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Immunomodulatory of sesquiterpenoids and sesquiterpenoid dimers-based toll-like receptor 4 (TLR4) from *Dysoxylum parasiticum* stem bark

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In recent decades, the interest in natural products with immunomodulatory properties has increased due to their therapeutic potential. These products have a wider range of pharmacological activities and demonstrate lower toxicity levels when compared to their synthetic counterparts. Therefore, this study aimed to investigate the immunomodulatory effects of sesquiterpenoids (SQs) and sesquiterpenoid dimers (SQDs) isolated from *Dysoxylum parasiticum* (Osbeck) Kosterm. stem bark on human and murine cells, particularly focusing on toll-like receptor 4 (TLR4). Utilizing the secreted alkaline phosphatase (SEAP) assay on engineered human and murine TLR4 of HEK-Blue cells, antagonist TLR4 compounds were identified, including SQs 6, 9, and 10, as well as SQDs 17 and 22. The results showed that 10-hydroxyl-15-oxo- α -cadinol (9) had a potent ability to reduce TLR4 activation induced by LPS stimulation, with minimal toxicity observed in both human and murine cells. The SEAP assay also revealed diverse immune regulatory effects for the same ligand. For instance, SQs 12, 14, and 16 transitioned from antagonism on human to murine TLR4. The SQs (4, 7, 11, and 15) and SQDs (18–20) offered partial antagonist effect exclusively on murine TLR4. Furthermore, these selected SQs and SQDs were assessed for their influence on the production of proinflammatory cytokines TNF- α , IL-1 α , IL-1 β , and IL-6 of the NF- κ B signaling pathway in human and murine macrophage cell lines, showing a dose-dependent manner. Additionally, a brief discussion on the structure-activity relationship was presented.

Keywords *Dysoxylum parasiticum* (Osbeck) Kosterm., Sesquiterpenoids and sesquiterpenoid dimers, Immunomodulatory TLR4, Proinflammatory cytokines

Abbreviations

SQs	Sesquiterpenoids
SQDs	Sesquiterpenoid dimers
TLR4	Toll-like receptor 4
hTLR4	Human TLR4
mTLR4	Murine TLR4
LPS	Lipopolysaccharide

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LBP	LPS-binding protein
LOS	Lipopolysaccharide
CD14	Cluster to differentiation 14
MD-2	Myeloid differentiation factor 2
SEAP	Secreted alkaline phosphatase
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B
THP-1	Tohoku hospital pediatrics-1
RAW 264.7	Ralph, raschke, Watson 264.7
TNF- α	Tumor necrosis factor
IL-1/6	Interleukin-1/6
ASC	Apoptosis-associated speck-like protein
ELISA	Enzyme-linked immunosorbent assay
pNPP	<i>para</i> -Nitrophenyl phosphate
RPMI	Roswell park memorial institute
DMEM	Dulbecco's modified eagle's medium
FBS	Fetal bovine serum
HRP	Horseradish peroxidase
TMB	Tetramethylbenzidine
OD	Optical density
CCK8	Cell counting kit 8

