Psidium guajava leaves decrease arthritic symptoms in adjuvant-induced arthritic rats

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ABSTRACT

BACKGROUND
Guava is an herbal with proven antioxidant and anti-inflammatory properties. The aim of this study was to investigate the anti-arthritic activity of the ethanol extract of Psidium guajava leaves (EEPG) against complete Freund’s adjuvant (CFA) induced arthritis in rats.

METHODS
An experimental study was conducted on 40 male Wistar Sprague Dawley rats, which were divided into 5 groups. Each group was induced with 0.2 mL CFA (1 mg/mL) on day 1 and 0.1 CFA mL booster injection on day 5. Group I served as an arthritic control, group II received dexamethasone (6.75 mg.kg-1 orally), group III, IV and V received EEPG at oral doses of 250, 500, and 750 mg/kg BW, respectively, on days 14 to 28. Anti-arthritic activity was observed from the arthritis score, the paw circumference was measured on days 0, 1, 4, 8, 12, 16, 20, 24, and 28, the mobility score was determined on days 12 and 28, and the histolopathology of the knee joint was examined on day 29.

RESULTS
Ethanol extract of Psidium guajava leaves significantly suppressed the swelling of the paws in chronic phase based on increasing of edema (%), while starting on day 20. EEPG at 250 mg/kg was most effective in significantly reducing arthritis scores (p<0.05). Histopathological examination showed repair of the knee joint synovial membrane and cartilage.

CONCLUSIONS
Psidium guajava leaf extract is effective in decreasing the inflammatory response and arthritic symptoms in rats with adjuvant-induced arthritis. Psidium guajava leaves can be developed into an alternative anti-arthritis treatment.

Keywords: Psidium guajava, antiarthritis, edema, adjuvant induced arthritis, rats
Daun Psidium guajava menurunkan respon inflamasi dan gejala artritis pada tikus terinduksi adjuvant induced arthritis

ABSTRAK

LATAR BELAKANG
Daun jambu biji merupakan salah satu tanaman herbal yang telah terbukti sebagai antioksidan dan antiinflamasi. Penelitian ini bertujuan untuk menyelidiki aktivitas anti artritis dari efek ekstrak etanol daun Psidium guajava (EEPG) terhadap artritis yang terinduksi complete Freund Adjuvant pada tikus.

METODE
Penelitian eksperimantal dilakukan pada 40 ekor tikus Sprague dewley wistar jantan. Mereka dibagi menjadi 5 kelompok dan tiap kelompok diinduksi dengan 0,2 ml CFA (1 mg/ml) pada hari ke-1 dan diberikan injeksi booster dengan 0,1 ml CFA (1 mg/ml) pada hari ke-5. Kelompok I sebagai control arthritis, II diberikan dexamethason (6,75 mg.kg-1 p.o.), kelompok III, IV dan V diberikan EEPG dengan dosis 250 mg/kg BB (p.o), 500 mg/kg BB (p.o) dan 750 mg/kg BB (p.o) pada hari ke 14-28. Aktivitas antiarthritis diamati melalui skor artritis, lingkar kaki diamati pada hari ke 0, 1, 4, 8, 12, 16, 20, 24, dan 28, skor mobilitas diamati pada hari ke 12 dan 28 serta histopatologi sendi lutut diamati pada hari ke-29.

HASIL
Ekstrak etanol daun Psidium guajava mampu menurunkan pembengkakan secara signifikan mulai hari ke 20. EEPG dosis 250 mg/kg BB paling efektif dalam menurunkan skor artritis secara signifikan (p<0,05). Hasil histopatologi sendi lutut menunjukkan perbaikan pada membran sinovial dan kartilago.

KESIMPULAN
Ekstrak daun Psidium guajava efektif menurunkan respon inflamasi dan gejala artritis pada tikus yang terinduksi adjuvant induced arthritis. Daun jambu biji dapat dikembangkan menjadi terapi alternatif antiarthritis.

