from various diseases. This study included questions on awareness of epilepsy. The respondents were of age group between 23 to 25 years and majority were female (n = 380, 81.4%). All the respondents had basic awareness of epilepsy while (71.9%) were aware of its kinds (Table 1). About one fourth of the students (21.8%) claimed that they have excellent awareness regarding epilepsy (Table 1). Books and leaflets were found as the

**Table 1.** Basic Knowledge Regarding Epilepsy

S/No	Question	Yes (%)	No (%)	Don't Know (%)
1	Do you know what epilepsy is?	467 (100)	0 (0)	-
2	Your awareness regarding epilepsy is			
	Excellent	102 (21.8)		
	Adequate	166 (35.5)	-	-
	Average	163 (34.9)		
3	Do you know someone suffering from epilepsy?	147 (31.5)	320 (68.5)	-
4	Have you ever seen someone having a seizure/fit?	282 (60.4)	185(39.6)	-
5	Do you know how to help a patient with epilepsy during the seizure?	220 (47.1)	247 (52.9)	-
6	Do you know how many types of seizures are there?	336 (71.9)	131 (28.1)	-
7	Do you think epilepsy is a contagious disease?	29 (6.2)	393 (84.2)	45 (9.6)
8	Do you think epilepsy is a chronic brain disease that cannot be cured or controlled?	127 (27.2)	314 (67.2)	26 (5.6)
9	Do you think all epileptic patients have the same symptoms?	96 (20.6)	316 (67.7)	55 (11.8)
10	Do you think all epileptics have to use an anti seizure drug?	265 (56.7)	134 (28.7)	68 (14.6)
11	Do you think people suffering from epilepsy need lifelong drug treatment?	339 (72.6)	72 (15.4)	56 (12)

as the major source of information (Figure 1) regarding epilepsy (n = 425, 91%) besides the lectures that are taught in their Pharmacy institutes according to the approved curriculum. Since university students symbolize a better-educated part of the population, therefore it is important that the students studying pharmacy in various universities should have the proper knowledge and suitable approach towards healthcare issues like epilepsy as the disease has considerable social implications. Earlier reports have also shown that there is a sturdy association between the age, level of education, etc towards epilepsy (Caveness and Gallup, 1980; Kim et al., 1994).

Among the respondents, (31.5%) knew some epilepsy patients personally and (60.4%) had witnessed the onset of an epileptic attack. Majority of the students (84.2%) were aware that epilepsy

is not contagious and (67.7%) students had knowledge that patients with epilepsy show different signs and symptoms (Table 1). Almost half of the respondents (47.1%) claimed that they knew how to help patients with epilepsy during attack and (67.2%) students knew that epilepsy is not chronic/uncontrollable (Table 1). The students were asked to choose main manifestation of epilepsy and according to (60.4%) students it was convulsions (Figure 2). Convulsions were found as the most common cause of mortality in epilepsy patients (Mac and Tran, 2007).

The results of the study have shown that (73.8%) students knew that treatment of epilepsy (Figure 3) is mainly medication. More than half (56.7%) of the students thought that all patients with epilepsy should use medication and most of them (72.6%) emphasized on treatment for extended time period (Table 1). Antiepileptic agents

can successfully manage epilepsy (World Health Organization, 2003) hence improving quality of life in patients with epilepsy. It has been observed that many patients with epilepsy have inadequate seizure control due to poor medication compliance (Jones and Butler, 2006). To help end a seizure, some respondents thought that eau de cologne (1.9%), shoe smell (47.3%) or onions (7.3%) would be helpful.

### Conclusion

The Pharmacy students stand for a better-educated group in the healthcare system as they have more knowledge regarding the use of drugs, hence it is essential for them to clasp proper information of the disease and drug use. There is a need to improve certain aspects of knowledge and

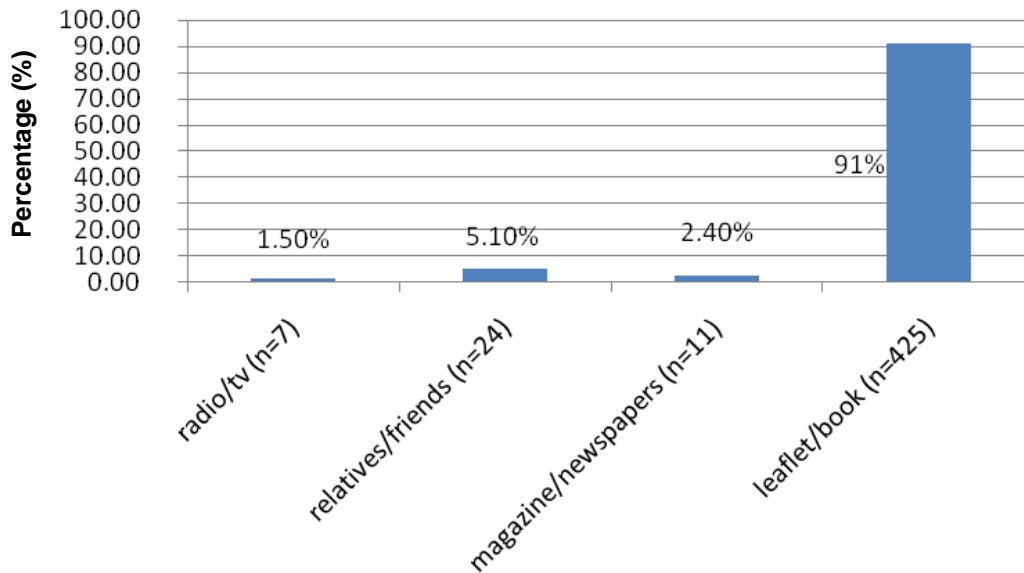


Figure 1. Source of knowledge about epilepsy.