The urgent need for the discovery of new therapeutic candidates is indicated by the unceasing emergence of immune health issues^{1,2}. Recently, there has been a huge interest in the utilization of immunomodulation drugs derived from natural products due to significant efficacy and minimal negative side effects^{3,4}. Toll-like receptors (TLRs) play a crucial role in recognizing conservative molecules of pathogenic microorganisms such as lipopolysaccharide (LPS), lipid A, and lipopolysaccharide (LOS) from Gram-negative bacteria^{5–7}. Among these receptors, TLR4 is the first member and is mainly secreted on monocytes, macrophages, dendritic cells, and hematopoietic cells^{8,9}. Through a multistep molecular recognition process, TLR4 responds quickly to minute amounts of circulating LPS. This process begins with the transfer of LPS monomers from aggregates in solution to LPS-binding protein (LPB), thereby causing the protein to cluster to differentiation 14 (CD14) and myeloid differentiation factor 2 (MD-2)¹⁰. The manipulation of immune responses through TLR4-mediated pathways shows promise as a pharmacological intervention approach¹¹. These strongly suggest that TLR4 regulation is a convincing target for therapeutic control of a wide range immunomodulatory-based diseases. Meanwhile, secondary metabolites such as terpenoids, flavonoids, and alkaloids promote alternative immunomodulating properties, specifically as anti-inflammatory agent¹². Sesquiterpenoid derivatives, constituting the most diverse subclass of the terpenoid group, are not only distinguished by their fascinating structures but are also intriguing in their bioactivities¹³. This has prompted the exploration of the potential of the derivatives as new lead compound in promoting health, particularly in immunomodulation^{12,14}. The Meliaceae family, a widely recognized medicinal plant discovered in tropical and subtropical regions, comprises the extensive genus *Dysoxylum*, which consists of over 200 species. Its origins span across Australia, New Zealand, India, Southeast Asia, and Indonesia^{15–17}. Numerous species of this genus are used in traditional medicine against skin irritations, digestive ailments, antiperiodic, and emmenagogue properties^{15,18}. Previous studies on the species reported various phytoconstituents with anti-inflammatory activities, including sesquiterpenoids^{19,20}, while their potential as immunostimulants shows great activation yet remains unexplored²¹.

Dysoxylum parasiticum (Osbeck) Kosterm. (Meliaceae) (<http://powo.science.kew.org/taxon/578268-1/>) is a species endemic to Indonesia and hold significance in spiritual activity among the Bali people. The species is popularly known as “Majegau” or divine tree due to its characteristic fragrances. The pioneer phytochemical investigations of this plant carried out by our group afforded 16 sesquiterpenoids (SQs) and six sesquiterpenoid dimers (SQDs), involving four SQs and six SQDs, first found from this plant^{22–24}. Since the potential of all SQs and SQDs as immunomodulatory remains unexplored, this study aimed to investigate the immunomodulatory activity based on TLR4 receptors. The evaluation of TLR4 activation was performed against human and murine HEK-Blue cells using a secreted alkaline phosphate (SEAP) assay in the presence or absence of LPS. The SEAP will be induced by TLR4 stimulation on the cell membrane of the downstream transcript of NF- κ B. Therefore, the level of SEAP in the cell supernatant relatively could represent the TLR4 and NF- κ B activations. An enzyme-linked immunosorbent assay (ELISA) was further utilized to test the potential of SQs and SQDs to modulate TLR4 signaling pathways against THP-1 human monocytes and RAW 264.7 murine macrophages, focusing on proinflammatory cytokines TNF- α , IL-1 α , IL-1 β , and IL-6. The study also included an examination of their structure-activity relationship (SAR) to identify the crucial molecular component influencing their immunomodulatory effects.

Results and discussions

The phytochemical investigation of the *n*-hexane extract of *D. parasiticum* yielded sesquiterpenoids (SQs): 10 β ,11-dihydroxy-1 β -hydroperoxide-4 α H,5 α H,7 β H-guaiane (**1**), (1S*,4S*,5R*,10S*)-guai-6-ene-10 β -ol (**2**), guaianediol (**3**), 4 α ,10 α ,11-trihydroxy-1 β H,5 β H-guai-7(8)-ene (**4**), spathulenol (**5**), 4 β -10 α -dihydroxyaromadendrane (**6**), 10 β -hydroxy-4 α ,4 β -dimethyl-5 α H,7 α H-eudesm-3-one (**7**), α -cadinol (**8**), 10-hydroxyl-15-oxo- α -cadinol (**9**), dysoticans A and B (**10**) and (**11**), 2,15-dihydroxycalamenene (**12**), schiffnerone B (**13**), aristegelon A (**14**), eudesm-4(15)-ene-1 β ,6 α -diol (**15**), and eudesm-4(15)-ene-1 β ,6 β -diol (**16**) and sesquiterpenoid dimers (SQDs): dysoticans C-H (**17–22**), including four SQs **1**, **7**, **10**, and **11** and six SQDs **17–22** that were first discovered from

