INVESTIGATING THE ANTI-INFLAMMATORY ACTIVITY OF AN ETHANOLIC EXTRACT FROM ARTOCARPUS TONKINENSIS LEAVES USING A COLLAGEN ANTIBODY-INDUCED ARTHRITIC MOUSE MODEL

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Received: 25 January 2018; Accepted for publication: 20 April 2018

ABSTRACT

This study was contrived for evaluating the in vitro and in vivo anti-inflammatory effects of an aqueous ethanolic leaf extract of the Vietnamese Artocarpustonkinensis A. Chev. ex Gagnep. using lipopolysaccharide-stimulated RAW 264.7 macrophages and a collagen antibody-induced arthritic mouse model as well. The obtained results here demonstrate that the 70% ethanolic leaf extract of A. tonkinensis (AT2), traditionally used in Vietnamese folk medicine for treating arthritic symptoms, has beneficial effects on pro-inflammatory cytokine inhibition and in an experimental arthritic mouse model. LPS-stimulated RAW 264.7 macrophages treated with AT2 showed a significant decrease in the production of IL-6 and TNFα at concentrations of 12.5, 25 and 50 µg/mL (P < 0.05), indicating its potential anti-inflammatory properties. The treatment of CAIA mice with AT2 also led to diminish the incidence of arthritis at doses of 200 and 300 mg/kg body weight.

Keywords: Artocarpustonkinensis, autoimmune, interleukin 6, inflammation, rheumatoid arthritis.

Classification numbers: 1.1.1; 1.2.1
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1. INTRODUCTION

Inflammation is involved in the development of conditions such as autoimmune disease [1, 2] and rheumatoid arthritis [3, 4]. The major classes of drugs used to suppress inflammation are nonsteroidal anti-inflammatory agents and corticosteroids, but their toxic adverse effects such as epigastric distress, peptic ulceration and osteoporosis have limited their use [5]. Considering that different medicinal compounds, derived from plant sources, such as flavonoids, flavonoid glycosides, stilbenoids and saponins, may represent new anti-inflammatory agents more efficacious, safer, affordable, and accessible for patients, this path of searching for new drugs should be taken in serious consideration.

Artocarpustonkinensis A. Chev. ex Gagnep. is a tree currently found in North Vietnam. The decoction of its leaves and roots is used in traditional knowledge to treat backache, arthritis and joint disorders. Our previous phytochemical analysis of the A. tonkinensis leaves revealed the presence of flavonoid compounds such as auronol, kaempferol, quercetin and their glycosides [6-8]. Of them, two auronol glucosides (1 and 2) were found to have potent immunomodulatory, anti-proliferative and anti-inflammatory activities [9-11]. For a possible use of 70% ethanolic extract of A. tonkinensis leaves as alternative herb medicines to treat human disease, this study was undertaken to evaluate its possible anti-inflammatory activities in vitro using LPS-stimulated RAW 264.7 macrophages and in vivo using a collagen antibody-induced arthritic BALB/c mouse model.

2. MATERIALS AND METHODS

2.1. Chemicals

Dulbecco’s Modified Eagle’s Medium (DMEM) and antibiotic - antimycotic were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS), lipopolysaccharides (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO., USA). TNF-alpha and IL-6 ELISA kits were purchased from Biovision (Chester Springs, PA, USA). The collagen antibody cocktail was purchased from Modi Quest Research (Nijmegen, The Netherlands).

2.2. Preparation of ethanolic extract of A. tonkinensis leaves (AT2)

Leaves of A. tonkinensis were collected in Hanoi, Vietnam (October 2016) and identified by the taxonomist – Ngo Van Trai (National Institute of Medicinal Materials, Hanoi, Vietnam). The voucher specimen (Nr. 1482-AT-2016) was deposited in the Institute of Chemistry, Vietnam Academy of Science and Technology (ICH, VAST) for further reference.