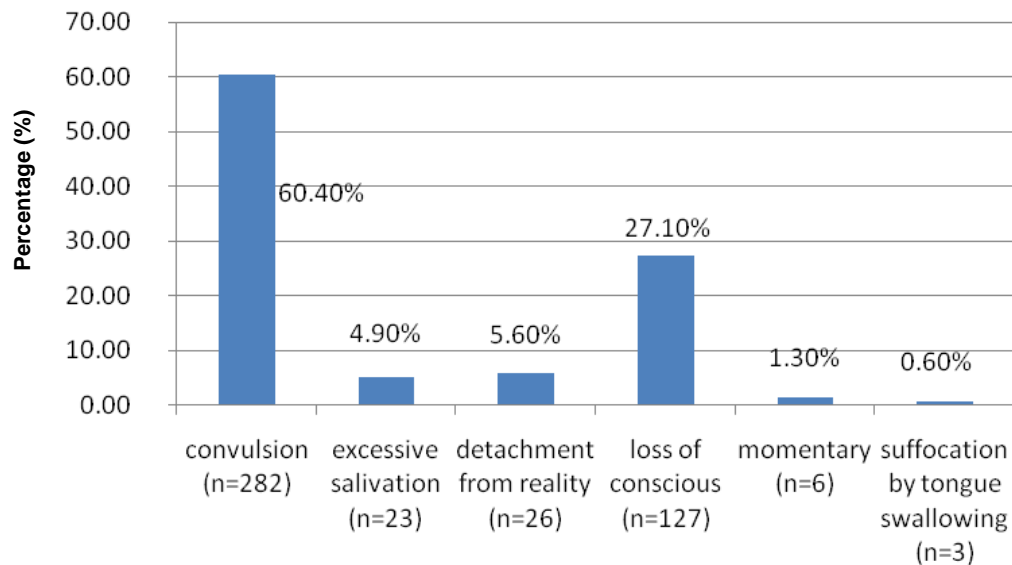


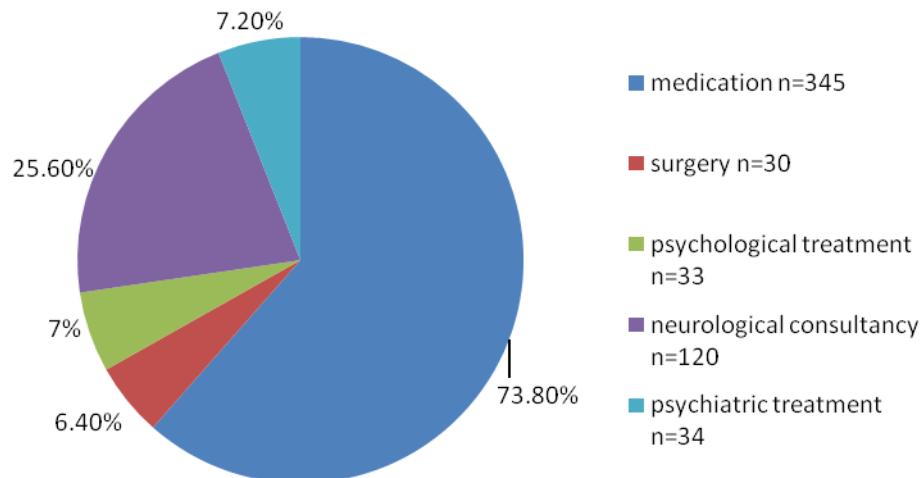
Figure 2. Manifestations of epilepsy.

understanding of epilepsy among pharmacy students especially for those selecting their career as clinical pharmacist. Besides, well-organized educational programs are required to develop proper public awareness about epilepsy.

**STUDY LIMITATIONS AND SUGGESTIONS**

This study covered only few universities in Karachi conducting pharmacy curriculum so in future studies, more

more pharmacy schools could be enrolled, even outside Karachi so as to have a comparison of knowledge among the students and identifying areas for improvement. Public knowledge and attitude towards epilepsy should also be assessed and students should be taught in the universities accordingly. The organizations like International League Against Epilepsy (ILAE) can play an important role in advancing and spreading knowledge about epilepsy. Such kind of organizations should also be founded in our country to promote research, education and training regarding epilepsy.



**Figure 3.** Treatment of epilepsy.

### Conflict of Interests

The author(s) declared that they do not have any conflict of interests.

### REFERENCES

- Awad A, Al-Ebrahim S, Abahussain E (2006). Pharmaceutical care services in hospitals of Kuwait. *J. Pharm. Sci.* 9(2):149-157.
- Aziz H, Ali SM, Frances P, Khan MI, Hasan KZ (1994). Epilepsy in Pakistan: a population based epidemiologic study. *Epilepsia* 35(5):950-958.
- Bluml BM (2005). Definition of medication therapy management: development of profession wide consensus. *J. Am. Pharm. Assoc.* 45:566-572.
- Canger R, Cornagsia C (1985). Public attitudes toward epilepsy in Italy: results of a survey and comparison with USA and west hormones data. *Epilepsia* 26:221-6.
- Carole B (2012). Improving quality of care for epilepsy patients using a pharmacist review service. *Progress Neurol. Psychiatr.* 16(5):12-16.
- Caveness WF, Gallup GH (1980). A survey of public attitudes towards epilepsy in 1979 with an indication of trends over the past thirty years. *Epilepsy* 21:509-18.
- Chen C, Lee DS, Hie SL (2013). The impact of pharmacist's counseling on pediatric patients' caregiver's knowledge on epilepsy and its treatment in a tertiary hospital. *Int. J. Clin. Pharm.* 35(5):829-34.
- Choi KS, Park KA, Lee HJ, Park MS, Lee CH, Cheon SE (2004). Familiarity with knowledge of and attitude toward epilepsy in residents of Seoul, South Korea. *Acta Neurol. Scand.* 110:39-45.
- Christensen DB, Farris KB (2006). Pharmaceutical care in community pharmacies practice and research in US. *Ann. Pharmacother.* 40(7-8):1400-1406.
- Chung MY, Chang YC, Lai YHC, Lai CW (1995). Survey of public awareness, understanding, and attitudes toward epilepsy in Taiwan. *Epilepsia* 36:488-93.
- Cuong LQ, Thien DD, Jallon P (2006). Survey of public awareness, attitudes, and understanding toward epilepsy in Nhan Chinh, Hanoi, Vietnam, in 2003. *Epilepsy Behav.* 8:176-180.
- Dugan BA (2006). Enhancing Community pharmacy through advanced pharmacy practice experiences. *Am. J. Pharm. Edu.* 70(1):21.
- Eickhoff C (2006). Pharmaceutical Care in Community Pharmacies Practice and Research in Germany. *Ann. Pharmacother.* 40(4):729-735.
- Gambhir SK, Kumar V, Singhi PD, Goel RC (1995). Public awareness, understanding and attitudes toward epilepsy. *Indian J. Med. Res.* 102:34-38.
- Hughes CM, Hawwa AF, Scullin C, Anderson C, Hersberger KE (2010). Provision of pharmaceutical care by community pharmacists: a comparison across Europe. *Pharm. World Sci.* 32(4):472-487.
- Jacoby A (2002). Stigma, epilepsy, and quality of life. *Epilepsy Behav.* 3:10-20.
- Jacoby A, Gorry J, Gamble C, Baker GA (2004). Public knowledge, private grief: a study of public attitudes to epilepsy in the United Kingdom and implications for stigma. *Epilepsia* 45: 1405-1415.
- Jensen R, Dam M (1992). Public attitudes toward epilepsy in Denmark. *Epilepsia* 33:459-463.
- Jones EJ, Mackinnon NJ, Tsuyuki RT (2005). Pharmaceutical care in community pharmacies practice and research in Canada. *Ann. Pharmacother.* 39(9):1527-1533.
- Jones RM, Butler JA (2006). Adherence to treatment in patients with epilepsy: associations with seizure control and illness beliefs. *Seizure* 15(7):504-508.
- Khatri IA, Iannaccone ST, Ilyas MS, Abdullah M, Saleem S (2003). Epidemiology of Epilepsy in Pakistan: review of literature. *J. Pak. Med. Assoc.* 53(12):594-7.
- Kim MK, Cho KH, Shin J, Kim SJ (1994). A study of public attitudes towards epilepsy in Kwang-Juarea. *J. Kor. Neurol. Assoc.* 12:410-27.
- Lawrence L, Sherman J, Adams E, Gandra S (2004). Pharmacy students' perceptions of pharmaceutical care in retail and clinic settings. *Am. J. Pharm. Educ.* 68(1):4.
- Mac TL, Tran DS (2007). Epidemiology, aetiology, and clinical management of epilepsy in Asia: a systematic review. *Lancet Neurol.* 6(6):533-543.
- McAuley James W, Miller MA, Klatte E, Shneker BF (2009). Patients with epilepsy's perception on community pharmacist's current and potential role in their care. *Epilepsy Behav.* 14:141-145.
- McAuley JW, Mott DA, Schommer JC, Moore JL, Reeves AL (1999). Assessing the needs of community pharmacists and primary care physicians in providing care to patients with epilepsy. *J. Am. Pharm. Assoc.* 39:499-504.
- Mirnic Z, Czikota G, Zavec T, Halasz P (2001). Changes in public attitudes toward epilepsy in Hungary: results of surveys conducted in 1994 and 2000. *Epilepsia* 42:86-93.
- Nyame PK, Biritwum RB (1997). Epilepsy: knowledge, attitude and practice in literate urban population, Accra, Ghana. *West Afr. J. Med.* 16:139-1345.
- Patsalos PN, Duncan JS (1993). Drug Safety. *Int. J. Med. Toxicol. Drug Exp.* 9(3):156-84.



