The Effect of Ursolic Acid from *Plantago Lanceolata* Leaves on Leukocytes Migration and Chemokines Level

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**ABSTRACT**

Initially considered as a normal body response to injury, inflammation is currently known as a major event contributing to the development of many human disorders. Many drugs and bioactive molecules have been discovered from medicinal plants and the number is still growing by time. Among those medicinal plants used in folk medicines, *Plantago lanceolata* is used to cure inflammatory-related diseases. In our previous study, we showed that the n-hexane insoluble fraction of *P. lanceolata* leaves (HIF) demonstrated a potent anti-inflammatory activity by inhibiting leukocytes migration in mice. This study aimed to identify the active compound from HIF which responsible for the inhibition of leukocytes migration in mice, and to investigate the effect of the active compound on the level of chemokine (IL-8 and MCP-1) responsible for leukocytes migration. *P. lanceolata* leaves were initially macerated with dichloromethane. The dried extract was partitioned using n-hexane to obtain n-hexane soluble fraction (HSF) and n-hexane insoluble fraction (HIF). Both fractions were evaluated for their anti-inflammatory activity in thioglycollate-induced leukocyte migration. The active fraction (HIF) was subjected to a preparative thin-layer chromatography to isolate the major compound. The structure of the isolated compound was identified based on NMR, IR, and Mass spectra. The compound was identified as ursolic acid, based on its spectral data. Ursolic acid at dose of 30, 60, and 120mg/kg BW inhibited leukocyte migration and reduced the level of IL-8 and MCP-1.

**Keywords:** *Plantago lanceolata*, anti-inflammatory agents, MCP-1, chemokine CCL2, interleukin-8

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**INTRODUCTION**

Leukocyte recruitment from the blood circulation to the site of inflammation plays an important role in inflammation. This process occurs during the development of inflammation and is mediated by pro-inflammatory mediators called chemoattractant cytokines (chemokines). These chemokines are produced to induce leukocyte migration. Inhibiting any of the events can severely interfere with leukocyte migration to the site of inflammation (Chavakis *et al.*, 2005; Muller, 2013). The leukocyte migration across endothelial cells to inflammatory sites is guided by a chemokine gradient. Important chemokines that play a crucial role in leukocyte migration are IL-8 (Gonzalez-Aparicio and Alfaro, 2019) and MCP-1 (Balamayooran *et al.*, 2011). The roles of chemokines in leukocyte migration in inflammation have been established in many studies (Charo and Taubman, 2004; Turner *et al.*, 2014). Thus, inhibiting leukocyte migration by reducing chemokine levels is a promising therapeutic target in inflammatory-related diseases (Del Prete *et al.*, 2015; Hopkin *et al.*, 2019).

Medicinal plants provide a broad spectrum of chemical structure diversity regarding bioactive molecules.
natural products. Natural compounds derived from herbal medicines are being increasingly explored to develop therapeutic agents for various diseases, including inflammatory disorders. *Plantago lanceolata* has been used traditionally as an herbal remedy for inflammatory disorders that occur in the mouth, skin, respiratory tract, pharyngeal mucosa, and throat (Dawid-Pać, 2013; Vogl *et al*., 2013). However, only a few scientific data are available concerning the anti-inflammatory compounds from *P. lanceolata*. Previous studies showed that dichloromethane extract of the *P. lanceolata* leaf showed potent anti-inflammatory activity by inhibiting NF-κB activation (Vogl *et al*., 2013).

Further separation of the extract with n-hexane yielded n-hexane insoluble (HIF) and n-hexane soluble (HSF) that represent relatively non-polar and polar fractions, respectively. An anti-inflammatory study of HIF in a murine model showed that HIF exerted anti-inflammatory activity by inhibiting cyclooxygenase-2 (COX-2) activity and reducing chemokine levels (Fakhrudin *et al*., 2017). However, the anti-inflammatory compound responsible for the activity has not been identified. Therefore, this study aimed to isolate and identify the anti-inflammatory compound from the HIF and to determine the anti-inflammatory activity of the HIF in thioglycollate-induced leukocyte migration. The effect of the active compound at the chemokine levels (MCP-1 and IL-8) was also evaluated.

