In vivo and in vitro anti-inflammatory activity of *Litchi chinensis* Sonn leaf extract and isolated compound procyanidin A2

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*Litchi chinensis* Sonn seeds and pericarp are used in popular medicine as antioxidant, anticancer, antiviral, analgesic, anti-inflammatory, immunomodulatory agent and others. However, literature on litchi leaves extract and the main compound procyanidin A2 (PA2) activities are scarce. Here, we investigated the anti-inflammatory activity of *L. chinensis* leaves extract and the main compound PA2 using in vitro and in vivo assays. We used the pleurisy and air-pouch model induced by carrageenan, substance P, histamine, bradykinin or lipopolysaccharide (LPS) in mice. The PA2 effects were also evaluated using carrageenan-induced pleurisy and LPS-induced air-pouch. In addition, LPS-induced NO₂⁻ production and cytotoxicity were quantified using peritoneal neutrophils recruited by oyster glycogen previously treated with *L. chinensis* extract or PA2. Animals orally treated with *L. chinensis* leaves extract exhibited a reduction on carrageenan-induced paw edema. Furthermore, the extract reduced the leukocyte migration and the protein leakage in the pleurisy model induced by carrageenan, substance P, histamine and bradykinin. The main compound in the leaves extract, PA2 reduced the paw edema and cell migration into the carrageenan-inflamed tissue. The data obtained in the LPS-induced air pouch model show that the extract and PA2 inhibit the in vivo neutrophil migration. In addition, the leaves extract and PA2 did not affect the neutrophil viability and reduces NO₂⁻ production. Taken together, the data herein obtained showed the modulatory actions of *L. chinensis* leaves extract on leukocyte migration to inflamed tissue, suggesting its therapeutic application to acute inflammatory process. In addition, it may be supposed that PA2 could be related with these actions, as it is the main compound of *L. chinensis* leaves extract.

Keywords: Air-pouch model, Carrageenan, Inflammation, Lichia, Lipopolysaccharide, Neutrophil migration, Nitric oxide, PA2, Pleurisy

Inflammation is a pathophysiological and complex biological process among cells and soluble factors that can occur in any tissue, characterized by heat, redness, pain, swelling and loss of function. It is a kind of host defense response that involves active inflammation, tissue destruction and tissue repair. The tissue exposed to infection, trauma, lipopolysaccharides (LPS) or other stimulus leads to resident cells to secrete cytokines, arachidonic acid metabolites and reactive nitrogen and oxygen species. Moreover, activated circulating leukocytes migrate into the inflamed area and, especially neutrophils and macrophages, release an array of pro-inflammatory mediators, as nitric oxide (NO). In this way, considering the deleterious action of neutrophils to the host inflamed tissue in case of non-controlled inflammation, blockade of leukocyte mobilization into focus of the inflammatory reaction is an important therapeutic strategy.

Seeds and pericarp from *Litchi chinensis* Sonn exhibit numerous biological activities, such as antioxidant, anticancer, antitussive, analgesic, anti-inflammatory, haemostatic, diuretic, antiviral and immunomodulatory activity. These effects are related with active compounds in litchi pericarp and seeds, as polyphenols polysaccharides, triterpenes, phytosterols and polyphenolic compounds. Although litchi leaves are extensively used in tea infusions, their biological properties are almost unknown.

Recently, our group demonstrated the presence of procyanidin A2 (PA2), procyanidin B2 (PB2) and epicatechin (EPI) in the ethanolic and methanolic extract of litchi leaves, which were related with significant antioxidant activity and central and peripheral antinociceptive action. However, the anti-
Inflammatory effect of leaves of *L. chinensis*, was early exploited only for lipophilic extract (petroleum ether extract) without phytochemical investigation or mechanism involved. Also, despite several studies demonstrating the anti-inflammatory activity from other procyanidins, as PB2, present in grape seeds, apples and cacao beans and other foods, there is no work available on this activity of PA2, the main component of the polar extract of *L. chinensis* leaves. Therefore, here we studied the oral anti-inflammatory activity of *Litchi chinensis* leaves extract and the main compound PA2, using *in vitro* and *in vivo* assays.