- Pearson GJ (2007). Evolution in the practice of pharmacy-not a revolution. *CMAJ* 176(9):1295-1296.
- Radhakrishnan K, Pandian JD, Santoshkumar T, Thomas SV, Deetha TD, Sarma PS (2000). Prevalence, knowledge, attitude and practice of epilepsy in Kerala, South India. *Epilepsia* 41:1027-1035.
- Rafique G, Azam SI, White F (2006). Diabetes knowledge, beliefs and practices among people with diabetes attending a university hospital in Karachi, Pakistan. *East Mediterr. Health J.* 12:590-8.
- Rwiza HT, Matumja WBP, Kilonzo GP, Hawe J, Mbera P, Mwangombola R (1993). Knowledge attitude and practice towards epilepsy among rural Tanzanian residents. *Epilepsia* 34:1017-1023.
- Saeed A, Ibrahim H (2005). Reasons for the Problems faced by Patients in Government Hospitals: results of a survey in a government hospital in Karachi, Pakistan. *J. Pak. Med. Assoc.* 55:45-7.
- Schommer JC, Pedersen CA, Gaither CA, Doucette WR, Kreling DH, Mott DA (2006). Pharmacists' desired and actual times in work activities: evidence of gaps from the 2004 National Pharmacist Workforce Study. *J. Am. Pharm. Assoc.* 46:340-7.
- Scott RA, Lhatto SP, Sander JWAS (2001). The treatment of epilepsy in developing countries and its problems: where do we go from here?. *Bull. World Health Organ.* 79(4):344-51.
- Tinelli M, Bond C, Blenkinsopp A, Jaffray M, Watson M, Hannaford P (2007). Patient evaluation of a community pharmacy medications management service. *Ann. Pharmacother.* 41:1962-70.
- Westerlund LT, Bjork HT (2006). Pharmaceutical care in community pharmacies practice and research in Sweden. *Ann. Pharmacother.* 40(6):1162-1169.
- World Health Organization (WHO) (1999). Fact sheet 217: The global burden of mental and neurological problems. Geneva, WHO. p. 99.
- World Health Organization (WHO) (2003). Adherence to long-term therapy. WHO, Geneva. P 211.

Full Length Research Paper

## Antiviral, antimicrobial and anti-inflammatory activities of *Ureia baccifera* (L.) Gaudich

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*Ureia baccifera* (Urticaceae) is a shrub used in folk medicine for rheumatic diseases and mycosis. This study aimed to test the antiviral, antinociceptive, anti-inflammatory and antimicrobial activities of *U. baccifera* leaves and roots. The cytotoxic and antiviral activity was evaluated against Herpes virus type 1, *in vitro*. The antimicrobial activity was determined by the broth microdilution method based on Clinical and Laboratory Standards Institute (CLSI) M27-A3, M38-A2 and M7-A6 standardized reference method. *In vivo* anti-inflammatory and antinociceptive activity were evaluated in ear edema measurement, complete Freund's adjuvant-induced nociception, measurement of cold allodynia and spontaneous nociception in male adult Swiss mice. *U. baccifera* roots and leaves presented anti-herpetic activity. The plant extract, predominantly the ethyl acetate and butanol fractions, also inhibit *Klebsiella pneumoniae*, *Prototheca zopfii* and *Saccharomyces cerevisiae* and did not show antinociceptive and anti-inflammatory effect on the tested experiments. The presence of biologically active products and the low cytotoxicity demonstrated by the extract and fractions of *U. baccifera* makes these extracts promising antiviral candidates. This nettle can also be considered a moderated antimicrobial agent against *K. pneumoniae*, *P. zopfii* and *S. cerevisiae*. However, antinociceptive and anti-inflammatory effects to the samples in this study were not observed.

**Key words:** Stinging nettle, herpes simplex type 1, Urticaceae, *Klebsiella pneumonia*, *Prototheca zopfii*, *Saccharomyces cerevisiae*.

### INTRODUCTION

*Ureia baccifera* (L.) Gaudich Ex. Wedd is a shrub with one meter and a half of height, having many stinging trichomes on the stem and leaves. The stinging hairs of

the plant gave it its folk names as nettles in English and ortigas in Spanish. This shrub is popularly used for inflammatory diseases, where leaves or roots are prepared

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by infusion to oral or topic use (Badilla et al., 1999a), and the rubefacient effect is also used for rheumatic pains and arthritis (Badilla et al., 1999a; Valadeau et al., 2009). By topic use, this plant is also used for dermal diseases. There are no references concerning the compounds of *U. baccifera* in the literature.

Herpes simplex type 1 is a pathogen of children and adults responsible for several disorders, including gingivostomatitis, pharyngitis, keratoconjunctivitis and encephalitis (Whitley, 2001). Despite the symptoms caused by herpes infections are self-limiting in healthy individuals, these can be extensive and prolonged in immunocompromised patients (Chen et al., 2000). Nucleoside analogues such as acyclovir and penciclovir are some drugs licensed for the treatment of Herpes simplex virus (Whitley, 2001). However, these agents may cause a variety of toxic side effects, and the emergence of viral strains resistant to these compounds have become a growing problem, especially in immunocompromised patients (Van De Perre et al., 2008). Thus, there is an increasing need for the discovery of more specific antiviral agents effective against the herpes simplex virus.

Natural products like plant extracts are very promising sources of compounds with antiviral activity, due to the very low toxicity that they show for cells, and also due to the great variety of chemical constituents they have (Jassin and Najj, 2003). Fractions obtained from aerial parts of *U. baccifera* showed a significant activity against HSV-1-ACVr (Martins et al., 2009). Moreover, studies of other species of Urticaceae also have demonstrated anti-HSV activity (Silva et al., 2010; Uncini et al., 2005).

The anti-inflammatory (Badilla et al., 1999a, b) tests already been performed with the aerial parts of *U. baccifera* obtained good results in the dosage of 25 to 100 and 500 mg/kg intraperitoneally administrated. Besides, *U. baccifera* aqueous extract in 250 and 500 mg/kg was capable of reducing the edema caused in mice by a snake venom intraperitoneally (Badilla et al., 2006). The roots and leaves of this nettle showed antimicrobial effect against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Onofre and Herkert, 2012), although the metanolic extract of leaves did not show effect against *E. coli* and *S. aureus* in other studies (Melendez and Capriles, 2006). Therefore, the aim of this study was a screening of cytotoxicity and antiviral activity against HSV-1, *in vitro*, besides that to test the antiviral, anti-inflammatory and antimicrobial activities of crude extract and chloroformic, ethyl acetate and butanol fractions from the roots and leaves of *U. baccifera*.