In a pre-experiment, it was found that application of 70 % ethanol to extract the dried leaves of A. tonkinensis possessed high total phenolic content. Thus, 70 % ethanol was used for this extraction. Dried ground of A. tonkinensis leaves (600g) were extracted with 70 % EtOH, each 3 litres, overnight, x3 times) at RT. The extract was filtered and concentrated under reduced pressure in a rotary evaporator at 50-60 °C until the aqueous volume reduced to one third of its original volume and then freeze-dried. The crude ethanolic extract (110 g) was a brown powder, of which the obtained yield was 18.3 % (w/w).

2.3. Phytochemical analysis of AT2
To obtain the purified flavonoids for the chemical structural analysis, a large-scale isolation was carried out as previously described and their structures were confirmed by comparing their ESI-MS, $^1$H and $^{13}$C NMR data with reported data[6, 8]. AT2 was fractionated by a classical chromatography methodology. Columns packed with DIAION HP-20 and Sephadex LH-20 adsorbents were efficient for the isolation of nine compounds. Two auronoglucosidemaesopin 4-O-β-glucopyranoside (hovetrichoside C, 1), alphiton-O-β-glucopyranoside (2), as well as kaempferol(3),astragalin (4), kaempferol 3-O-β-rutinoside (5), kaempferol 3-O-β-neohesperidoside(6), quercetin, quercetin 3-O-β-glucopyranoside (7),afzelechin-(4α→8°)-catechin-3-O-β-glucopyranoside(8) and catechin(9) were identified [6, 8]. The AT2 extract was analyzed for the presence of various phytochemical constituents employing screening TLC. The detailed qualitative analyses of AT2 extract was performed with HPLC method. The presence of flavonoids 1-9 (Figure 1) in AT2 was examined by high-performance liquid chromatography coupled with mass spectrum (HPLC-MS), with UV detection.

Table 1. The MS data of isolated compounds from ethanolic extract of A. tonkinensis leaves (AT2)\(^a\)

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Name of compounds(^b)</th>
<th>Molecular formula</th>
<th>Molecular ion (m/z)</th>
<th>Rt(^c)(min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hovetrichoside C</td>
<td>C_{23}H_{22}O_{11}</td>
<td>473[M+Na](^+)</td>
<td>10.89</td>
</tr>
<tr>
<td>2</td>
<td>alphiton-O-β-D-glucopyranoside</td>
<td>C_{31}H_{22}O_{12}</td>
<td>489[M+Na](^+)</td>
<td>9.41</td>
</tr>
<tr>
<td>3</td>
<td>kaempferol</td>
<td>C_{15}H_{10}O_{6}</td>
<td>287[M+H](^+)</td>
<td>16.29</td>
</tr>
<tr>
<td>4</td>
<td>astragalin</td>
<td>C_{23}H_{20}O_{11}</td>
<td>449[M+H](^+)</td>
<td>14.70</td>
</tr>
<tr>
<td>5</td>
<td>kaempferol 3-O-β-rutinoside</td>
<td>C_{27}H_{30}O_{15}</td>
<td>617[M+Na](^+)</td>
<td>c</td>
</tr>
<tr>
<td>6</td>
<td>kaempferol 3-O-β-neohesperidoside</td>
<td>C_{27}H_{30}O_{15}</td>
<td>593[M-H](^-)</td>
<td>c</td>
</tr>
<tr>
<td>7</td>
<td>quercetin 3-O-β-D-glucopyranoside</td>
<td>C_{23}H_{20}O_{12}</td>
<td>465[M+H](^+)</td>
<td>13.85</td>
</tr>
<tr>
<td>8</td>
<td>afzelechin-(4α→8°)-catechin-3-O-β-glucopyranoside</td>
<td>C_{36}H_{36}O_{16}</td>
<td>747[M+Na](^+)</td>
<td>12.60</td>
</tr>
<tr>
<td>9</td>
<td>catechin</td>
<td>C_{15}H_{14}O_{6}</td>
<td>291[M+H](^+)</td>
<td>16.88</td>
</tr>
</tbody>
</table>

\(^a\)UPLC/MS/MSXEvo-TQ, Waters, USA. UPLC C18 column (150 mm×2.1 mm i.d.) at 25°C. UV spectrum was recorded from 190 nm to 400 nm. The mobile phase consisted of A, water containing 0.1 % formic acid, and B, methanol containing 0.1 % formic acid. The elution gradient was performed using a pump from 10 % to 100 % B for 25 min at a flow rate of 0.25 mL/min. Detection was carried over 30 min at a flow rate of 0.25 mL/min. Detection was carried out at 290 and 294 nm. The injection volume was 5 μL. ESI-MS (positive ion), fullscan(m/z from 100 to 1300).