Kata kunci : Psidium guajava, antiarthritis, edema, adjuvant induced arthritis, tikus

INTRODUCTION
Rheumatoid arthritis (RA) is an autoimmune disease that attacks the joints of the body and causes chronic inflammation in the synovium. Inflammation in RA joints is a very complex process and involves the interaction of a variety of inflammatory cells, autoantibodies and cytokines. Conventional treatments that have been used to overcome arthritis and inhibit the development of RA involve the disease modifying anti-rheumatic drugs (DMARDs). Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world’s population, especially in the developing world. Recently, the use of plant extracts for arthritis treatment is being promoted in the USA, especially after the withdrawal of FDA-approved anti-inflammatory drugs. One of the plants that have the potential to be developed as an antiarthritic drug is the guava plant (Psidium guajava). Guava contains tannins, phenolic compounds, flavonoids, volatile oils, sesquiterpenes and triterpenoids. Flavonoids and phenolic compounds contained in guava have been proven to be antioxidant and anti-inflammatory. Jahagirdar et al. reported that a hydroalcoholic extract of guava leaves at a dose...
of 200 mg/kg/day showed antiarthritic activity. Dutta and Das (7) observed that an ethanol extract of guava leaves at doses of 250 mg/kg and 500 mg/kg body weight significantly inhibited chronic inflammation and reduced the arthritis index. Ojewole (8) suggested that the aqueous extract of guava leaves in the dose range of 50-800 mg/kg administered intraperitoneally had analgesic and anti-inflammatory activity in rats with egg albumin-induced arthritis. The aqueous extract of guava leaves at doses of 125, 250, and 500 mg/kg had antiinflammatory activity and decreased edema in rats, with percentage inhibition of 40.81%, 55.45%, and 43.61%, respectively.(9)

The Freund’s adjuvant model was chosen in this study, as it develops chronic swelling in multiple joints, with influence of inflammatory cells with erosion of joint cartilage and bone destruction. Freund’s complete adjuvant-induced arthritis is a well established rat model and has been widely used for many years for evaluation of the anti-inflammatory and anti-arthritic potential of various agents.(10,11)

Guava leaves has the potential to be developed as an antiarthritic drug. The present study aimed to evaluate the anti-arthritic activity of the ethanol extract of the leaves of *Psidium guajava* (EEPG) against complete Freund’s adjuvant (CFA) induced arthritis in rats, especially with reference to its anti-inflammatory properties, using dexamethasone as reference drug.

**METHODS**

**Research design**

This study used a completely randomized design of undirectional pattern. Freund’s adjuvant induced arthritis model was used to assess the anti-arthritic activity of the ethanolic extract of *P. guajava* in rats. The research was carried out in the Laboratory of Pharmaceutical Biology, Department of Pharmacy, Faculty of Medicine and Health Sciences, Universitas Jenderal Sudirman and the Laboratory of Pathology and Anatomy, Medical Faculty, Gadjah Mada University, Yogyakarta. The study was conducted from August 2013 to February 2014.

**Animals**

The study was conducted in male Sprague Dawley (SD) rats weighing 130-150 g obtained from the animal house in the Faculty of Pharmacy, Gadjah Mada University. Animals were acclimatized to experimental conditions in cages and kept under standard environmental conditions (22 ± 3°C; 12/12 h light/dark cycle). Rats were allowed to feed and water ad libitum.

**Plant material**

Leaves were collected from Karangwakal, Purwokerto. Taxonomic identification of the plant was made by the Laboratory of Taxonomy, Faculty of Biology.

**Preparation of extract**

The powdered leaves (1000g) were successively extracted with ethanol (70-80ºC) for 3x24 h. The macerate was then evaporated in a rotary evaporator for ± 90 minutes in the temperature range of 70-80°C, then evaporated over a water bath until a thick extract was produced, which was stored in the refrigerator until used in the study.