**MATERIAL AND METHODS**

**Animals**

Male mice (Balb/C strain, 6 months old, 20-30g weight,) were from the Laboratory of Pharmacology and Toxicology, Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, UGM. The animals were given free access to both a standard commercial pellet diet and water. They were kept in the animal house with a 12h light/dark cycle at an ambient temperature of 25°C. The protocols were in accordance with the guideline of the Ethic Committee at the Integrated Research and Testing Laboratory, UGM (Ethical clearance number 102/KEC-LPPT/V/2013).

**Plant material**

The leaves of *P. lanceolata* were collected from Tawangmangu district, Karanganyar, Jawa Tengah, Indonesia. The identity of plant species was authenticated (certificate number BF/78/Ident/Det/III/2013) by Dr. Djoko Santosa, the botanist in the Faculty of Pharmacy, Universitas Gadjah Mada. Fresh leaves of *P. lanceolata* were washed with water, cut into smaller-sized pieces, and allowed to overnight at room temperature. The leaves were further dried in the oven (50°C for 48h). Finally, the dried leaves were pulverized for further processing.

**Extraction, fractionation, isolation and identification**

The powdered leaves (1.7kg) were macerated in 5L of dichloromethane for 24h. The filtrate was separated in a vacuum-assisted Buchner funnel. The maceration process was done three times (each with 5L dichloromethane), and the filtrates were combined and evaporated in a rotary evaporator (Heidolph; Schwabach, Germany) to obtain a dry extract (46g). The extract (2g) was dissolved in 20mL n-hexane assisted by intensive stirring to give n-hexane soluble (HSF) and n-hexane insoluble (HIF) fractions. This process was repeated three times until colorless HSF was obtained. The HIF was collected and dried (0.8g).

After anti-inflammatory evaluation, the active fraction (HIF) was subjected to vacuum column chromatography on silica gel (Emerck; Darmstadt, Germany). The column was eluted first with n-hexane followed by n-hexane:ethyl acetate (6:1 v/v); next by the portion of 4:1; and 2:1 v/v; and then by ethyl acetate and finally, by methanol. The fractions were collected according to the order of separation to give six combined fractions (F1–F6): F1 (460mg); F2 (145mg); F3 (364mg); F4 (245mg); F5 (555mg); and F6 (429mg). The chemical profile of each fraction was monitored by a TLC on a silica gel 60 F254 (Emerck; Darmstadt, Germany) with n-hexane:ethyl acetate (4:1) as the mobile phase and Cerium(IV) sulfate (Sigma-Aldrich; Missouri, USA) as a reagent to visualize the spots. F3–6 showed similar TLC profiles and contained the same major compound characterized by a dominant spot on the TLC profile. This major compound was isolated using preparative TLC.

In the preparative TLC, the fractions were applied as a band into glass-supported preparative TLC plates. The band on the plates was eluted with n-hexane:ethyl acetate (4:1) as the mobile phase in the preparative TLC chamber saturated previously with the mobile phase. The silica-containing band of the major compound was scraped gently and collected. The compound was extracted from the silica using methanol assisted...
by intensive magnetic stirring. The silica was separated by filtering to give the isolated compound (174mg). The structure of the isolated major compound was elucidated by interpreting the UV, NMR, IR, and mass spectra. UV spectra was recorded on UV spectrophotometer (Hitachi U-2800; Tokyo, Japan). DELTA2 500MHz spectrometer (Jeol; Massachusetts, USA) was used to gain NMR spectra. The compound was dissolved in CDCl$_3$ and the instrument was operated at 500MHz and 125.76MHz for $^1$H- and $^{13}$C-NMR, respectively, with tetramethylsilane as an internal standard. The IR spectra was recorded on a Perkin Elmer Spectrum 100 FTIR Spectrometer (Massachusetts, USA) in the range of 4000-400cm $^{-1}$. The LC-MS data were generated using Mariner Biospectrometry (Cambridge Scientific; Massachusetts, USA) integrated with the high performance liquid chromatography (HPLC) instrument (Hitachi L 6200; Tokyo, Japan) equipped with a Q-TOF mass spectrometer fitted with an ESI source ($m/z$ 39 to 1200).