## METHODS AND MATERIALS

### Preparation of the sample extracts and fractions

The leaves and the roots of *U. baccifera* were collected in São

Francisco de Assis, RS, Brazil (S 29° 37.115' W 054° 53. 970'; precision: 2.5 m; altitude 150 m) in May, 2010. Prof. Dr. Renato Aquino Záchia identified the plant and a voucher deposited on the University Herbarium under number 13.070. The material (1,000 g of roots and 400 g of leaves) was dried, milled and taken to maceration with ethanol 70% (30 g of plant in 100 ml solvent), for 28 days, with daily agitation. Once every week, each extract were filtrated, giving the respective hydro-ethanolic extract and fresh extraction solvents were added. The extracts were pooled and the ethanol was eliminated on rotary evaporator, giving the aqueous extracts (roots and leaves separately). Part of the aqueous extract was totally dried, becoming the crude extract (CE), and part was fractionated with solvents of crescent polarity: chloroform (CHCl<sub>3</sub>), ethyl acetate (EtOAc) and butanol (BuOH). In the fractionation process, to each 400 ml of aqueous extract, 200 ml of solvent were added, which was renovated many times until the exhaustion of the extract, when the next solvent is added in crescent order of polarity. The fractions were equally dried. The income of the leaves and roots were, respectively: CE: 31.5 and 22.3%; CHCl<sub>3</sub>: 2.2 and 0.54%; EtOAc: 1.1 and 0.3%; BuOH: 2.1 and 1.8%.

### Animals

Male adult Swiss mice (25 to 35 g), 56 animals, maintained in home cages under a 12:12 h light-dark cycle (lights on 06:00 h) and constant room temperature (22 ± 2°C) were used. The animals were acclimatized in the laboratory for at least 1 h before testing. The experiments were performed with the agreement of Ethics Committee of the Federal University of Santa Maria (Protocol number 67/2010) and were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experiments in conscious animals (Zimmermann, 1983). The number of animals and intensity of noxious stimuli were the minimum necessary to demonstrate consistent effects of drug treatments.

### Cells and viruses

HEp-2 cells were maintained in minimum essential medium (MEM – GIBCO Invitrogen Corporation, Grand Island, NY, USA) containing 10% fetal bovine serum (SFB – GIBCO Invitrogen Corporation, Grand Island, NY, USA), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). For the cytotoxicity and antiviral activity tests, cell cultures were prepared in 96 well plates and incubated at 37°C and 5% CO<sub>2</sub>. The strain KOS of HSV-1 was kindly provided by Dr. Paulo Roehe from the Federal University of Rio Grande do Sul (UFRGS). The virus stocks were prepared and titrated as previously described (Simões et al., 1999), and the aliquots were kept at -70°C.

### Cytotoxicity assay

The cytotoxic and antiviral tests were performed through the MTT colorimetric assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] according to Freitas et al. (2009), with minor modifications. HEp-2 cells were seeded into 96-well plates (2 × 10<sup>4</sup> cells/well) and incubated for 24 h at 37°C. After that, the minimum essential medium with 10% of fetal bovine serum and 200 µl/well of different concentrations of the crude extracts and fractions (1.95 to 250 µg/ml) was added onto subconfluent cells in six replicates for each concentration of the samples. Control wells without the extracts, containing only the culture medium, and the culture medium plus 1% ethanol were included. After 3 days of incubation

at 37°C and 5% CO<sub>2</sub>, the growth medium was removed and 50 µl of MTT solution (1 mg/ml) was added. The plates were re-incubated for 4 h and after removal of the MTT solution, dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals (100 µl/well). The supernatant was transferred to a new plate and the readings performed in the enzyme linked immunosorbent assay (ELISA) reader at a wavelength of 540 nm. The viable cells percentage for each compound was calculated according to the formula: absorbance of the compound/absorbance of the cell control × 100%. The cytotoxic concentration of the sample that reduced the viable cell number by 50% (CC<sub>50</sub>) was determined from dose-response curves. Additionally the maximum noncytotoxic concentration (MNCC) of each compound was determined for subsequent use in antiviral tests (Freitas et al., 2009).

#### Antiviral evaluation

Briefly, 100 µl/well of MNCC of the compounds were added to preformed monolayers of HEp-2 cells and diluted 1:2 in 96 well plates. After that, a suspension of 100 µl/well containing 10<sup>4</sup> TCID<sub>50</sub>/ml of virus was added to all the wells with the exception of the cell control. Control virus wells were kept without addition of the compound. After 72 h of incubation at 37°C and 5%, MTT was added following the procedure described. Acyclovir (10 µg/ml) was used as positive control for HSV-1 inhibition. The inhibition for each compound was calculated according to the formula: (compound absorbance – viral control absorbance) / (cell control absorbance – viral control absorbance) × 100%. The 50% inhibitory concentration (IC<sub>50</sub>) was defined as the concentration that inhibited 50% of viral replication when compared to virus controls and was determined from dose-response curves. The selectivity index (SI) was calculated as CC<sub>50</sub>/IC<sub>50</sub> (Freitas et al., 2009).

#### Antimicrobial activity

The crude extract and the chloroform, ethyl acetate and butanol fractions were individually evaluated against *Aspergillus flavus* (clinical isolate), *Candida parapsilosis* (American Type Culture Collection - ATCC 90018), *Candida tropicalis* (ATCC 750), *Candida glabrata* (ATCC 2301), *Candida dubliniensis* (clinical isolate), *Candida albicans* (ATCC 28967), *Saccharomyces cerevisiae* (ATCC 2601), *Cryptococcus neoformans* (ATCC 2857), *Cryptococcus gattii* (ATCC 56990), *Malassezia pachy* (clinical isolate), *Prototheca zopfii* (clinical isolate), *Micrococcus* sp. (ATCC 7468), *Proteus mirabilis* (ATCC 7002), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Aeromonas* sp. (clinical isolate), *Enterococcus faecalis* (ATCC 51299), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus agalactiae* (clinical isolate) and *Escherichia coli* (clinical isolate). The minimal inhibitory concentration (MIC) of each fraction against the tested microorganisms was determined by the broth microdilution method based on CLSI M27-A3, M38-A2 and M7-A6 standardized reference method. The experiments were performed in duplicate. Each extract and fraction was serially diluted in DMSO to generate stock solutions. After that, it was serially diluted in RPMI 1640 broth for fungi and Brain Heart Infusion (BHI) broth for bacteria, to obtain final concentrations ranging from 2000 to 15.625 µg/ml. The inoculums of bacteria were prepared from 24 h-culture in BHI agar; filamentous fungi from 7-day-culture in potato dextrose agar; yeasts from 48-h-culture in Sabouraud dextrose agar. After 24 h of incubation at 37°C for bacteria and 48 h at 30°C for fungi, MIC endpoint was considered the lowest concentration of extract and fraction that inhibited 100% growth. The results of the samples

were compared to the standard antibiotic amphotericin B.