**Materials and Methods**

**Animals**

Male Swiss mice (25-30 g) were obtained from the Central Animal House of the Universidade do Vale do Itajaí. The animals were housed in standard cages, at room temperature (25±3°C), with 12:12 h dark/light cycles, and supplemented with food and water *ad libitum*. All procedures were performed according to the Brazilian Society of Science of Laboratory Animals’ guidelines for the proper care and use of experimental animals, the experiments were approved by the local ethics committee (protocol number CEUA/UNIVALI 291/2008).

**Plant material**

Leaves of *Litchi chinensis* were collected in May 2009 in Itajaí, in the State of Santa Catarina, Brazil, and identified by Prof. Dr. Rene Artur Ferreira (Universidade do Vale do Itajaí-UNIVALI). A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (BRH, Itajaí-SC, Brazil) under number 52829.

**Preparation of extract**

*L. chinensis* leaves were dried at room temperature, powderized, and submitted to methanol (MeOH) extraction at room temperature for 7 days. The MeOH extract of *L. chinensis* leaves was put to liquid-liquid partitioning using n-hexane, CH2Cl2 and ethyl acetate (EtOAc). The complete procedure used for obtaining the compounds procyanidin B2, epicatechin and procyanidin A2 which is evaluated in the present study, from ethyl acetate fraction was previously described by Castellain and collaborators.

**Chromatography analysis**

High performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-10AD LC system (Shimadzu, Tokyo, Japan) consisting of a binary pump (LC-10ADvp), column oven (CTO-10Avp), automatic injector (SIL-10AF) and a Shimadzu SPD-M10A photo diode array detector. The injections (20 µL) were carried out on a Phenomenex® (Torrance, California, USA) Luna C18 5 μm (250 × 4.6 mm) at 30°C, with detection at 278 nm. The mobile phase consisted in a gradient of acetonitrile (A): acidified water pH 3.0 with sulfuric acid (B) of 10:90 (A:B) (0-5 min); 15:85 (5-7 min); 20:80 (7-13 min); 25:75 (13-20 min); 30:70 (20-30 min); 10:90 (30 min) maintaining this composition until 40 min, as previously developed and validated to ethanolic extract of *L. chinensis*. The methanolic extract (5 mg) was dissolved in 1 mL of methanol, sonicated for 15 min and diluted 1:1 (with 1:9 v/v acetonitrile: acidified water at pH 3.0) in view of obtain a concentration of 2.5 µg/mL. Reference solution was prepared by dissolving PA2 in methanol followed by a dilution with 1:9 v/v acetonitrile: acidified water at pH 3.0 to obtain a concentration of 50 µg/mL.

**Paw edema model**

The animals were orally treated with doses of crude methanolic extract of *L. chinensis* (25, 50 or 100 mg/kg, p.o.) or procyanidin A2 (PA2) (3 or 6 mg/kg, p.o.) dissolved in 0.9% saline solution. The control group (vehicle) received only saline solution (0.9%) and on the positive control group a subcutaneous injection of dexamethasone (0.5 mg/kg). One hour after treatments, the carrageenan (300 μg/50 μL) was administered in their right hind paw. The same volume of saline solution was administered into the left hind paw. Edema was measured with a paw plethysmometer (model 7150, Hugo Basile) and expressed as the differences in the values of the paws at 0, 5, 2 and 4 h after carrageenan application.

**Pleurisy model**

Methanolic extract of *L. chinensis* (25, 50 or 100 mg/kg, p.o.) or PA2 (3 or 6 mg/kg, p.o.) were administered orally 1 h before carrageenan pleural injection. The positive control group orally received indomethacin (10 mg/kg). Pleurisy was induced by applying 0.1 mL of a suspension of carrageenan 1% (diluted in saline solution) to the pleural cavity of the mice. Four hours after the pleurisy induction, the animals were euthanized, and the pleural inflammatory exudate was collected through pleural lavage with 1 mL of PBS and the total leukocytes number was determined using Neubauer chamber. Differential cell counts were performed on smears stained with May Grünwald-Giemsa. In addition, the
exudate was collected in order to evaluate the protein extravasation.

