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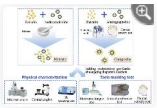

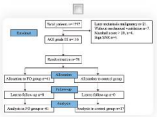

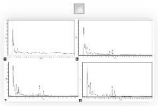

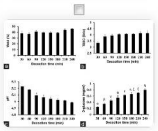

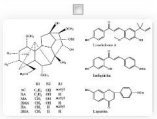

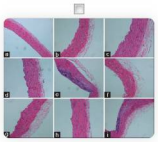

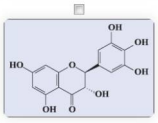

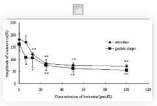

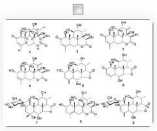

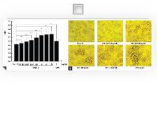

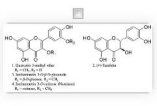

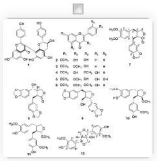

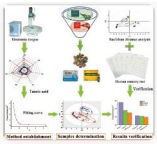

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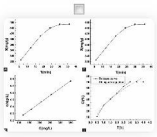

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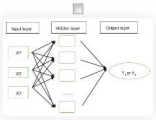

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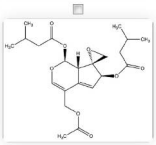

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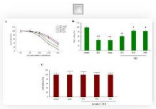

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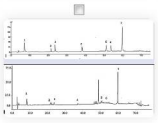

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Effects of *Plantago major* extracts and its chemical compounds on proliferation of cancer cells production of lipopolysaccharide-activated THP-1 macrophages

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Abstract

Background: *Plantago major* has been reported to have anticancer and anti-inflammatory properties. However, its antiproliferative and anti-inflammatory mechani elucidated. Moreover, which plant parts are more suitable as starting materials has not been explored. **Objectives:** To investigate the antiproliferative activity of *P* MCF-7, MDA-MB-231, HeLaS3, A549, and KB cancer cell lines as well as their effects on inflammatory cytokines (tumor necrosis factor [TNF]-a, interleukin [IL]- [IFN]-g) production by lipopolysaccharide (LPS)-stimulated THP-1 macrophages. **Materials and Methods:** The methanol and aqueous extracts of *P. major* from its chemical compounds, i.e., ursolic acid (UA), oleanolic acid (OA), and aucubin were tested in this experiment. **Results:** Methanol and aqueous extracts of *P. ma* greatest antiproliferative activity. The methanol extracts of seeds also demonstrated the highest inhibition of TNF-a, IL-1β, IL-6, and IFN-g production. Interestingly commonly discarded, exhibited comparable activities to those of leaves and petioles. Furthermore, UA exhibited stronger activities than OA and aucubin. **Concl** being proposed as the main source for further development of anticancer and anti-inflammatory products, whereas the roots could be included in the preparat products with respect to anti-inflammatory. **Abbreviations used:** TNF: Tumor Necrosis Factor; IL: Interleukin; IFN: Interferon; HPTLC: High Performance Thin Laye Ursolic Acid; OA: Oleanolic Acid; AUC: Aucubin.

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Summary

Amongst the parts of Plantago major, seeds exhibited the greatest antiproliferative activity against MCF-7, MDA-MB-231, HeLaS3, A549, and KB cell lines as well on TNF-α, IL-1β, IL-6, and IFN-γ productionThe roots, which were commonly discarded, exhibited comparable antiproliferative and cytokines inhibition activities petiolesUrsolic acid, a chemical compound of Plantago major, exhibited stronger activities than oleanolic acid and aucubinThe seeds are being proposed as the development of anticancer and anti inflammatory products, whereas the roots could be included in the preparation of Plantago major derived products with respect t