this plant (Fig. 1) after separation using normal and reverse techniques of column chromatography^{22–24}. The potential immunomodulatory effect of 22 pure compounds was assessed in an experimental model of macrophage activation through the TLR4 signaling pathway. Various modulators have demonstrated distinct effects on human and murine TLR4. In certain cases, compounds showed a completely different from an antagonistic to agonistic effects when transitioning from human to murine TLR4/MD-2/CD-14 system¹¹. In this study, the impact of SQ and SQD derivatives on human and murine macrophage cells by the stimulation of endotoxin lipopolysaccharide and without LPS, was examined. The results presented that among all the tested compounds (1–22), some had potent immunomodulatory effect on TLR4, including immunosuppressive/antagonist (6, 9, 10, 22) and partial immunosuppressive mTLR4 agents (4, 7, 11, 15, 18, 19, 20). Meanwhile, compounds 12, 14, and 16 had an antagonistic effect on human TLR4, which completely switched to an agonistic effect on murine TLR4 (Table 1).

Modulation of SQs and SQDs on TLR4 signaling in HEK-Blue cells

In order to evaluate the influence of isolated SQs and SQDs on the TLR4 signaling, we investigated the modulation of compounds 1–22 to antagonize TLR4 activation in LPS-stimulated cells, as well as examined their ability to act as TLR4 agonists in the absence of LPS. The immunomodulatory activity on a reporter gene cell line for human (HEK-Blue™ hTLR4) and a separate murine TLR4 (HEK-Blue™ mTLR4) was screened. These cells were engineered to stably express the human (hTLR4, hMD-2, and hCD14) and murine receptors (mTLR4, mMD-2, and mCD14) of the endotoxin recognition complex, as well as a SEAP reporter gene, respectively. The SEAP was controlled by NF- κ B and AP-1 of TLR4-dependent transcription factors. Since assay optimization indicated saturation at 10 ng/mL on HEK-Blue mTLR4 cell (Fig. S1), the concentrations used in this experiment were 10 ng/mL and 1 ng/mL LPS on human and murine TLR4, respectively. As shown in Fig. 2a,b, SQs 6, 9, and 10 and