**Leukocyte migration assay**

The leukocyte migration assay was done according to the protocol from previous studies (Fakhrudin et al., 2014). Briefly, the mice were divided into control and treatment groups (5 mice per group). The treatment groups were given intraperitoneal (i.p., 0.2mL) injections of the fractions, isolated compound, or indomethacin (Sigma-Aldrich; Missouri, USA) whereas the control group received solvent (DMSO–saline). After 30min, 0.5mL of sterile thioglycollate (4%) was injected (i.p.) to induce leukocyte migration. The animals were sacrificed after 4h and the peritoneal lavage was collected and centrifuged (10min, 1200rpm at 4°C) to obtain cell pellets. The cells were resuspended in 1mL PBS and stained with methylene blue. The number of cells was counted by direct microscopic counts using a Neubauer chamber (Assistent; Sandenheim, Germany).

**Determination of chemokine levels**

The levels of MCP-1 and IL-8) in the peritoneal lavage were detected using an ELISA kit (Cusabio CSB-E07274m and CSB-E07430m, respectively), and were measured in an ELISA plate reader (Pioway RT-2100C; Nanjing). The assay protocol was done according to the instructions recommended by the manufacturer. The activity of the fractions or compound to reduce chemokine levels was expressed as a percent of reduction compared with the solvent-treated group.

**RESULT AND DISCUSSION**

Inflammation plays a crucial role in the development of inflammatory disorders such as obesity, arthritis, atherosclerosis, asthma, diabetes, sarcopenia, hepatitis and Alzheimer’s disease (Chung et al., 2011). The discovery of inflammatory agents from natural products, especially medicinal plants remains challenging as many of the modern drugs developed today are derived from plants (Calixto, 2019). Our previous study showed that the dichloromethane extract of the P. lanceolata leaf showed anti-inflammatory activity by inhibiting NF-$\kappa$B (Vogl et al., 2013). NF-$\kappa$B represents a promising target in inflammation and this protein regulate the production of pro-inflammatory cytokines and mediate inflammatory development. In the previous study, we found that the n-hexane insoluble fraction (HIF) of P. lanceolata dichloromethane extract is the active fraction with anti-inflammatory activity in mice (Fakhrudin et al., 2017). In this study, we isolated the major compound from the HIF and evaluated its anti-inflammatory activity by employing thioglycollate-induced mice peritonitis as pharmacological evidence. This method represents a simple bioassay to confirm the anti-inflammatory of a compound based on the inhibition of leukocyte migration (Cash et al., 2009; Hermida et al., 2017).

The HIF and HSF were evaluated for their anti-inflammatory activities and the chemical profiles of both fractions were presented in our previous study (Fakhrudin et al., 2017). The HIF demonstrates a higher activity compared with the HSF at 400mg/kg BW (Figure 1 and Table I). This finding indicates that the most active anti-inflammatory compound might be present in the HIF. Thus, we focused on the HIF and further separated it by using vacuum column chromatography that resulted in six fractions. The TLC profile of the fractions indicated the presence of a major compound (Figure 2). We further isolated this major compound using a preparative TLC. The structure of the isolated major compound was determined based on spectral data ($^{13}$C- and $^1$H- NMR, MS, IR, UV). We identified that compound as ursolic acid (174 mg) (Figure 2). The spectra of the isolated compound were compared with those of ursolic acid in the literature. The isolated ursolic acid appeared as a white powder with a m.p. of 197°C (as a reference (Hossain and Ismail, 2013) m.p 195°C).
Table I. The anti-inflammatory activity of n-hexane insoluble fraction (HIF) and n-hexane soluble fraction (HSF) (400mg/kg BW) in thioglycollate-induced leukocyte migration in mice. The mice (5/group) were pretreated (i.p.) with the fractions (HIF or HSF), indomethacin (36mg/kg BW), or solvent (DMSO–saline) for 30min before stimulation of leukocyte migration using thioglycollate (i.p.). After 4h, the mice were sacrificed, and the total leukocytes were counted. HIF: n-hexane insoluble fraction; HSF: n-hexane soluble fraction of the *P. lanceolata* leaf.