**Experimental animals**

Animals were randomly divided into five groups (n=8). The sample size determination was based on Federer’s formula and the randomization of the test animals in this research used a completely randomized design (CRD) of unidirectional pattern. Group I receiving 1 mg/mL complete Freund’s adjuvant (CFA) served as an arthritic control, group II receiving dexamethasone (6.75 mg.kg-1 p.o.) served as the reference standard, while group III, IV and V received ethanolic extract at doses of 250 mg/kg BW (p.o), 500 mg/kg BW (p.o) and 750 mg/kg BW (p.o), respectively. Each group was induced by 0.2 mL CFA(1 mg/mL) on day 1 and received
a booster injection of 0.1 mL CFA (1 mg/mL) on day 5. To assess the anti-arthritic activity of *P. guajava*, the extracts were given on days 14-28.

**Evaluation of arthritic score**

Each paw was scored on a scale of 0–4 for the degree of swelling, erythema and deformity (maximum score 16 per animal) as follows: 0 = normal, 1 = slight erythema and/or swelling of the ankle or wrist, 2 = moderate erythema and/or swelling of ankle or wrist, 3 = severe erythema and/or swelling of ankle or wrist and 4 = complete erythema and swelling of toes or fingers and ankle or wrist and inability to bend the ankle or wrist. The arthritic score was measured on days 0, 1, 4, 8, 12, 16, 20, 24, and 28.

**Evaluation of mobility score**

Whole animal mobility was scored between 0 and 4 according to the following definitions: 0 = normal, 1 = slightly impaired, 2 = major impairment, 3 = does not step on paw, and 4 = no movement. The mobility score was measured on days 12 and 28.

**Evaluation of paw edema**

The paw edema was measured on days 0, 1, 4, 8, 12, 16, 20, 24, and 28. The mean changes in injected paw edema with respect to initial paw volume, were calculated on respective days and the percentage of inhibition of paw edema with respect to the untreated group was calculated on respective days by the following formula: \[ \text{Increasing of edema (\%)} = \left( \frac{V_t - V_0}{V_0} \right) \times 100\% \]

Vt =Paw edema on day t
Vo = Paw edema on day 0

**Histological processing and assessment of arthritic damage**

Rats were killed by ether anesthesia. Knee joints were removed and fixed for 4 days in 10% formalin. After that the specimens were processed for preparation of paraffin-embedded tissue sections (7 µm thick), which were stained with hematoxylin and eosin.

**Statistical analysis**

The data were expressed and analyzed using SPSS software. Statistical analysis of difference between groups was evaluated by one-way ANOVA followed by LSD test. The value of p<0.05 was regarded as statistically significant.

**Ethical clearance**

This study was accorded ethical clearance by the Commision on Research Ethics in Medicine and Health Sciences, Faculty of Medicine, Universitas Jenderal Soedirman.

**RESULTS**

Injection of CFA increased edema, with a peak increase of 29.46% ± 5.58 on day 12 following the injection of CFA in the arthritic control group (Table 1). Over the 28-day study, rats treated with extract at doses of 250, 500 and 750 mg/kg showed a significant decrease in edema (p<0.05) starting on day 20 up to day 28, as compared to control rats (Figure 1). Dexamethasone, the standard anti-arthritic drug, also showed a significant decrease in edema.

There was an increase in arthritic scores after induction with CFA that indicated systemic inflammation due to the arthritic condition (Figure 1). In the arthritic control group, the arthritis scores remained at their peak level from day 8 to day 24, and started to decrease on day 28. Following the CFA injections, the rats developed arthritis beginning on day 4 and reached a peak level on day 8. Rats treated with dexamethasone and *P. guajava* leaf extract showed a significant decrease in arthritis score.