In addition, substance P (20 nmol/cavity), histamine (100 μg/cavity) or bradykinin (10 nmol/cavity) were employed in another set of experiments as flogistic agents to investigate the mechanism of action presented by the extract.

Air-pouch model

Animals were anesthetized with xylazine/ketamine (80/8 mg/kg, i.p.) and 3 mL of sterile air was injected subcutaneously into the dorsal region. Six days after, the pouch was refilled with 3 mL of air. On the 10th day following the first air injection, the animals were divided into six groups and received treatment orally by gavage: (i) Sham; (ii) vehicle (PBS); (iii) indomethacin (30 mg/kg; positive control); (iv) L. chinensis extract (100 mg/kg); or (v) PA2 (6 mg/kg). After 4 h, the lipopolysaccharide (LPS) from E. coli (serotype 026:B6, 100 μg/mL PBS, 2 mL/animal; Sigma) was injected directly into the pouches. Four hours later, the animals were re-anesthetized and euthanized. The pouches were washed with 3 mL of ice-cold PBS, and the total leukocyte number was determined using a Neubauer chamber. Differential cell counts were performed on smears stained with May Grünwald-Giemsa.

Neutrophil isolation

Neutrophils were obtained from male Swiss mice 4 h after an intraperitoneally (i.p.) injection of 3 mL of 1% sterile oyster glycogen solution in PBS. Following this period, the animals were anesthetized and the cells were collected by rinsing the abdominal cavity with 3 mL of sterile PBS. The number of viable cells was counted in a Neubauer chamber using a light microscope (Nikkon, Japan).

Cell viability

Neutrophils (1×10^6 cell/well) were incubated with L. chinensis extract or procyandin A2 in the presence or absence of LPS (5 μg/mL) for 18 h (37°C, 5% CO2). Then, 10 μL of neutrophil suspension from each well was mixed with 10 μL of 0.4% trypan blue solution. The suspension (10 μL) was placed in Neubauer chamber, 200 cells were counted and the percentage of viable cells was calculated.

Determination of nitrite levels

Neutrophils, collected as described above, were incubated (1×10^6 cell/well) in the absence or presence of LPS (5 μg/mL) and L. chinensis extract or PA2 (1, 10 and 100 μg/mL) during 18 h (37°C, 5% CO2). Culture supernatant was used as a measurement of NO2⁻ production by neutrophils. Samples (100 μL) were incubated for 10 min with 100 μL of Griess reagent (1% sulfanilamide, 0.1% naphthyl-ethylenediamine dihydrochloride in 5% phosphoric acid) and absorbance at 550 nm were determined using a multiwell plate reader. The results were reported as μM of NO2⁻.

Statistical analysis

The results are presented as mean ± SEM of 5-6 rats per group. Statistical comparison of the data was performed using analyses of variance (ANOVA) followed by Tukey’s test. The P values less than 0.05 (P <0.05) were considered significant.

Results

Chromatographic analysis

The phytochemical profile obtained by HPLC analysis of the MeOH L. chinensis extract showed the major component, PA2 (20.98 ± 0.29 mg/g), at 23.8 min of retention time (Fig. 1 A and B), with typical phenolic UV absorption profile, as observed for the ethanolic extract.

L. chinensis extract inhibit paw edema induced by carrageenan

As shown in the Fig. 2, after 2 and 4 h of carrageenan injection in the right hind paw was observed an edema in the animals pre-treated with vehicle. However, the animals orally pre-treated with L. chinensis extract at the doses of 25, 50 or 100 mg/kg inhibit the paw edema formation at 4 h. However, after 2 h of carrageenan administration just the dose of 100 mg/kg of L. chinenses extract was able to decrease the edema. The animals pre-treated with dexamethasone, the positive control, showed a significant reduction at all period evaluated (Fig. 2).