Introduction

Plantago L. (Plantaginaceae) is a genus widely distributed all over the world, and more than 275 species have been reported.[1]Plantago major [Figure 1] is an er Indonesia. This plant has both perennial and annual growth habit, with ovoid or elliptic leaves. The leaves are normally green, sometimes with purple shading and the surface, with 5–9 veins in parallel venation. Flowering of P. major is a spike which arises up to 30 cm in length. The fruit is a capsule (5 mm in length) which pro centimeter of spike contains 23–26 capsules, and each capsule carries 4–15 seeds.[2],[3],[4] It is used traditionally and commercially available.[5] This herb ca different geographical origins. Previous studies have indicated a variety of beneficial pharmacological effects of P. major such as anticancer,[6],[7],[8],[9] anti immunomodulatory.[6],[12] Concerning its anti-inflammatory activity, several in vivo experiments were carried out to prove this property.[13],[14] Inflammatory re immune cells, macrophages, which are able to produce various inflammatory mediators including cytokines (tumor necrosis factor [TNF]-a, interleukin [IL]-1aβ, IL-6 eicosanoid derived from arachidonic acid, and reactive oxygen species.[15] Various in vitro studies demonstrated the anti-inflammatory potency of P. major an through inhibition of cyclooxygenases and lipoxygenases, enzymes involved in arachidonic acids pathways.[16],[17],[18] Anti-inflammatory activity of P. major inflammatory cytokines, however, has not been well characterized.{Figure 1}

In terms of plant parts, leaves of P. major were most frequently used mainly as aqueous preparations, whereas roots were utilized in limited country, especiall countries, including Indonesia, roots are commonly discarded without any utilization. Nevertheless, our previous studies showed that root extracts exerted cytotoxic cell lines as well as possessed free radical scavenging activity which were comparable to those of the other plant parts.[5] To more understand the anti-inflammator investigated the effect on cytokines (TNF-a, IL-1β, IL-6, and interferon [IFN]-g) production in LPS-activated THP-1 macrophages cells by P. major extracts and it Their antiproliferative effect against KB, MCF-7, MDA-MB-231, A-549, and HeLaS3 cell lines was determined as well.

Materials and Methods

Chemicals

Silica gel, alumina, and high-performance thin-layer chromatography (HPTLC) plates precoated with silica gel 60 F254 were purchased from Merck KGaA (Darms acid (UA), oleanolic acid (OA), aucubin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium pyruvate, glucose, phorbol 12-myristate lipopolysaccharide (LPS, O127:B8) were obtained from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and cytokine kit (MILLIPLEX ® MAP Kit, prod 60K-05) were obtained from Merck KGaA (Darmstadt, Germany). RPMI-1640 medium, Modified Eagle Medium (MEM), fetal bovine serum (FBS), 2-mercaptoetl saline, and penicillin-streptomycin solution were purchased from Gibco Life Sciences (GibcoThai, Thailand). All solvents used were analytical grade and obtai (Bangkok, Thailand).

Plant materials

P. major samples were collected from two locations in Indonesia, i.e. Lumajang and Tawangmangu. Whole plant materials were harvested and cleaned with tap v was separated into leaves (L1), petioles (P1), roots (R1), and seeds (S1) which were used for bioactivity assays. The aerial part of the sample from Tawangma isolation of the chemical compounds. Authentication of the plants (No. 1101/D.T/XI/2013) was conducted by Prof. Sutarjadi (Center of Information and Developm University of Surabaya, Indonesia). All samples were dried and ground as described in previous work.[5]

Preparation of the extracts

P. major extracts were prepared according to the previous study.[5] Briefly, 50 g of plant materials was extracted twice by boiling in 500 ml distilled water for 1 h e filtered and concentrated in vacuo by a rotary evaporator (Buchi, Switzerland) and then were lyophilized to dryness. For the methanol extracts, 10 g of plant materi methanol (3 x 100 ml, 3 x 24 h) at room temperature. The extracts were combined, filtered, and evaporated under vacuum to dryness. The aqueous and meth petioles, roots, and seeds were obtained.

All crude extracts were determined for the antiproliferative activity against several cancer cells in comparison with doxorubicin, an anticancer drug. Moreover, the extracts on cytokines production of THP-1 macrophages were also investigated.