Throughout the 28-day study, all arthritis groups showed a decrease in mobility scores that was statistically not significant (Table 2). Rats treated with *P. guajava* extract with the dose of 250 mg/kg showed the greatest decrease in mobility scores.
Table 1. The increasing of edema (%) in the control and treated animals groups with *P. guajava* extracts on freund’s adjuvant complete induced arthritis

| Day after adjuvant induction | Control arthritis | Standard drug (Dexamethasone) | *P. guajava* 250 mg/kg | *P. guajava* 500 mg/kg | *P. guajava* 750 mg/kg | P |
|-----------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|---|
| 0                           | 7.81 ± 4.41       | 2.36 ± 0.05       | 2.36 ± 0.05       | 2.36 ± 0.05       | 2.35 ± 0.03       | 0.840 |
| 4                           | 7.81 ± 4.41       | 12.14 ± 3.82      | 14.90 ± 3.84      | 8.99 ± 4.31       | 8.08 ± 3.21       | 0.627 |
| 8                           | 21.13 ± 5.7       | 13.77 ± 3.44      | 17.01 ± 5.72      | 15.81 ± 5.09      | 15.42 ± 3.10      | 0.781 |
| 12                          | 29.46 ± 5.88      | 14.81 ± 2.58      | 17.01 ± 10.55     | 17.44 ± 5.60      | 15.42 ± 4.27      | 0.175 |
| 16                          | 16.33 ± 3.90      | 8.51 ± 3.94       | 14.95 ± 2.72      | 13.29 ± 3.07      | 13.95 ± 3.77      | 0.592 |
| 20                          | 17.39 ± 4.90b     | 4.78 ± 1.50a      | 4.32 ± 1.36a      | 8.02 ± 2.78a      | 14.99 ± 3.94b     | 0.020 |
| 24                          | 20.58 ± 5.68      | 2.49 ± 1.16      | 6.97 ± 1.33a      | 7.99 ± 2.62a      | 11.80 ± 4.02b     | 0.009 |
| 28                          | 17.39 ± 3.76b     | 1.63 ± 1.60b      | 5.37 ± 1.96a      | 7.99 ± 2.74a      | 11.25 ± 4.11b     | 0.009 |

Values expressed in average ± SEM of 8 rats; a= significance at the level of p<0.05 compared by control arthritis; b= significance at the level of p<0.05 compared by standard drug.

Histopathologically, after treatment with dexamethasone (Figure 2B), there was a reduction in the inflammatory condition of the synovial membranes, accompanied by cartilage repair, compared to the arthritis in the knee joints of the controls. The group treated with guava leaf extract at the dose of 250 mg/kg (Figure 2C) also showed a reduction in the inflammatory condition of the synovial membranes, accompanied by cartilage repair, while treatment with guava leaf extract at the doses of 500 mg/kg (Figure 2D) and 750 mg/kg (Figure 2E) showed repair of the synovial membranes.
Table 2. Distribution of mean of mobility score by treatment groups after 12th and 28th day on freund’s adjuvant complete induced arthritis

| Day after adjuvant induction | Arthritic control | Standard drug | P. guajava 250 mg/kg | P. guajava 500 mg/kg | P. guajava 750 mg/kg | p   |
|------------------------------|-------------------|---------------|----------------------|----------------------|----------------------|-----|
| 12th day                    | 1.75 ± 0.16       | 1.37 ± 0.18   | 1.51 ± 0.19          | 1.37 ± 0.18          | 1.37 ± 0.18          | 0.524|
| 28th day                    | 1.62 ± 0.18       | 1.12 ± 0.12   | 1.12 ± 0.12          | 1.25 ± 0.16          | 1.125 ± 0.12         | 0.091|

Values of mobility score expressed in average ± S.E.M of 8 rats

Figure 2. Histopathology of rat knee joint tissues; A (arthritic rat): inflammation of the synovial membrane and cartilage destruction; B (rat on dexamethasone): reduction of synovial membrane inflammation and cartilage repair; C (rat on EEPG 250 mg/kgBB): reduction of synovial membrane inflammation and cartilage repair; D (rat on EEPG 500 mg/kg): synovial membrane repair; E (rat on EEPG 750 mg/kgBB): synovial membrane repair. HE staining, magnification 40x. Synovial membrane (ms), joint cavity (rs), cartilage (k)
DISCUSSION