Fig. 1 — Representative HPLC chromatogram of A) L. chinensis leaves methanolic extract (3 mg/mL) and B) PA2 (50 μg/mL) detected at 278 nm with insert of UV absorption profile of PA2.
L. chinensis extract reduced leukocyte migration in pleurisy-model induced by carrageenan, substance P, histamine and bradykinin

The in vivo anti-inflammatory effect of L. chinensis extract was firstly evaluated in pleurisy induced by carrageenan 1%. Different doses were tested and the number of cells migrated into the pleural cavity was quantified 4 h after the carrageenan injection. The results obtained show that the extract (25, 50 or 100 mg/kg) decreases the leukocyte migration into the inflammatory site, especially polymorphonuclear cells influx. In addition, the extract was able to decrease the protein leakage in the pleural cavity (Table 1). As expected, animals pre-treated with indomethacin (30 mg/kg) showed a significant decrease in all parameters evaluated (Table 1).

Following the experiments, an intrapleural injection of Substance P (SP, 20 nmol) in the pleural cavity was used to induce inflammatory process. The SP injection promotes 4 h later a significant influx of inflammatory cells into pleural cavity. In fact, the data obtained show that after 4 h animals pre-treated with vehicle presents large cell migration associated with a protein extravasation (Table 1). However, in animals pre-treated with 25, 50 or 100 mg/kg of L. chinensis extract was possible to verify a decrease in the leukocyte influx and exudation into the pleural cavity (Table 1). The same condition was observed when

![Graph](image)

**Fig. 2 — Effects of oral administration of L. chinensis extract on carrageenan-induced paw edema in mice. Animals received the extract of L. chinensis (25, 50 and 100 mg/kg, p.o.), dexamethasone (5 mg/kg, s.c.) or vehicle. One hour after treatments, the carrageenan (300 μg/50 μL) was administered in their right hind paw. Edema was measured and expressed as the differences in the values of the paws at 0.5, 2 and 4 h after carrageenan application. [Data are expressed as mean ± SEM of 5-6 animals in each group. Statistical analysis was performed using ANOVA followed by Tukey’s test. **P <0.01 versus vehicle]**

### Table 1—Anti-inflammatory effects of oral administration of Litchi chinensis leaf extract

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose mg/kg</th>
<th>Flogistic agent</th>
<th>Protein (µg/mL)</th>
<th>Total leukocytes (10^6 cells/mL)</th>
<th>Mononuclear cells (10^6 cells/mL)</th>
<th>Polymorphonuclear cells (10^6 cells/mL)</th>
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[Animals received leaf extract of L. chinensis (25, 50 and 100 mg/kg, p.o.), indomethacin (30 mg/kg, p.o.) or vehicle. One hour after treatments, the carrageenan (1%), substance P (20 nmol/cavity), histamine (100 μg/cavity) or bradykinin (10 nmol/cavity) were administered into the pleural cavity of the mice. 4 hours later the pleurisy induction the animals were euthanized and the pleural inflammatory exudate were collected to measure the protein extravasation, total leukocyte, mononuclear and polymorphonuclear cell migration into the pleural cavity. Data are expressed as mean ± S.E.M. of 5-6 animals in each group. Statistical analysis was performed using ANOVA followed by Tukey’s test. *P <0.05 versus vehicle]
was employed as flogistic agent histamine (100 μg/cavity), however, just the major and intermediate dose of *L. chinensis* extract presented significant reduction in leukocyte migration and protein extravasation, when compared to vehicle treated animals (Table 1).

Finally, the bradykinin (10 nmol/cavity) induced-pleurisy was assessed. Data obtained on this experiment demonstrate that only at 100 mg/kg of *L. chinensis* extract was able to inhibit the leukocyte migration into the pleural cavity (Table 1). The positive control, indomethacin presented inhibitory effects on leukocyte migration and protein exudation in all different flogistic agents employed (Table 1).

**PA2 reduced paw edema and cell migration into carrageenan-inflamed tissue**

The PA2 is the main compound present in the litchi leaves extract, as demonstrated in the Fig. 1. The PA2 was evaluated on carrageenan-induced paw edema and pleurisy models. The results obtained show that the PA2 at 6 mg/kg was able to reduce the paw edema (Fig. 3) and the total leukocytes, as well as polymorphonuclear migration (Fig. 4) and protein leakage into the lesion site on pleurisy model.