#### In vivo anti-inflammatory and antinociceptive activity

Only the crude extracts of leaves and roots of *U. baccifera* were used in the *in vivo* anti-inflammatory and antinociceptive assays.

#### Ear edema measurement

Edema was expressed as an increase in ear thickness, which was measured before and after induction of the inflammatory response, using a digital micrometer (Starret Series 734) in animals anesthetized with isoflurane. The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges. The thickness was recorded in µm. To minimize variation, a single investigator performed the measurements throughout each experiment. The root (10 to 10000 µg/ear) or leaf (1000 to 10000 µg/ear) of *U. baccifera* or vehicle (acetone) was applied to the right ear by topical administration according to the method described previously, with some modifications (Silva et al., 2011).

#### Nociceptive parameters

##### CFA- induced nociception

To produce a nociceptive response, mice were lightly anaesthetized with 2% halothane via a nose cone and received 20 µl of complete Freund's adjuvant [CFA-1 mg/ml of heat killed *Mycobacterium tuberculosis* in paraffin oil (85%) and mannide monooleate (15%)] subcutaneously on the plantar surface (intraplantar, i.pl.) of the right hind paw (Ferreira et al., 2001). To evaluate the nociceptive response, 48 h after CFA administration, the animals received oral administration of leaf and root extract of *U. baccifera* (100 mg/kg) or saline (10 ml/kg) used as vehicle.

##### Measurement of cold allodynia

Cold allodynia was used as a parameter of nociception, which was characterized by a nocifensive reaction of animals after evaporative cooling of topically applied acetone (Caspani and Heppenstall, 2009). For this, 20 µl of acetone was applied on the dorsal hind paw, ipsilateral to the injury, and the behavior was assigned as an arbitrary score. The nociceptive score was assigned as follows: 0 = no behavioral response, 0.5 = licking response, 1 = flinching and brushing of the paw, 2 = strong flinching, 3 = strong flinching and licking. Behavior was observed during the first 30 s after acetone application and evaluated before (basal) and several times after treatments or CFA injection.

##### Spontaneous nociception

Briefly, the animals that received previous injection of CFA were placed individually in chambers (transparent glass cylinders of 20 cm in diameter) for an adaptation period before treatment, and spontaneous nociception induced by only CFA was observed as described. The behavior of spontaneous nociception was observed during 30 s before (basal) and at 0.5, 1 and 2 h after treatment with leaf and root extract of *U. baccifera* (100 mg/kg) or saline solution (10 ml/kg).

**Table 1.** Results of cytotoxicity and antiviral activity of the roots and leaves.

Parameter	CC <sub>50</sub> µg/ml (±SD)	IC <sub>50</sub> µg/ml (±SD)	SI (CC <sub>50</sub> /IC <sub>50</sub> )	
Roots	CE	>1083.32±102.34	27.39±4.52	39.55
	EtOAc	283.54±10.93	8.08±2.37	35.09
	CHCl <sub>3</sub>	131.36±8.43	9.23±3.58	14.23
	BuOH	>385.55±9.85	25.97±3.26	14.85
Leaves	CE	162.04±6.79	17.97±2.54	9.02
	EtOAc	176.04±8.54	25.30±4.69	6.96
	CHCl <sub>3</sub>	98.72±6.48	10.59±3.22	9.32
	BuOH	181.63±6.81	6.21±2.41	29.25
ACV (10 µg/ml)	>138.23	1.25±0.32	>110.58	

CE: Crude extract; CHCl<sub>3</sub>: Chloroform fraction; EtOAc: Ethyl acetate fraction; BuOH: butanol fraction; ACV: acyclovir.

### Statistical analysis

Statistical analysis was carried out by Student's t-test or one-way or two-way analysis of variance (ANOVA) followed by Student-Newman-Keuls' or Bonferroni post tests when appropriate, using GraphPad Software 4.0 (GraphPad, USA).

## RESULTS

### Antiviral activity

The results of the cytotoxicity and viral inhibition tests are shown in Table 1. *U. baccifera* best results was obtained with the CE and EtOAc fraction of the roots, and the BuOH fractions of the leaves. The BuOH fraction of the roots showed very low toxicity to the cells, however its IC<sub>50</sub> was high, implying a lower SI as compared to the fractions already cited. The cytotoxicity of this fraction was even lower than the cytotoxicity of the ACV. Although the remaining fractions of both the roots and the leaves showed higher cytotoxicity, they also demonstrated antiviral activity (Table 1). The cytotoxicity in this method can be evaluated by the CC<sub>50</sub> (cytotoxic effects on 50% of cultured cells) value, such that the lower it gets, the more toxicity the extract brings to the cells. Thus, the CE of roots can be considered a promising candidate of an antiviral phytochemical, due its high CC<sub>50</sub> (>1083.32 ± 102.34) and moderate IC<sub>50</sub> (27.39 ± 4.52) which resulted in the higher SI of the samples (39.55).

### Antimicrobial activity

The large amount of microorganisms tested in this study have the finality of making a screening analysis, to verify the possible action of *U. baccifera* extracts against a specific microbe. The serial dilution has the objective of evaluation, which is the smaller concentration that

was effective against the microorganisms. Usually, quantitative MIC determination is well accepted for proper evaluation of the antimicrobial activity of a plant extract (being the MIC of a plant extract below 1000 µg/ml) considered as significant (Rios and Recio, 2005). Therefore, only the samples that showed minimal inhibitory concentration (MIC) lower or equal to 1000 µg/ml for most of the microorganisms were listed on Table 2. The fractions obtained from the leaves were the only samples that have action against *K. pneumoniae*. EtOAc of the roots was the only sample that inhibited *S. cerevisiae*. However, to the alga *P. zopfii*, the fractions BuOH and EtOAc from both parts of the plant presented a MIC value considered significant, similarly to the roots CE.

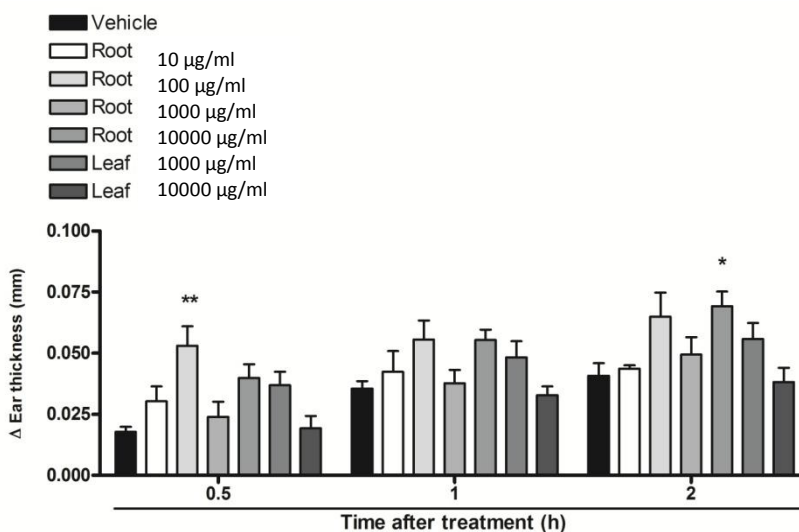
### Anti-inflammatory activity

On the ear edema evaluation, a graphic with time-course and dose-response curves of mouse ear edema induced by topical application of the plant was obtained (Figure 1). The data show that there is no occurrence of edematogenic effect, suggesting that *U. baccifera* leaves and roots does not have irritating effect. In the CFA-induced nociception test, it was observed that the roots and the leaves of the plant was not capable to reduce the paw withdrawal threshold induced by CFA at all time tested (data not shown). Therefore, *U. baccifera* did not present antinociceptive effect in this model of inflammatory pain. The antinociceptive pain against allodynia induced by acetone is shown in the Figure 2. The plant extract was not capable to revert the nociception induced by acetone on the animals who received CFA. In the spontaneous nociception experiment, the animals did not present spontaneous pain (Figure 3). The test shown that the crude extracts of the plant did not decrease the spontaneous pain of the mice in comparison