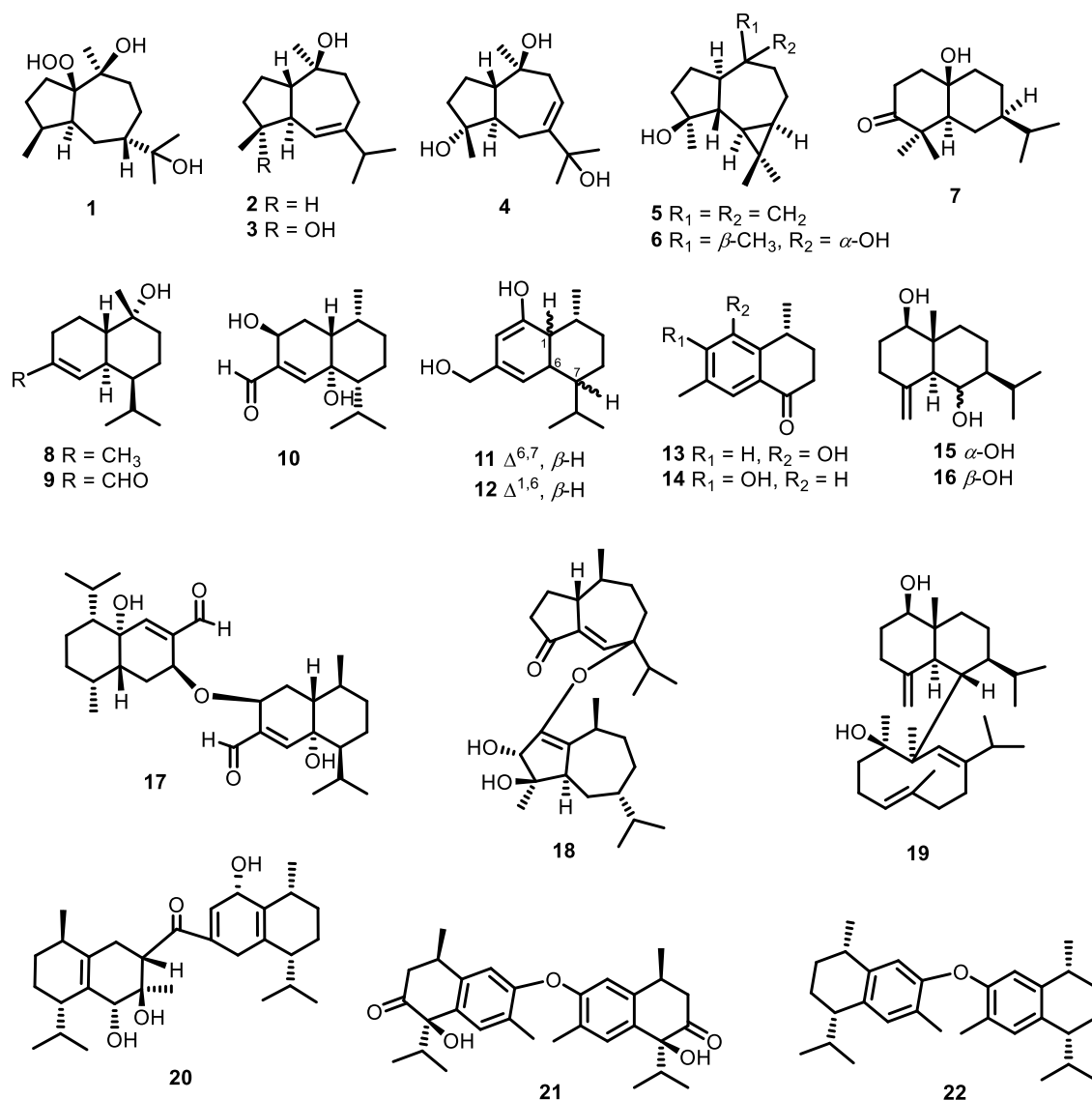


Figure 1. The structures of all isolated compounds 1–22.

Compounds	Type	Activity
10 β ,11-dihydroxy-1 β -hydroperoxide-4 α H,5 α H,7 β H-guaiane (1)	Guaiane	Inactive
(1S*,4S*,5R*,10S*)-guaia-6-ene-10 β -ol (2)		
Guaianediol (3)		Partial antagonist mTLR4
4 α ,10 α ,11-trihydroxy-1 β H,5 β H-guai-7(8)-ene (4)		
Spathulenol (5)	Aromadendrane	Inactive
4 β -10 α -dihydroxyaromadendrane (6)	Eudesmane	Antagonist TLR4
10 β -hydroxy-4 α ,4 β -dimethyl-5 α H,7 α H-eudesm-3-one (7)		
α -cadinol (8)	Cadinane	Inactive
10-hydroxyl-15-oxo- α -cadinol (9)		Partial antagonist TLR4
Dysotican A (10)		Antagonist mTLR4
Dysotican B (11)		Contrasting effect
2,15-dihydroxycalamenene (12)		
Schiffnerone B (13)	Tris- <i>nor</i> -cadinane	Inactive
Aristegelson A (14)		Contrasting effect
Eudesm-4(15)-ene-1 β ,6 α -diol (15)	Eudesmane	Partial antagonist mTLR4
Eudesm-4(15)-ene-1 β ,6 β -diol (16)		Contrasting effect
Dysotican C (17)	Asymmetrical cadinane-dimer	Antagonist TLR4
Dysotican D (18)	Asymmetrical guaiane-dimer	Partial antagonist mTLR4
Dysotican E (19)	Hybrid eudesmane/germacrene-dimer	
Dysotican F (20)	Asymmetrical cadinane-dimer	
Dysotican G (21)	Symmetrical cadinane-dimer	Inactive
Dysotican H (22)		Antagonist TLR4

Table 1. The chemistry of isolated sesquiterpenoids (SQs) and sesquiterpenoid dimers (SQDs) along with their activity against human and murine TLR4.