\(^b\)Confirmed by comparison to authentic standards; c Overlapped.

2.4. Biological tests

2.4.1. Cell culture

The RAW 264.7 mouse macrophage cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle’s Medium.
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(DMEM) supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin. Cells were incubated in a humidified incubator with an atmosphere of 5% CO₂ at 37 °C.

2.4.2. Cell viability assay

The MTT assay was used to evaluate the effects of AT2 on cell viability. In brief, cells were seeded into 96-well plates at a density of 1 × 10⁵ cells/mL. Cells were treated with different concentrations (0, 3.125, 6.25, 12.5, 25, and 50 μg/mL) of AT2 for 1 h, followed by stimulation with LPS (1 μg/mL) for 24 h. After incubation, 30 μL MTT (5 mg/mL) was added to each well and incubated for another 4 h. After replacing the culture supernatant with 100 μL dimethyl sulfoxide (DMSO), the optical density of the plates was read at a wavelength of 490 nm using a microplate reader (TECAN, Austria).

2.4.3. Pro-inflammatory cytokine determination assay

RAW 264.7 cells were pretreated with various concentrations (0, 3.125, 6.25, 12.5, 25, and 50 μg/mL) of AT2 for 24 h before stimulating with LPS (1 μg/mL). After 18 h incubation, the levels of pro-inflammatory cytokines TNF-alpha and IL-6 were measured in the supernatant using commercial ELISA kits (Biovision, Chester Springs, PA, USA) following the manufacturer’s protocols.

2.4.4. Evaluating AT2 extract-reduced arthritis using a model of collagen antibody-induced arthritis (CAIA)

The male mice used in this study were of 10-11 weeks of age. The anti-collagen antibody cocktail was purchased from Modi Quest Research (Nijmegen, The Netherlands) and used according to the manufacturer’s instructions. Briefly, mice were intraperitoneally (IP) injected with 3 mg of the cocktail of anti-collagen antibodies. On days 3 and 9, the mice were IP injected with 25 μg LPS (Sigma, St. Louis, MO) to trigger the development of arthritis. Dosing began on day 4 after the mice had initial symptoms of arthritis. Thirty mice were assigned equally into five groups: the positive control (Group 1) orally received dexamethasone at a dose of 0.5 mg/kg body weight (bw) daily for 9 days; the negative control (Group 2) received water daily for 9 days; The experimented groups, of which were group 3, 4 and 5, was orally administered with AT2 at different doses for 9 days. Accordingly, group 3 was treated with AT2 at a dose of 300 mg/kg bw daily; Group 4 was treated with AT2 at a dose of 200 mg/kg bw daily; and Group 5 was treated with AT2 at a dose of 100 mg/kg bw daily. The mice were scored for clinical arthritis by two observers who were blinded to the group assignments. Each paw was scored on a scale of 0-2 based on the signs of swelling and inflammation. Serum was collected from each mouse injected with anti-collagen antibodies on days 0, 4, 6, 8, 10, and 12 and stored at -20 °C until use. The levels of TNF-alpha and IL-6 in the sera were measured as already described.