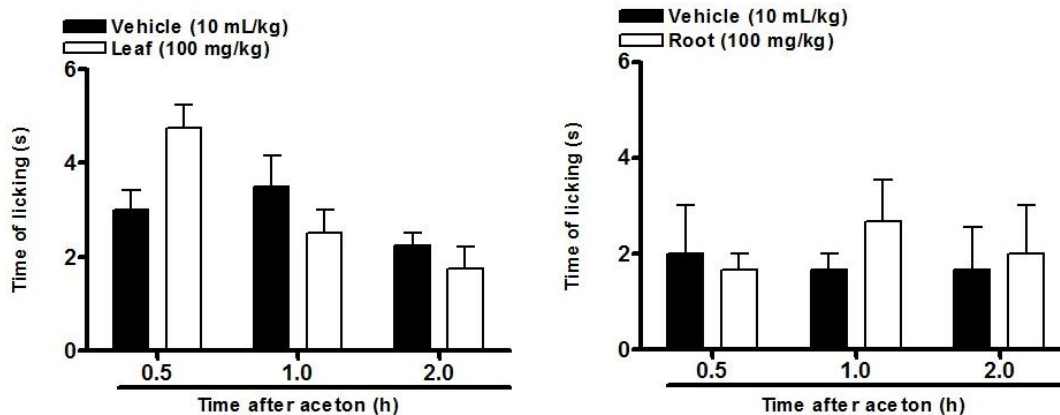
**Table 2.** Results of antimicrobial activity.

Parameter	CE (µg/ml)	CHCl <sub>3</sub> (µg/ml)	EtOAc (µg/ml)	BuOH (µg/ml)
<b>Leaf</b>				
<i>S. cerevisiae</i>	>1000	>1000	>1000	>1000
<i>P. zopfii</i>	>1000	>1000	250	250
<i>K. pneumoniae</i>	>1000	312	312	156
<b>Root</b>				
<i>S. cerevisiae</i>	>1000	>1000	250	>1000
<i>P. zopfii</i>	500	>1000	500	250
<i>K. pneumoniae</i>	>1000	>1000	>1000	>1000

CE: Crude extract; CHCl<sub>3</sub>: Chloroform fraction; EtOAc: Ethyl acetate fraction; BuOH: butanol fraction.



**Figure 1.** Time-course and dose-response curves of mouse ear edema induced by topical application of root and leaf of the *Uretra baccifera*. The effects of these extracts are expressed as a change in ear thickness. Each column represents the mean ± S.E.M. for 6 animals. \*P < 0.05 and \*\*P < 0.01 vs. vehicle.



**Figure 2.** Evaluation of antinociceptive pain against cold allodynia induced by acetone.

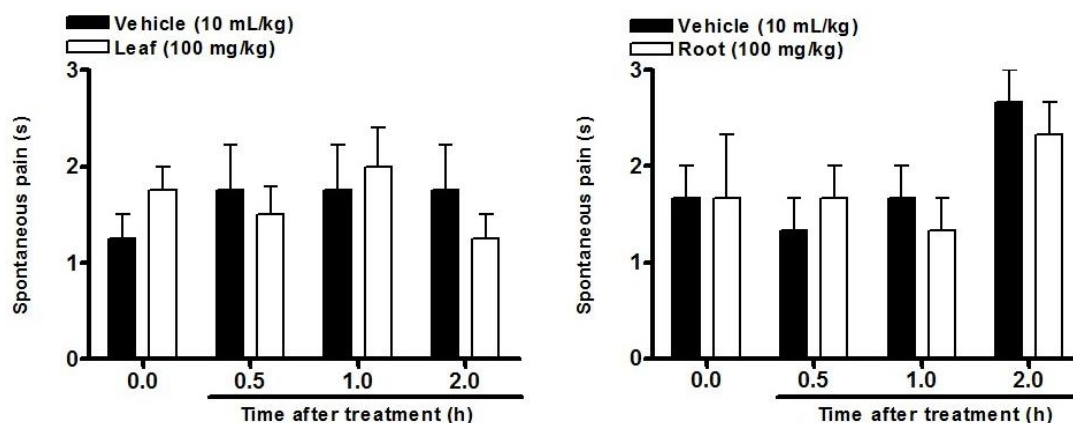


Figure 3. Evaluation of spontaneous pain.

with the vehicle.

## DISCUSSION

### Antiviral activity

The MTT method allows the evaluation of cytotoxicity of the samples in parallel with their antiviral activity, what is particularly important, since cytotoxic compounds sometimes behave as if they were active (Takeuchi et al., 1991). All samples tested showed anti-herpetic activity according to the parameters proposed by Amoros et al. (1992) and Cos et al. (2006) in order to consider a product as a potential antiviral candidate. *U. baccifera* is a plant classified in the Urticaceae family. The antiviral activity of other Urticaceae species has been confirmed in several studies (Silva et al., 2010; Uncini et al., 2005). The crude extract and the fractions methanol and butanol of *Cecropia glaziovii* Sneth leaves showed high activity against HSV-1 and HSV-2 (Silva et al., 2010). In addition, another study has shown that the species *Urtica dioica* was very effective to inhibit the syncytium formation by the feline immunodeficiency virus (FIV), which is regarded as a model for HIV (Uncini et al., 2005).

The plants have a wide variety and complexity of chemicals that are responsible for various biological effects, such as flavonoids, terpenoids, lignans, polyphenols, coumarins, saponins, alkaloids, thiophenes, proteins, peptides, and others (Jassin and Naji, 2003). The presence of phenolic compounds and flavonoids was previously confirmed in qualitative and quantitative tests for the roots and leaves of *U. baccifera* (Mannion and Menezes, 2010). The samples tested in this study have their content analyzed in a previous work (Gindri et al., 2014). The results showed  $29.98 \pm 1.27$ ,  $35.54 \pm 0.45$  and  $49.05 \pm 0.88$  mg/g of poly-phenols and  $16.13 \pm 0.22$ ,  $15.38 \pm 0.75$  and  $26.61 \pm 0.25$  of flavonoids, to the CE

CE and EtOAc of the roots, and the BuOH of the leaves, respectively. The phenolic compounds (Hudson, 1990; Sakagami et al., 1995; Van Den Berghe et al., 1986), particularly the flavones and flavonoids (Amoros et al., 1992), have been reported as having antiviral activity.

The proposed mechanisms by which the plant extracts and its compounds act on the viruses are variable, however several studies has shown that polyphenols act before virus entry, directly on the virus particle or avoiding the virus binding to the cells (Hudson, 1990; Sakagami et al., 1995; Van Den Berghe et al., 1986).