*L. chinensis* extract and PA2 did not affect cell viability and reduces NO$_2^-$ levels in LPS-stimulated neutrophils *in vitro*

In order to verify the potential cytotoxicity of *L. chinensis* extract and PA2, neutrophils viability was assessed using trypan blue assay. Data obtained showed that both, extract and PA2, in the concentration range used, did not promoted cell death, neither in basal or inflammatory conditions (LPS-stimulated neutrophil) (Fig. 5A).

The potential of *L. chinensis* extract and PA2 to inhibit NO$_2^-$ production was measured in the...
supernatant of cultivated LPS-stimulated neutrophils. As expected, LPS stimulation increased NO$_2^-$ levels in the supernatant of the neutrophils incubated for 18 h, which it was markedly reduced by the *L. chinensis* extract (100 μg/mL) and PA2 treatment (10 and 100 μg/mL) (Fig. 5B). In addition, the data obtained in air pouch model show that extract and PA2 inhibited the *in vivo* neutrophil migration induced by LPS (Fig. 6).

**Discussion**

In the innate and adaptive immune response, the leukocytes, especially neutrophils, have a relevant role in the defense against pathogens. The polymorphonuclear cells are the first cells that arrive at the lesion site, where they kill and phagocytose the pathogens. Therefore, in case of exacerbated reactions, blockage of their functions represents a therapeutic strategy, because uncontrolled inflammatory processes can lead to host tissue damage. Here, we show that *in vivo* treatment with *L. chinensis* leaves extract significantly impaired neutrophil migration into inflamed exudates and reduce the edema. In addition, we show for the first time that PA2 is responsible for these actions, as it is the main compound of *L. chinensis* leaves extract.

Edema and neutrophil migration into the lesion site is mediated by chemical mediators, locally secreted or generated by resident or migrated cells. In this way, we performed the carrageenan-induced paw edema and pleurisy models to study the effects of *L. chinensis* leaves extract. The sub-plantar carrageenan paw injection induces an acute inflammatory process, promoting edema formation, which involves three different phases. In the first hour after the carrageenan injection, there was an increase in vascular permeability mediated predominantly by histamine and serotonin. In the second hour, there was an increase in the permeability, possibly induced by kinins release. After the third hour, we observed the alterations induced by prostaglandin actions.

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Fig. 5 — Effects of *L. chinensis* extract and PA2 on cell viability and nitrite levels. Neutrophils were obtained from peritoneal cavity of male Swiss mice 4 h after intraperitoneal injection of oyster glycogen solution (1%, 3 mL). Neutrophils (1x10$^6$) were incubated in the presence or absence of LPS (5 μg/mL) with HBSS, *L. chinensis* extract or PA2 (1, 10 or 100 μg/mL) for 18 h. (A) Viability was quantified by trypan blue assay in optical microscopy; and (B) nitrite (NO$_2^-$) concentration was assayed using Griess reaction. [The results are expressed as the mean ± S.E.M. of cells obtained from 3-6 animals. *P <0.05 vs. LPS-stimulated basal]

Fig. 6 — Effects of *L. chinensis* extract and PA2 on *in vivo* leukocyte migration induced by LPS. Air pouch animals received orally: vehicle, indomethacin (30 mg/kg), *L. chinensis* extract (100 mg/kg) or PA2 (6 mg/kg). LPS from *E. coli* 026:B6 (1 mg/2 mL) or PBS were injected after 1 h directly into the air pouch, and 4 h later, the number of cells in the pouch was quantified. The dotted line indicates the number of neutrophils in the non-inflamed tissue. (A) Number of total leukocytes in the air pouch; and (B) Number of neutrophils in the air pouch. [Data are expressed as mean ± S.E.M. of 5-6 animals in each group. Statistical analysis was performed using ANOVA followed by Tukey’s test. **P <0.01 and ***P <0.001 vs. vehicle]
results obtained show that *L. chinensis* leaves extract at 100 mg/kg reduced the edema formation in the second and the fourth hour after carrageenan injection, but the doses 50 and 25 mg/kg just reduced edema in the fourth hour. Our results are in agreement with Besra and collaborators\textsuperscript{12} that previously demonstrated the anti-inflammatory effect of petroleum ether extract obtained from *L. chinensis* leaves. As expected, the positive control, dexamethasone was able to reduce the edema formation at all periods evaluated.