High-performance thin layer chromatography profiling and isolation of the chemical compounds from Plantago major

High-performance thin layer chromatography profiling

To check the TLC profile of P. major extracts, chromatography was performed with two different solvent systems, i.e., (A) toluene: acetone: formic acid (78:22:0.1 xylene: propan-2-ol: 12.5% NH3 (1:2:5:2). Samples were spotted by Camag Linomat 5 sample applicator on the HPTLC plates precoated with silica gel 60 F25 under the first solvent system (A), spotted samples were prederivatized with iodine vapor as described earlier.[5] Development was carried out with 10 ml of ea Camag Twin Trough Chamber (10 cm × 10 cm) previously equilibrated with the mobile phase for 20 min at room temperature. After development, the plates were d subjected to derivatization by dipping in 5% sulfuric acid in methanol. TLC plates were further dried in a fume hood and then heated on a Camag TLC Plate Heate photographs were recorded by Camag Reprostar 3 under white light.

Isolation of the chemical compounds

Two kilograms of aerial part of the sample from Tawangmangu (A1) was extracted with methanol (6 × 10 L) by maceration at room temperature for 6 × 24 h. The then evaporated under vacuum to dryness (333 g). A portion (67 g) of this crude extract was subjected to a vacuum silica gel column chromatography dichloromethane, and methanol by increasing polarity. The like fractions were combined, evaporated in vacuo to dryness, and yielded five fractions (F1–F5). F4 and further subfractionated in an alumina column and eluted by increasing polarity of ethyl acetate and methanol yielding 100 fractions. The combination of fractio then subjected to another silica gel column and development with ethyl acetate and methanol by increasing polarity afforded 177 subfractions. Subfraction 150 exh TLC plate at Rf value of 0.33 in the 1,4-dioxane: xylene: propane-2-ol:12.5%NH3 (1:2:5:2) solvent system. After recrystallization in methanol: ethyl acetate, the whi was obtained. The identification of this isolated compound was confirmed by comparison of the spectral data of infrared (IR), proton nuclear magnetic resonance nuclear magnetic resonance (13C-NMR), and electrospray ionization mass spectrometry (ESI-MS) with those in literature.

Antiproliferation assay on cancer cells

Cell culture

Human breast cancer (MCF-7, ATCC® HTB-22™ and MDA-MB-231, ATCC® HTB-26™), human cervical cancer (HeLaS3, ATCC® CCL-2.2™), human lung HTB-22™), and human nasopharynx carcinoma (KB, ATCC® CCL-17™) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA maintained in MEM or DMEM, supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin, and incubated at 37°C in a humidifiec 5% CO_2 and 95% air.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay

Cellular viability in the presence or absence of P. major extracts and three pure compounds, i.e. UA, OA, and aucubin, was examined using the MTT colorime previously.[19] Briefly, cancer cells under log phase of growth at the density of 1 × 103 cells/ml in 100 ml of culture medium were seeded onto 96-well culture pla MA, USA). After 24 h of preincubation, various concentrations of the tested extracts or pure compounds were added and then incubated for a further 72 h. At the en MTT solution (5 mg/ml) was added to each well and further incubated at 37°C for 3 h. The MTT formazan product was dissolved in DMSO and evaluated by meas 550 nm using a microplate reader (Benchmark 550, Bio-Rad, USA).

Quantification of pro-inflammatory cytokines production in lipopolysaccharide-activated THP-1 macrophages

Cell culture and differentiation

THP-1 (human monocytic leukemia, ATCC® TIB-202™) cell line was grown in suspension in RPMI-1640 medium, supplemented with 0.05 mM 2-mercaptoethanol, sodium pyruvate, 990 mg/L glucose, 10% FBS, and 100 U/ml penicillin-100 µg/ml streptomycin. The cells were maintained in a humidified incubator (5% CO2 changed once every 3–4 days. Cells were disposed and changed by frozen cells every 20 passages.