According to Hudson (1990), the viral inactivation is due to the *in vitro* binding of the polyphenols to the virus coat proteins. There are also studies showing that the antiviral activities of many natural products may occur by polyphenols binding either to the virus or to the proteins of the host cell membrane, thus preventing viral adsorption (Van Den Berghe et al., 1986). This view is reinforced by studies performed by Sakagami et al. (1995) which suggests that polyphenols act through direct inactivation of the virus and/or inhibition of the binding of the virus to the cells. Thus, the polyphenols may have an important role in the antiviral activity of the extracts of *U. baccifera*, although further studies are necessary to identify the mechanisms.

Furthermore, in our conditions, the BuOH fraction of the leaves of *U. baccifera* showed high antiviral activity against HSV-1, differently from the other fractions of the leaves (Table 1). Similar results have been observed by Martins et al. (2009) who reported that the butanol fractions of the aerial parts of *U. baccifera* showed high antiviral activity against HSV-1- ACVr (99.4%) *in vitro*, although in the same study the extracts did not show expressive percentage of viral inhibition against HSV-2- ACVr. It is important to highlight that in the *Urera* genus, there is the unique study available concerning the antiviral activity. Afterward, the probable presence of biologically

active compounds and the low cytotoxicity demonstrated by the extract and fractions of *U. baccifera* make this plant a promising antiviral candidate. Even though the indication for treatment is not yet possible, the observed activity justify further studies on the elucidation of the mechanisms of virus inhibition. Moreover, the relatively high toxicity of the drugs approved for the treatment of herpetic infections and the continuous propagation of resistant viral strains emphasize the need for new antiviral agents (Van De Perre et al., 2008).

### Antimicrobial activity

CHCl<sub>3</sub>, EtOAc and BuOH fractions of the leaves showed a good activity against *Klebsiella pneumoniae*, gram-negative bacteria with a prominent polysaccharide capsule, which can cause important hospital infections among adult and pediatric populations, showing that the fractionation of the crude extract can evidence very important antimicrobial activities, not accessible when only crude extracts are tested. The best value was obtained for BuOH (MIC: 156 µg/ml), and based on the criteria mentioned earlier, the fractions of the extracts of *U. baccifera* leaves could be considered active against this important pathogen. Being a well-known raw material in the baking and brewing industry, *Saccharomyces cerevisiae* is also used as a probiotic in humans. However, this fungus can be a singular cause of infections in humans by different forms of invasive infection after ingestion (Sidrim et al., 2003). Taking this to account, EtOAc and BuOH fractions of the roots can be a promising inhibitor of this microorganism, reaffirming the importance of fractionation of the crude extract.

In the same way, the EtOAc and BuOH fractions of leaves and roots and the crude extract of roots presented activity against *Prototheca zopfii*, algae, particularly difficult to eradicate with drugs and without protocol to treatment (Sidrim et al., 2003). Many plant constituents are related to antimicrobial activities as phenolics and polyphenols, terpenoids and essential oils, alkaloids, lectins and polypeptides (Cowan, 1999). The flavonoids are described to possess antifungal, antiviral and antibacterial activity in several studies (Cowan, 1999; Cushnie and Lamb, 2005; Hernandez, Tereschuk and Abdala, 2000; Oh et al., 2011) and can be responsible of the *U. baccifera* activity against *K. pneumonia*, *S. cerevisiae* and *P. zopfii*.

### Anti-inflammatory activity

In this study were tested the anti-nociceptive and anti-inflammatory activity of *U. baccifera* in rats, using the concentrations of 100 and 10 to 1000 mg/kg of animal

weight administered orally, to the roots and the leaves, respectively, and these extracts did not show antinociceptive and anti-inflammatory effect in these experiments. Conversely, the plant showed anti-inflammatory and antinociceptive effects (Badilla et al., 1999a, b, 2006) only when the extracts were administered intraperitoneally, and in the last two studies, the dosages used were higher than those used in the present work, indicating that the plant can display anti-inflammatory and antinociceptive activities, but only when administrated intraperitoneally and in high concentrations. Alkaloids (Barbosa-Filho et al., 2006; Matu and Staden, 2003) and diterpenes (Matu and Staden, 2003), as well as flavonoids (Ferrándiz and Alcaraz, 1991; Guardia et al., 2001), were cited as anti-inflammatory constituents of plants. Alkaloids and flavonoids were quantified in this plant, even though *U. baccifera* did not show anti-inflammatory and antinociceptive activities.

This study tested the antiviral, antinociceptive, anti-inflammatory and antimicrobial activities of *U. baccifera* leaves and roots. The antinociceptive and anti-inflammatory effects were not evidenced when orally administrated at the tested doses. On the other hand, this work provides new information about the antimicrobial activity of *U. baccifera*, showing that the fractions of this plant could be considered active against *K. pneumoniae*, *P. zopfii* and *S. cerevisiae*. Additionally, the plant showed a very good anti-herpetic activity, and the best results were obtained with the crude extract and ethyl acetate fractions of the roots and the butanol fractions of the leaves. The probable presence of biologically active compounds and the low cytotoxicity displayed by the extract and fractions of *U. baccifera* make these compounds promising antiviral candidates. This study became important due to the fact that there are few studies concerning the pharmacological activities of *U. baccifera*, and no study regarding activities of other species of *Urera* genus, which makes the genus a promising source of study.

### Conflict of interest

No competing interests were disclosed.

### ABBREVIATIONS

**ACV**, Acyclovir; **ATCC**, american type culture collection; **BuOH**, butanol fraction; **CC<sub>50</sub>**, cytotoxic concentration 50% (µg/ml); **CE**, crude extract; **CFA**, complete freund's adjuvant; **CHCl<sub>3</sub>**, chloroform fraction; **DMSO**, dimethyl sulfoxide; **EtOAc**, ethyl acetate fraction; **FBS**, fetal bovine serum; **HSV**, herpes simplex virus; **HSV-1**, Herpes simplex type 1; **HSV-1-ACVR**, acyclovir-resistant Herpes simplex virus type 1; **IC<sub>50</sub>**, inhibitory concentration; (µg/ml); **i.pl.**, intraplantar (plantar surface); **MNCC**, maximum noncytotoxic concentration; **SD**, standard deviation; **SI**, selectivity index; **TCID<sub>50</sub>**, 50% tissue culture infectious



doses.