3. RESULTS AND DISCUSSION

3.1. Phytochemical analysis

In our previous studies, we prepared an ethyl acetate (EtOAc) extract from the leaves of the A.tonkinensis plant and tested its anti-inflammatory properties in a collagen-induced arthritis
Phytochemical analysis indicated that the EtOAc extract contains of potentially bioactive glucosides such as hovetrichoside C (1), alphitonin-\(\beta\)-D-glucoside (2), artonkin-4′-\(\beta\)-D-glucopyranosid and astragalin (4). All four compounds were found to have potent anti-proliferative and anti-inflammatory effects in vitro model [7, 9]. The main compound 1 inhibited also the growth of OCI-AML cells [10]. Moreover, gene expression profiling identified 19 genes modulated by compound 1 and among them, HMOX1 and SRXN1 genes were consistently and highly up-regulated [10]. To examine the more polar components from A. tonkinensis, AT2 was prepared from 70 % ethanol extraction of A. tonkinensis leaves. The use of 70 % ethanol for extraction resulted in higher yield of crude extract (18.3 %, w/w). On the other hand, AT2 contains significant amounts of the same glycosides as in EtOAc extract, which are responsible for anticancer and anti-inflammatory activities [8, 11]. Thus, in this study, AT2 was evaluated for potential at reducing arthritis using a CAIA model.

Phytochemical studies using the 70 % ethanolic extract of AT2 revealed the presence of nine flavonoids1-9. Their structures were elucidated by electrospray ionization mass spectrometry (ESI-MS) and \(^1\)H, \(^{13}\)C NMR spectral analysis. Nine compounds 1-9 are identified, including maesopsin 4-\(\beta\)-glucopyranoside (1), alphitonin 4-\(\beta\)-glucopyranoside (2), kaempferol(3), astragalin (4), kaempferol 3-\(\beta\)-rutinoside (5), kaempferol 3-\(\beta\)-neohesperidoside (6), quercetin 3-\(\beta\)-glucopyranoside (7), afzelechin-(4\(\alpha\)-\(\beta\)-catechin-3-\(\beta\)-glucopyranoside (8) and catechin (9) (Figure 1). The detailed qualitative analyses of AT2 extract were performed with HPLC and HPLC-MS methods. Constituents of AT2 were confirmed by comparing HPLC-MS data of standard isolated compounds (Table 1) [8]. At present, it is difficult to attribute the observed effects of the leaves of A. tonkinensis to any one particular chemical moiety.

![Figure 1. Structures of flavonoids (1-9) from 70% ethanolic extract of A. tonkinensis leaves (AT2).](image-url)
3.2. *A. tonkinensis* leaf extract (AT2) inhibited pro-inflammatory cytokines releasing in LPS-stimulated RAW 264.7 macrophages

There were no significant changes in cell viability following AT2 treatment at the tested concentrations (Figure 2). Therefore, non-toxic concentrations (3.125, 6.25, 12.5, 25, and 50 µg/mL) of AT2 were used in the cytokine inhibition experiment, allowing the determination of the effects of AT2 on the production of pro-inflammatory cytokines in LPS-stimulated macrophages. Treatment with LPS resulted in a significant increase in TNF-alpha and IL-6 production compared with the control. However, treatment with AT2 (12.5, 25 and 50 µg/mL) remarkably inhibited LPS-induced TNF-alpha and IL-6 production in a dose-dependent manner (P < 0.05) (Figure 3). This led us to conclude that AT2 might have anti-inflammatory properties caused by the inhibition of cytokine production in LPS-induced macrophages.

![Figure 2](image2.png)

**Figure 2.** Effects of AT2 extract on cell viability in LPS-treated RAW 264.7 mouse macrophages. RAW 264.7 cells were cultured with AT2 extract in the presence of LPS (1 µg/mL) for 24 h. Cell viability was tested by MTT reduction assays. Values are presented as the mean ± standard deviation (SD) of three independent experiments.

![Figure 3](image3.png)

**Figure 3.** Effects of AT2 extract on the production of IL-6 (A) and TNF-alpha (B) in LPS-stimulated RAW 264.7 cells. Cells were treated with LPS alone or LPS plus various concentrations (3.125, 6.25, 12.5, 25, and 50 µg/mL) of AT2 extract for 24 h. *P < 0.05, **P < 0.01 compared to LPS stimulated cells. Values represent the mean ± SD of three independent experiments.
3.3. The *in vivo* effect of *A. tonkinensis* leaf extract on CAIA

For a possible use of 70 % ethanolic extract of *A. tonkinensis* leaves as alternative herb medicines to treat human disease, it was undertaken to evaluate its potential at reducing arthritis using a CAIA model. Previous toxicological studies have shown that AT2 exerted no effects on survival, clinical observations, macroscopic examination of organs, body weight or food, or water consumption in mice up to 14.50 g/kg bw (oral administration) dose. In addition, from sub-chronic toxic tests, AT2 did not change the behavior, body weight or histopathological assessment of the kidney or liver at doses of 0.6 and 1.8 g/kg body weight for 8 weeks (data not shown); hence doses of 100, 200 and 300 mg/kg bw were selected for the present study.