| mice | control | solvent | indomethacin | HSF | HIF |
|------|---------|---------|--------------|-----|-----|
| 1    | 7.63    | 100.00  | 11.13        | 88.55 | 32.74 |
| 2    | 6.80    | 100.00  | 11.19        | 86.86 | 31.33 |
| 3    | 7.03    | 100.00  | 12.13        | 88.65 | 34.21 |
| 4    | 7.89    | 100.00  | 10.32        | 90.19 | 34.09 |
| 5    | 7.21    | 100.00  | 10.89        | 89.11 | 32.47 |
| mean | 7.31±0.44 | 100.00  | 11.13±0.66   | 88.67±1.20 | 32.97±1.20 |

*the number of migrated leukocytes in the solvent-treated group was set as 100% of leukocyte migration upon thioglycollate induction.

Figure 1. Hexana insoluble fraction inhibited leukocyte migration in thioglycollate-induced mice. The mice were pretreated with fractions (HSF or HIF at dose of 400 mg/kg BW), solvent (DMSO–saline), or indomethacin (36 mg/kg BW) 30min before the induction of leukocyte migration using thioglycollate (i.p.). The data were means ± standard errors (**p < 0.01; ANOVA/Dunnett) and were relative to the solvent-treated group (n = 5). HSF: n-hexane soluble fraction; HIF: n-hexane insoluble fraction of the *P. lanceolata* leaf.

Figure 2. The structure of ursolic acid and TLC profile of ursolic acid in the HIF. HIF (1), and ursolic acid (2) were applied on the silica gel F254 TLC plate and developed using n-hexane:ethyl acetate (4:1). The TLC plates were visualized at UV366 nm (A); and sprayed with Cerium sulfate reagent, documented under visible light (B); and UV366 nm (C).
The Effect of Ursolic Acid on Leukocyte Migration and Chemokines Level

ESI-MS m/z 457 [M+H]+ (calc. for C30H48O3); UV: 210 nm; IR (KBr) vmax in cm⁻¹: 3406 (OH), 1092 (C-O), 2924 (CH), 1690 (C=O) 1458 (C=C); ¹H-NMR (500 MHz, CDCl₃): δ 3.21 (1H, dd, J = 2.2, 9.1 Hz, H-3); 5.25 (1H, t, J = 1,5; H-12), 2.17 (1H, d, J = 11.0 Hz, H-18), 1.28 (3H, s, H-23), 0.91 (3H, s, H-24), 1.13 (3H, s, H-25), 0.98 (3H, s, H-26), 1.25 (3H, s, H-27), 0.94 (3H, d, J = 6.5 Hz, H-29), 1.08 (3H, d, J = 6.0 Hz, H-30). ¹³C-NMR (500 MHz, CDCl₃): δC 39.2 (C-1), 27.4 (C-2), 79.2(C-3), 38.9 (C-4), 77.4 (C-5), 17.2 (C-6), 33.1 (C-7), 48.1 (C-8), 76.9 (C-9), 37.2 (C-10), 17.1 (C-11), 126.0 (C-12), 138.1(C-13), 52.8(C-14), 29.4 (C-15), 23.8 (C-16), 55.3 (C-17), 77.2 (C-18), 39.6 (C-19), 47.6 (C-20), 29.9 (C-21), 38.9 (C-22), 28.3 (C-23), 15.6 (C-24), 14.3 (C-25), 15.7 (C-26), 23.5 (C-27), 180.4 (C-28), 21.4 (C-29), 18.5 (C-30). The ¹³C-NMR, ¹H-NMR, and mass spectra data of the isolated ursolic acid (Figure 3).