## REFERENCES

- Amoros M, Simões CMO, Girre L (1992). Synergistic effect of flavones and flavonols against herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis. *J. Nat. Prod.* 55:1732-1740.
- Badilla B, Mora G, Lapa AJ, Emim JAS (1999a). Anti-inflammatory activity of *Ureia baccifera* (Urticaceae) in Sprague-Dawley rats. *Rev. Biol. Trop.* 47:365-371.
- Badilla B, Mora G, Poveda LJ (1999b). Anti-inflammatory activity of aqueous extracts of five Costa Rican medicinal plants in Sprague-Dawley rats. *Rev. Biol. Trop.* 47:723-727.
- Badilla B, Chaves F, Mora G, Poveda JL (2006). Edema induced by *Bothrops aspes* (Squamata: Viperidae) snake venom and its inhibition by Costa Rican plant extracts. *Rev. Biol. Trop.* 54:245-252.
- Barbosa-Filho JM, Piuevam MR, Moura MD, Silva MS, Batista Lima KV, Leitão da-Cunha EV, Fachine IM, Takemura OS (2006). Anti-inflammatory activity of alkaloids: A twenty-century review. *Rev Bras. Farmacogn.* 16:109-139.
- Caspani O, Heppenstall PA (2009). TRPA1 and cold transduction: an unresolved issue? *J. Gen. Physiol.* 133:245-249.
- Chen Y, Scieux C, Garrait V, Socié G, Rocha V, Molina JM, Thouvenot D, Morfin F, Hocqueloux L, Garderet L, Espérou H, Sélimi F, Devergie A, Leleu G, Aymard M, Morinet F, Gluckman E, Ribaud P (2000). Resistant herpes simplex virus type 1 infection: an emerging concern after allogeneic stem cell transplantation. *Clin. Infect. Dis.* 31:927-935.
- CLSI - Clinical and Laboratory Standards Institute (2008). Reference method for broth dilution antifungal susceptibility testing yeasts. Approved standard M27-A3, 3rd ed., CLSI, Wayne, PA.
- CLSI - Clinical Laboratory Standards Institute (2008). Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard, 2nd ed. M38-A2. CLSI, Wayne, PA.
- CLSI - Clinical Laboratory Standards Institute (2013). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A6. National Committee for Clinical Laboratory Standards, Wayne, PA.
- Cos P, Vlietinck AJ, Berghe DV, Maes L (2006). Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. *J. Ethnopharmacol.* 106:290-302.
- Cowan MM (1999). Plant Products as Antimicrobial Agents. *Clin. Microbiol. Rev.* 12:564-582.
- Cushnie TPT, Lamb AJ (2005). Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* 26:343-356.
- Ferrández ML, Alcaraz MJ (1991). Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. *Agents Actions* 32:283-288.
- Ferreira J, Campos MM, Pesquero JB, Araújo RC, Bader M, Calixto JB (2001). Evidence for the participation of kinins in Freund's adjuvant-induced inflammatory and nociceptive responses in kinin B1 and B2 receptor knockout mice. *Neuropharmacology* 41:1006-1012.
- Freitas AM, Almeida MT, Andrighetti-Fröhner CR, Cardozo FT, Barardi CR, Farias MR, Simões CM (2009). Antiviral activity-guided fractionation from *Araucaria augustifolia* leaves extract. *J. Ethnopharmacol.* 126:512-517.
- Guardia T, Rotelli AE, Juarez AO, Pelzer LE (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *II Farmacologie* 56: 683-687.
- Hernandez NE, Tereschuk, ML, Abdala LR (2000). Antimicrobial activity of flavonoids in medicinal plants from Tafí del Valle (Tucumán, Argentina). *J. Ethnopharmacol.* 73:317-322.
- Hudson JB (1990). *Antiviral Compounds from Plants*. Boston; Boca Raton, Ann Arbor, CRC Press.
- Jassim SA, Naji MA (2003). Novel antiviral agents: a medicinal plant perspective. *J. Appl. Microbiol.* 95:412-427.
- Matu EN, van Staden J (2003) Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. *J. Ethnopharmacol.* 87:35-41.
- Melendez PA, Capriles VA (2006). Antibacterial properties of tropical plants from Puerto Rico. *Phytomedicine* 13:272-276.
- Mannion F, Menezes FS (2010). Antioxidant activity of *Ureia baccifera* Gaud extracts. Trinity College Dublin: *J. Pharm. Pharm. Sci.* 2:8-9.
- Martins FO, Gomes MMR, Nogueira FLP, Martins GR, Romanos MTV, Kaplan MAC, Menezes FS (2009). *In vitro* inhibitory effect of *Ureia baccifera* (L.) Gaudich. extracts against herpes simplex. *Afr. J. Pharm. Pharmacol.* 1:581-584.
- Oh I, Yang W, Chung S, Kim T, Oh K, Shin J (2011). *In Vitro* Sortase: a Inhibitory and Antimicrobial Activity of Flavonoids Isolated from the Roots of *Sophora flavescens*. *Arch. Pharm. Res.* 34:217-222.
- Onofre SB, Herkert PF (2012). Antimicrobial Activity of Extracts Obtained from *Ureia baccifera* (L.) Gaudich. *Adv. Life Sci.* 2:139-143.
- Rios JL, Recio MC (2005). Medicinal plants and antimicrobial activity. *J. Ethnopharmacol.* 100:80-84.
- Sakagami H, Sakagami T, Takeda M (1995). Antiviral properties of polyphenols. *Polyphenol Actual.* 12:30-32.
- Sidrim JJC, Rocha MFG, Câmara LMC, Brilhante RSN, Diogenes MJN, Oliveira AMA (2003). Onycholysis caused by a mixed infection of *Prototheca zopfii* and *Candida albicans*. *Clin. Microbiol. Newslett.* 25:69-71.
- Silva IT, Costa GM, Stoco PH, Schenkel EP, Reginatto FH, Simões CMO (2010). *In vitro* antiherpes effects of a C-glycosylflavonoid-enriched fraction of *Cecropia glaziovii* Sneth. *Lett. Appl. Microbiol.* 51:143-148.
- Silva CR, Oliveira SM, Rossato, MF, Dalmolin GD, Guerra GP, Prudente AS, Cabrini DA, Otuki MF, André E, Ferreira J (2011). The involvement of TRPA1 channel activation in the inflammatory response evoked by topical application of cinnamaldehyde to mice. *Life Sci.* 20:88, 25-26, 1077-1087.
- Simões CM, Falkenberg M, Mentz LA, Schenkel EP, Amoros M, Girre L (1999). Antiviral activity of south Brazilian medicinal plant extracts. *Phytomedicine* 6:205-214.
- Takeuchi H, Baba M, Shigeta S (1991). An application of tetrazolium (MTT) colorimetric assay for the screening of anti-herpes simplex virus compounds. *J. Virol. Methods* 33:61-71.
- Uncini Manganelli RE, Zaccaro L, Tomei PE (2005). Antiviral activity in vitro of *Urtica dioica* L., *Parietaria diffusa* M. et K. and *Sambucus nigra* L. *J. Ethnopharmacol.* 98:323-327.
- Valadeau C, Pabon A, Deharo E, Joaquina AC, Yannick E, Augusto LF, Gamboa Dionicia RR, Michel S, Denis C, Geneviève B (2009). Medicinal plants from the Yanasha (Peru): Evaluation of the Leishmanicidal and antimalarial activity of selected extracts. *J. Ethnopharmacol.* 123:413-422.
- Van De Perre P, Segondy M, Foulongne V, Ouedraogo A, Konate I, Huraux JM, Mayaud P, Nagot N (2008). Herpes simplex virus and HIV-1: deciphering viral synergy. *Lancet Infect. Dis.* 8:490-497.
- Van Den Berghe DA, Vlietinck AJ, Van Hoof L (1986). Plant products as potential antiviral agents. *Bull. Inst. Pasteur* 84:101-107.
- Zimmermann M (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16:109-110.
- Whitley RJ (2001). Herpes simplex virus. pp. 2461-2509. In: Knipe DM, Howley PM, Griffin DF (eds.) *Fields virology*, 4th ed, V. 2. Lippincott Williams & Wilkins, Philadelphia, PA.

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