![Figure 4](image)

*Figure 4.* Effect of AT2 administration at high doses caused statistically significant reductions in the arthritis scores compared with the vehicle-treated controls (*P*<0.05) (A). TNF-alpha was measured in the sera of AT2-treated mice at different doses (100, 200 and 300 mg/kg body weight), together with negative (vehicle-treated) and positive (dexamethasone) groups (B). IL-6 levels in compliance with TNF-alpha measured from AT2-treated groups and other control groups (C).

The effect of the AT2 extract on signs of arthritis in the CAIA model was evaluated. Initial experiments were performed using the BALB/c mouse model because of its higher susceptibility to CAIA. On days 0 and 3, BALB/c mice were injected with 3 mg collagen antibody cocktail and 25 μg LPS, respectively. Animals were treated with AT2 at different concentrations from day 4. AT2 administration at doses of 300 mg/kg and 200 mg/kg bw caused statistically significant reductions in the arthritis scores compared with the vehicle-treated controls (*p* < 0.05) (Figure 4A). The arthritis scores were reduced from 9 in the control group to 5.75 and 7 at doses of 300 mg/kg and 200 mg/kg bw, respectively. Accordingly, these scores showed the average reduction of arthritis symptomatic scores to be 36.11 % and 22.22 %, respectively. In addition, mice treated with a daily oral dose of dexamethasone at a dose of 0.5 mg/kg bw (positive control) had excellent dose responsive inhibition of paw swelling over time, with activity peaking at 63.89 % inhibition of arthritis scores (Figure 4A). The IL-6 and TNF-alpha levels in the collected sera were also significantly decreased by AT2 extract at doses of 300 mg/kg and
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200 mg/kg bw compared with those of untreated mice (Figure 4B and 4C). The data were entirely consistent with the level of rheumatoid arthritis symptoms shown in those mouse groups.

The results indicate that the aqueous EtOH extract from A. tonkinensis leaves exerts its anti-inflammatory activity through decreasing arthritis scores. Moreover, reductions in arthritis scores were dose-related and statistically significant at doses of 200 and 300 mg/kg bw. Accordingly, the level of IL-6 and TNF-alpha pro-inflammatory cytokines in the sera of AT2 extract-treated mice showed a relevant reduction.

Flavonoids and their glycosides exhibit their anti-inflammatory effects by several mechanisms [14], along with a wide spectrum of other pharmacological effects such as analgesic, antioxidant, antimicrobial, antiviral, anticancer, anti-diabetic, and antiplatelet activities [15, 16]. Kaempferol, quercetin, alphinonin and their glycosidic derivatives are considered potent immunomodulatory, anticancer, anti-inflammatory activities and antioxidant ability [17, 18]. Therefore, the anti-inflammatory activity of AT2 may be attributed to its total flavonoids. These results suggest that AT2 can inhibit the inflammatory response and may represent a potential therapeutic candidate for the treatment of chronic inflammatory diseases.

4. CONCLUSIONS

The results of the present study indicate that the 70 % ethanol extract from A.tonkinensis leaves exerts anti-inflammatory activity when tested in vitro and in vivo. The in vivo tests indicate that the arthritis scores and pro-inflammatory cytokines were significantly reduced at doses of 200 and 300 mg/kg body weight ($P < 0.05$). These promising results demonstrate that this plant is valuable for preparing functional foods and pharmaceutical medicines to treat inflammatory, rheumatism and joint disorders.

Acknowledgements. The research was supported by a grant (NDT.33.ITA/17) from Vietnam Ministry of Science and Technology and by the Italian MinisterodegliAffariEsteriedellaCooperazioneInternazionale (MAECI).

REFERENCES


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