The ursolic acid isolated in this study has a similar spectrum to that identified in previous publications (Geană et al., 2014; Hossain and Ismail, 2013). The presence of ursolic acid in the P. lanceolata leaf was also reported previously (Baumgartner et al., 2011). Interestingly, ursolic acid was also found in P. major, a plant from the same Genus that has a wider distribution (Ringbom et al., 1998).

Figure 3. The spectra of ursolic acid isolated from the leaves of P. lanceolata obtained from NMR spectroscopy and Mass spectrometry analysis. (A) ¹H-NMR; (B) ¹³C-NMR spectra; (C) Mass-Spectra.
lic acid inhibited leukocyte migration induced by thioglycollate. The ursolic acid inhibited leukocyte migration (Figure 4). The migration of leukocytes to inflammatory sites is directed by the chemokines, such as MCP-1 and IL-8 (Gerszten et al., 1999; Shtein et al., 2012). To better understand the mechanism of action of ursolic acid in inhibiting leukocyte migration, we evaluated whether chemokines (IL-8 and MCP-1) expression was inhibited. Indeed, ursolic acid demonstrated a potent reduction in chemokine levels (Figure 5). Interestingly, ursolic acid reduced the MCP-1 level with a higher potency than IL-8. This result was in line with the previous study demonstrating that MCP-1 is the main chemokine responsible for leukocyte migration (Fakhrudin et al., 2017; Takahashi et al., 2009).

This suggests that ursolic acid might be further examined for its efficacy against inflammatory-related diseases in which MCP-1 plays crucial roles such as diabetic nephropathy (Tesch, 2008), cardiovascular diseases (Epstein et al., 2009), rheumatoid arthritis (Ellingsen et al., 2000), and angiogenesis (Chang et al., 2005). This study calculated the total migration of leukocytes as a readout of inflammatory development. There are five different types of leukocytes (neutrophils, basophils, eosinophils, monocytes, and lymphocytes) with specific roles in inflammation. Among these cells, neutrophil plays a dominant role in the development of inflammatory related diseases (Wright et al., 2010). Interestingly, the dynamic expression of leukocyte innate immune genes influenced the pathogenesis of acute and chronic inflammation (Vinther et al., 2015). Further study might be required to reveal the dynamic changes of leukocytes composition upon ursolic acid treatment and its consequences for inflammatory development.

Consistent with our findings, previous studies also showed the in vivo anti-inflammatory activities of ursolic acid in different models (Checker et al., 2012; Kashyap et al., 2016; Tapondjou et al., 2003). Nevertheless, our study provides an update that ursolic acid inhibited leukocytes migration by reducing the level of chemokines (IL-8 and MCP-1). Since targeting leukocyte migration induced by chemokines is a promising approach to resolve inflammation (Hopkin et al., 2019), ursolic acid might represent a potential compound to be developed as an anti-inflammatory agent. Although ursolic acid has been reported to exert anti-inflammatory activity by inhibition of pro-inflammatory transcription factor such as NF-κB,
The Effect of Ursolic Acid on Leukocyte Migration and Chemokines Level

NF-AT, and AP-1, this result reveals an additional anti-inflammatory mechanism of action of ursolic acid (Checker et al., 2012). We also provides a scientific evidence for the traditional practice of utilizing the *P. lanceolata* leaf as an herbal remedy to treat inflammatory disorders (Dawid-Pač, 2013).

CONCLUSION

In summary, we reported that ursolic acid from *P. lanceolata* leaves demonstrated an anti-inflammatory activity by inhibiting leukocyte migration in mice. Additionally, the level of chemokines responsible for the leukocyte migration, IL-8 and MCP-1 were also reduced after ursolic acid treatment. Ursolic acid is an important pharmacological marker for *P. lanceolata*, which represents a promising plant for combating inflammatory-related disorders.

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258 Volume 30 Issue 4 (2019)
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