The present study was carried out to evaluate the effect of *P. guajava* leaf extract in inflammatory disease. The Freund’s adjuvant model was chosen as it induces chronic swelling in multiple joints, with influence of inflammatory cells with erosion of joint cartilage and bone destruction. Freund’s complete adjuvant-induced arthritis is a well-established rat model and has been widely used from many years for evaluation of the anti-inflammatory and anti-arthritic potential of various agents. Nagakura et al. reported that the induction of arthritis in the Freund’s adjuvant model in rats caused joint inflammation, inflammatory cell infiltration, and cartilage and bone destruction. Freund’s adjuvant contains *Mycobacterium tuberculosis* H37Ra or *Mycobacterium butyricum*, which release endotoxins that trigger the production of nitric oxide (NO). NK cells, mast cells, eosinophils, neutrophils, cells and tissues dendritic endothelium are important sources of NO production associated with the immune response, because the activation of macrophages results in the production of nitric oxide (NO). Various inflammatory mediators including cytokines (IL-1, IL-6, interferon γ (IFN γ) and TNF α), which have been implicated in immune arthritis. Various inflammatory mediators including cytokines (IL-1B and TNF-α), MCSF, interferons, and platelet derived growth factor (PDGF) are responsible for the swelling joints, bone deformations, and disability of joint function.

The progress of the arthritic condition was evident around day 12, indicating systemic inflammation. The swelling was found to be increasing in the initial phase of inflammation and becoming constant within 2 weeks (beginning on day 4). These increases in arthritic and mobility scores were found to be associated with chronic inflammation. *Psidium guajava* extract at doses of 250 mg/kg, 500 mg/kg and 750 mg/kg significantly suppressed the swelling of the paws in chronic inflammation over day 28. The *P. guajava* leaf extract was found to be effective in decreasing arthritic scores, mobility scores, and inflammatory responses.

Several studies have shown the antioxidant and anti-inflammatory properties of flavonoids. Porwal et al. reported that *P. guajava* leaf extract contained essential oils, flavonoids, triterpenoids, vitamin C, tannins, and phenolics. Although the actual mechanism of suppression of the arthritic condition is not known, it can be correlated with the presence of flavonoids in suppressing the inflammation and antioxidant activity. Quercetin in *P. guajava* suppressed and inhibited nitric oxide (NO) production, catalyzed by inducible nitric oxide synthase (iNOS). Chen et al. reported that quercetin had antioxidant activity by suppressing the NF-κB signaling pathway. Quercetin-3-O-glucopyranoside and morin in *P. guajava* leaves have antioxidant activity. Vyas et al. reported that *P. guajava* had antioxidant activity and may have the potential to be developed as an anti-inflammatory drug. In a previous study, aqueous extract of *P. guajava* leaves at a dose of 250 mg/ml had anti-inflammatory activity in male albino rats with arthritis induced by carrageenan. From the results observed in the current investigation, it may be concluded that *P. guajava* leaves have a potentially in vivo anti-arthritic activity, and warrants more in-depth investigations on the mechanism of action of its anti-arthritic activity. In practice, it can be developed as an alternative anti-arthritis drug. It is suggested that *P. guajava* leaves may be useful in the treatment of the arthritic condition in rheumatoid arthritis. One limitation of this study was indicated by the animal ethical clearance. The study would have provided better conclusions if validated with a larger sample size.

CONCLUSION

*Psidium guajava* leaf extract is found to be effective in decreasing the inflammatory response and arthritic symptoms in adjuvant-induced arthritic rats.
CONFICT OF INTEREST

All authors declare that there was no actual or potential conflict of interest.

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