The macrophage-like state was obtained by treating THP-1 monocytes for 48 h with 40 nM PMA in FBS-free medium at a density of 5 × 105 cells/ml in 96- and 24-v ml, respectively). Furthermore, PMA-stimulated THP-1 cells (referred to as THP-1 macrophages) were washed with FBS-free medium and rested for another 24 h and treatments were then initiated.

Cytotoxicity test

Cytotoxicity of P. major extracts and its pure compounds on THP-1 macrophages was assessed using the MTT method. THP-1 macrophages were incubated with a extract (1–1000 µg/ml) in 96-well plate for 48 h. Cells were subsequently incubated with 100 µl of 1 mg/ml MTT in FBS-free medium for 3 h. After centrifugation (12(the medium was aspirated. The formazan product was dissolved in 100 µl DMSO, and the absorbance was further measured at 550 nm using a microplate rea formazan formed in control cells (without extracts or pure compounds) was referred as 100% viability.

Multiplex quantification of pro-inflammatory cytokines production in lipopolysaccharide-activated THP-1 macrophages

THP-1 monocyte cells were plated at a density of 5 × 105 cells/ml in 24-well plate, differentiated with PMA, and subsequently incubated with the corresp compounds in appropriate concentrations. One hour later, LPS was added to each well at the final concentration of 5 µg/ml and incubated for another 47 h. At th plates were centrifuged (1200 rpm) for 5 min at 4°C. The supernatant in each well was aspirated and kept in −80°C until cytokines determination. TNF-α, concentrations in the supernatants were quantified simultaneously using MILLIPLEX® MAP kit according to the manufacturer's instruction (Merck KGaA, Darmstadt,

Statistical analysis

All values were presented as mean ± standard error mean or mean ± standard deviation, n = 2–3. For multiple variables comparison, data were analyzed by ANOV multiple comparison and Tukey's test when necessary using GraphPad Prism statistical software (GraphPad Software Inc., Sandiego, California, Windows Version considered statistically significant at $P < 0.05$.

Results

High-performance thin layer chromatography profiling and chemical structure of the isolated compound

To evaluate the chemical composition of P. major, HPTLC profiling of each extract was carried out using two different systems [Figure 2]. It was shown that all value: 0.53), whereas OA (Rf value: 0.71) could be found in petioles and seeds only. Isolation of UA from the aerial part as well as determination of UA and OA c parts of P. major has been reported in our previous study.[5] Contrastingly, aucubin (Rf value: 0.39) was detected in all extracts [Figure 2]b.{Figure 2}

The isolated compound from this study was a white crystal with melting point 174–177°C, UV lmax (MeOH) 225.5 nm. IR nmax(KBr) 3289.83, 2914.38, 2882.50, 1480.94 cm−1; ESI-MS m/z 369.1169 [M + Na]+;1 H NMR (DMSO-d5) d 4.99 (1H, m, H-1), 6.27 (1H, dd, H-3), 4.81 (1H, d, H-4), 2.49 (1H, m, H-5), 4.27 (1H, br H-7), 2.71 (1H, br t, H-9), 3.93 (1H, d, H-10A), 4.13 (1H, d, H-10B), 4.48 (1H, d, H-1'), 2.94–3.65 (1H, H-2'-6'B);13 C NMR (DMSO-d5) d 95.4 (C-1), 140.1 (C-3), 80.5 (C-6), 129.2 (C-7), 146.2 (C-8), 46.5 (C-9), 59.6 (C-10), 98.2 (C-1'), 73.5 (C-2'), 76.2 (C-3'), 70.2 (C-4'), 77.2 (C-5'), 61.1 (C-6'). These results were in c previously reported for aucubin.[20] Therefore, it can be concluded that the isolated compound was aucubin [Figure 3].{Figure 3}

Effects of Plantago major extracts and its chemical compounds on cancer cells proliferation

Antiproliferative activity of P. major extracts and its chemical compounds against various cancer cells [Table 1] was evaluated in vitro by the MTT colorimetric ass tested, methanol extracts of P. major seeds exhibited the most potent antiproliferative activity against five cancer cell lines used with IC50 values between 153.3 terms of pure compounds, UA showed the strongest antiproliferative activity against all cancer cell lines used with IC50 values between 6.27 and 18.33 µg/ml, follo between 17.63 and >100 µg/ml), whereas aucubin had the lowest effects.{Table 1}