The carrageenan-induced pleurisy model assay results showed that the extract at all doses reduced the exudation and cell migration, especially polymorphonuclear cells into the lesion site. Together, these results showed that the *L. chinensis* extract could blockade the carrageenan-induced inflammation. However, there are other pathways and chemical mediators involved in the inflammatory cascades that are responsible for induction of the process, as substance P, histamine and bradykinin.

In this way, the pleurisy was performed using different flogistic agents. We employed substance P, the peptide that binds to the neurokinin receptor (NKR) and induces the release of mediators, cytokines, arachidonic acids derivatives, histamine and oxygen radicals, which potentiate tissue damage and stimulate the recruitment of leucocytes, amplifying the inflammatory response\textsuperscript{19,20}. Substance P induces leukocyte/endothelial cell adhesion by translocation of CD62 and upregulation of CD62E, inducing accumulation of leukocytes at injury sites\textsuperscript{21}. The data shows that the *L. chinensis* leaves extract at all doses evaluated here, inhibits the leukocyte migration and reduces the exudation induced by substance P. These effects are crucial to prevent the tissue damage, whereas the downregulation on neutrophil degranulation into the lesion site leads a consequently decreased chemical mediators release.

Similar results were observed in the pleurisy induced by histamine and bradykinin, the extract was able to inhibit the leukocyte migration and exudation induced by both chemical mediators, but just in the major dose (100 mg/kg). Histamine and bradykinin plays an important role in inflammation, promoting events, such as vasodilatation, increased vascular permeability and increased production of eicosanoids\textsuperscript{22}.

As demonstrated in the chemical profile, the major compound in the *L. chinensis* extract is PA2. PA2 is a procyandin, also known as protoanthocyanidin, is a polymer composed of flavan-3-ol, catechin, or epicatechin. Procyandin can be classified as an A, B, or C type. The A- and B-type procyandins are dimers of flavan-3-ol, while C-type procyandins are trimmers\textsuperscript{23}. Here, we have demonstrated for the first time the anti-inflammatory activity of PA2 isolated from *L. chinensis* leaves extract. PA2 was able to reduce the carrageenan-induced paw edema in all periods observed, and reduced the exudation and the polymorphonuclear cell migration carrageenan-induced pleurisy model.

Nitric oxide is another important pro-inflammatory mediator involved in exudation and leukocyte migration in the inflammatory process. In this context, it was evaluated for the effects of *L. chinensis* extract and PA2 in the nitric oxide production by LPS-stimulated neutrophils. The mechanism of LPS action is the activation of Toll Like Receptor-4 (TLR-4), as expressed on the neutrophil cell membrane and activates inflammatory pathways, as via activation of the nuclear transcription factor factor-\(\kappa\)B (NF-\(\kappa\)B). NF-\(\kappa\)B is the most important regulator of pro-inflammatory gene expression and induces a release of inflammatory mediators that induce the leukocyte chemotaxis\textsuperscript{24}.

The results have shown that both extract and PA2 could reduce the nitric oxide release by LPS-stimulated neutrophils. It is important to emphasize that *L. chinensis* extract and PA2 were non-cytotoxic to neutrophils. In addition, the *L. chinensis* extract and PA2 were able to inhibit the leukocyte migration stimulated by LPS in air-pouch model.

**Conclusion**

Taken together, data presented here from *in vivo* and *in vitro* studies have shown the mechanisms of the anti-inflammatory actions of *Litchi chinensis* leaf extract and the PA2 in the early phases of inflammation. Based on these findings, we have highlighted the inhibitory actions of *L. chinensis* extract and PA2 in the neutrophil migration into inflamed exudates and reduction of edema.

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Conflict of Interest

The authors declare that there is no conflict of interests associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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