Effects of Plantago major extracts and its chemical compounds on pro-inflammatory cytokines production in lipopolysaccharide-activated THP-1 macrophages

Cytotoxicity against THP-1 macrophage cells

In this study, methanol extracts of leaves, petioles, roots, and seeds of P. major were used for cytokines production assay. Before cytokines assay, potential cytotox pure constituents on THP-1 macrophages was evaluated. Leaves, petioles, and seeds extracts reduced significantly cell viability at concentrations ≥300, respectively. However, root extracts did not show any cytotoxic effect under the concentrations applied. Both UA and OA reduced significantly cells viability at co whereas cytotoxicity due to aucubin was observed at 1000 µg/ml [Figure 4]. Considering this cytotoxic effect, the cytokines production assay was conducted at th noncytotoxic effects.{Figure 4}

Pro-inflammatory cytokines production in lipopolysaccharide-activated THP-1 macrophages

THP-1 macrophage cells were incubated with various concentrations of either extracts or pure compounds for 48 h in the presence of LPS. In these exposed cells was analyzed and compared to LPS-stimulated cells. [Figure 5] shows that treatment of leaves, petioles, and roots extracts at low concentrations increased the le decreased it at high concentrations. Seeds extract slightly suppressed TNF-a production at both concentrations. UA, OA, and aucubin suppressed TNF-a producti used, in which the effect of UA was in a dose-dependent manner.{Figure 5}

Regarding IL-6 secretion [Figure 6], treatment of activated cells with any of P. major extracts increased this cytokine at low concentrations but inhibited concentrations. IL-6 secretion was reduced significantly by UA at all concentrations, and treatment with high concentration reached the basal production of nonact the inhibition effect of UA was in a dose-dependent manner. OA and aucubin slightly decreased IL-6 secretion.{Figure 6}

IL-1β secretion [Figure 7] was stimulated when activated cells were incubated with leaves, petioles, and roots extracts. On the contrary, seeds extract slightly inh IL-1β. All pure compounds tested decreased significantly IL-1β production.{Figure 7}

In general, all of P. major extracts decreased the IFN-g production [Figure 8]. The only one sample had a contrary effect, i.e., roots extract at the concentration of 10 was observed as well as in UA, OA, and aucubin.{Figure 8}

Discussion

P. major is one of the herbs widely used in cancer and pain relief management.[3] The quality of herbs may fluctuate depending on plant parts, growing environ solvents and methods of extraction, as well as many other postharvest factors.[21] In this study, we evaluated antiproliferative activity of P. major extracts and i against various cancer cell lines. We investigated as well as their effect on the production of inflammatory cytokines. P. major extracts were prepared from dif methanol and water as extracting solvents.

HPTLC profiles, [Figure 2], exhibited that UA, OA, and aucubin are clearly found in P. major. Anticancer activity of UA and OA has been actively investigated in [24],[25] Moreover, these two compounds along with aucubin have been known as anti-inflammatory agents.[26],[27],[28],[29],[30] In this recent work, we isolated part of P. major, whereas the isolation of UA was reported in our previous study.[5]

In terms of antiproliferative activity, methanol extracts of all parts of P. major demonstrated cytotoxicities on five cancer cell lines in various potencies [Table 1], a was shown by the seeds extract (IC50 value was between 153.38 and 247.41 μg/ml). Water extract of P. major seed was active against KB, MCF-7, MDA-MB-2 whereas the aqueous extracts of leaves, petioles, and root were active on MCF-7 only. These results were in concordance with the observations of Chiang et al. that aqueous extract of the whole plant of P. major showed cytotoxicities against various carcinoma cells with IC50 values ranged from 283 to 1809 μg/ml.[6] We roots had comparable cytotoxic effect with leaves and petioles. This finding supported our previous report[5] that roots could be included in the preparation of medicines. Further, toxicity and bioactivity tests are required to ensure its safety and efficacy.

Among the three pure chemicals utilized, UA showed the strongest cytotoxicity against five cancer cells tested, followed by OA. This finding confirmed our previou UA as one of the active markers for quality assessment of P. major.[5] Contrastingly, aucubin did not express cytotoxic effect (IC50 >100 μg/ml). This finding was in al. whose investigation showed that aucubin did not have a cytotoxic effect toward leukemia P388, but its aglycone (i.e., aucubigenin) did.[31]

Cytokines are soluble messengers having a fundamental role for cell–cell communication and regulation within the immune system. Macrophages release cytokine other cells during inflammation or as direct killing agents.[32] However, excessive production of this mediator in an inflammatory site may lead to chronic diseases effects of P. major extracts on the production of pro-inflammatory cytokines (TNF-α, IL-1 β, IL-6, and IFN-g) were determined.

Studies have shown that P. major extracts were able to alter the production of inflammatory cytokines. Levels and patterns of inflammatory cytokines production we for different parts of P. major, which may be due to the content of chemical compounds. Leaves, petioles, and roots extracts showed a similar effect on TNF-a and I 5] and [Figure 8]. These samples enhanced the production of TNF-a at low concentrations but decreased it at high concentrations and decreased the level of IFN- Leaves extract increased IL-6 at all concentrations, whereas petioles and roots increased this cytokine at low concentrations and decreased it at high concentra and roots extracts induced production of IL-1β at all tested concentrations, whereas petioles extract increased its production at low concentrations and decreased [Figure 7]. Our results seemed to be in concordance with the observations of Chiang et al. whose results revealed that P. major extracts enhanced the immu concentrations, whereas inhibited these effect at high concentrations. Our findings support the concept of dual immunomodulatory activity owned by P. major.[6]

Seeds extract exhibited different patterns. It inhibited secretion of TNF-a, IL-1β, and IFN-g in the range of concentrations used; however, it slightly increased IL- whereas decreased it at high concentrations. If we compare among P. major parts at equivalent concentrations, seeds caused the lowest production of TNF-a, IL-1 effectiveness might be caused by its high content in UA [5] which showed the greatest inhibition activity among the chemical compounds tested.

Various trends in the modulation of cytokines levels were exhibited by the pure compounds. UA was capable of inhibiting the production of TNF-a, IL-6, IL-1 β, an triterpenoid, having contrary anti- and pro-inflammatory activities that are dependent on the biological status of cells and tissues.[26] Our recent results, UA inh cytokines production in LPS-induced THP-1 macrophages, were in concordance with the previous study.[26]

We observed that OA and aucubin inhibited TNF-a, IL-1 β, and IFN-g; however, they did not exhibit any effect on IL-6. These findings were in agreement with t showed that a series of OA analogs had weak inhibition toward IL-1β production in LPS-induced RAW 264.7 and J774A.1 cells.[33]

From this study, we observed that roots extracts possessed comparable antiproliferative and cytokines production activities to the other parts (leaves and petioles) roots could be included in the preparation of P. major derived herbal medicines. Seeds extract showed the highest antiproliferative effect as well as inhibition of production. Among the pure constituents investigated, UA exhibited the strongest activities. Our previous study showed that UA was found abundantly in seeds, wh [Figure 2]b showed that roots contain a high amount of aucubin. Therefore, in addition to UA,[5] we proposed aucubin as another marker for standardization of P. m

Conclusions

The results of this study support the existing data and the empirical uses of P. major. Five cancer cell lines were used in antiproliferative assay to strengthen t cancer management. Anti-inflammatory assay of P. major through modulation of cytokines production was carried out to afford its comprehensive mechanism. We methanol extract of seeds possessed the greatest activity both on antiproliferative and cytokines production assay. Roots extract exhibited comparable activities The seeds are being proposed as the main source for further development of anticancer and anti-inflammatory products. The roots could be included in the prepar products with respect to anti-inflammatory. It could also be considered as the source of aucubin.

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Conflicts of interest

There are no conflicts of interest.

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