SQDs **17** and **22** inhibited signaling against HEK-Blue cells. The relative inhibition was lower than 60% of activation at a concentration of 20 μ M by the presence of LPS, yielding a concentration-dependent response ranging from 0.1 to 20 μ M (Fig. 2c,d). Compound **9**, identified as 10-hydroxyl-15-oxo- α -cadinol exhibited the strongest antagonist with relative inhibition of TLR4 activation ranging from 70 to 85% at concentration of 20 μ M, without adversely affecting cell viability. Additionally, 4 β -10 α -dihydroxyaromadendrane (**6**) and another SQ, first originated from *D. parasiticum*, namely dysotican A (**10**) had strong antagonistic effect (70%) against these two cells and slightly showed lower cell viability (<80%) against both HEK-Blue cells at 20 μ M. The SQDs, first derived from this plant, particularly dysotican H (**22**) displayed moderate antagonistic with negligible toxicity to cells (>80%) at 20 μ M (Fig. 2a,b). Although dysotican C (**17**) showed toxicity effects at 10 μ M, the evaluation in lower concentrations suggested that **17** moderately inhibited the LPS stimulation at 1 μ M and strong inhibition at 5 μ M without significant cytotoxicity, especially against a separate murine TLR4 (Fig. 2c,d, Figs. S2, S3). Several compounds performed inconsistency effects on human and murine TLR4 receptors. The SQs **12**, **14**, and **16** switched from antagonism hTLR4 to agonism mTLR4, while **4**, **7**, **11**, and **15** as well as SQDs **18**, **19**, and **20** presented partial antagonism against HEK-Blue mTLR4 (Fig. 2c–g). The viability assay confirmed that these compounds did not affect cell viability (Fig. 2a,b, Figs. S2, S3).

The effect of potent SQs and SQDs on proinflammatory cytokines in human and murine TLR4 cells

Cytokines play an important role in innate immunity as mediators engaged in the elimination of invading foreign pathogens through the TLRs²⁵. Macrophages contribute to enhancing both adaptive and innate immune responses²⁶. Activated macrophages secrete proinflammatory cytokines, such as TNF- α , IL-1, and IL-6, leading to the induction of NF- κ B proteins²⁷. As the two HEK-Blue cells are transgenic cell lines, to further confirm the modulation effects of potent SQs and SQDs on human and murine TLR4, human monocyte THP-1 and murine macrophage RAW 264.7 cells served as in vitro models, using the natural TLR4 signaling system, by an ELISA experiment. Initially, the differentiation of the monocyte THP-1 cell lines into macrophage-like cells was performed by PMA-induction before compound treatment.

The effects of antagonists (**6**, **9**, **10**, **17**, **22**), contrasting effects (**12**, **14**, **16**), and partial antagonists (**4**, **7**, **11**, **15**, **18**, **19**, and **20**) of SQs and SQDs on PMA-treated THP-1 and RAW 264.7 cells, were evaluated. These two cells were then exposed to different concentrations of SQ and SQD variants (0.1–20 μ M) in the presence and absence of LPS for up to 24 h. Cell viability was tested after treatment by using a CCK-8 reagent. As shown in Figs. S4, S5, all the SQs and SQDs of interest did not show any cytotoxicity in THP-1 and RAW 264.7 cells even at a high concentration of 20 μ M, except for **17**, which indicates toxicity at 10 μ M. The three major proinflammatory cytokines (TNF- α , IL-1, and IL-6) were used to determine whether the potent SQs and SQDs isolated from the stem bark of *D. parasiticum* were engaged in the TLR4 activation. TNF- α and IL-1 are crucial cytokines in

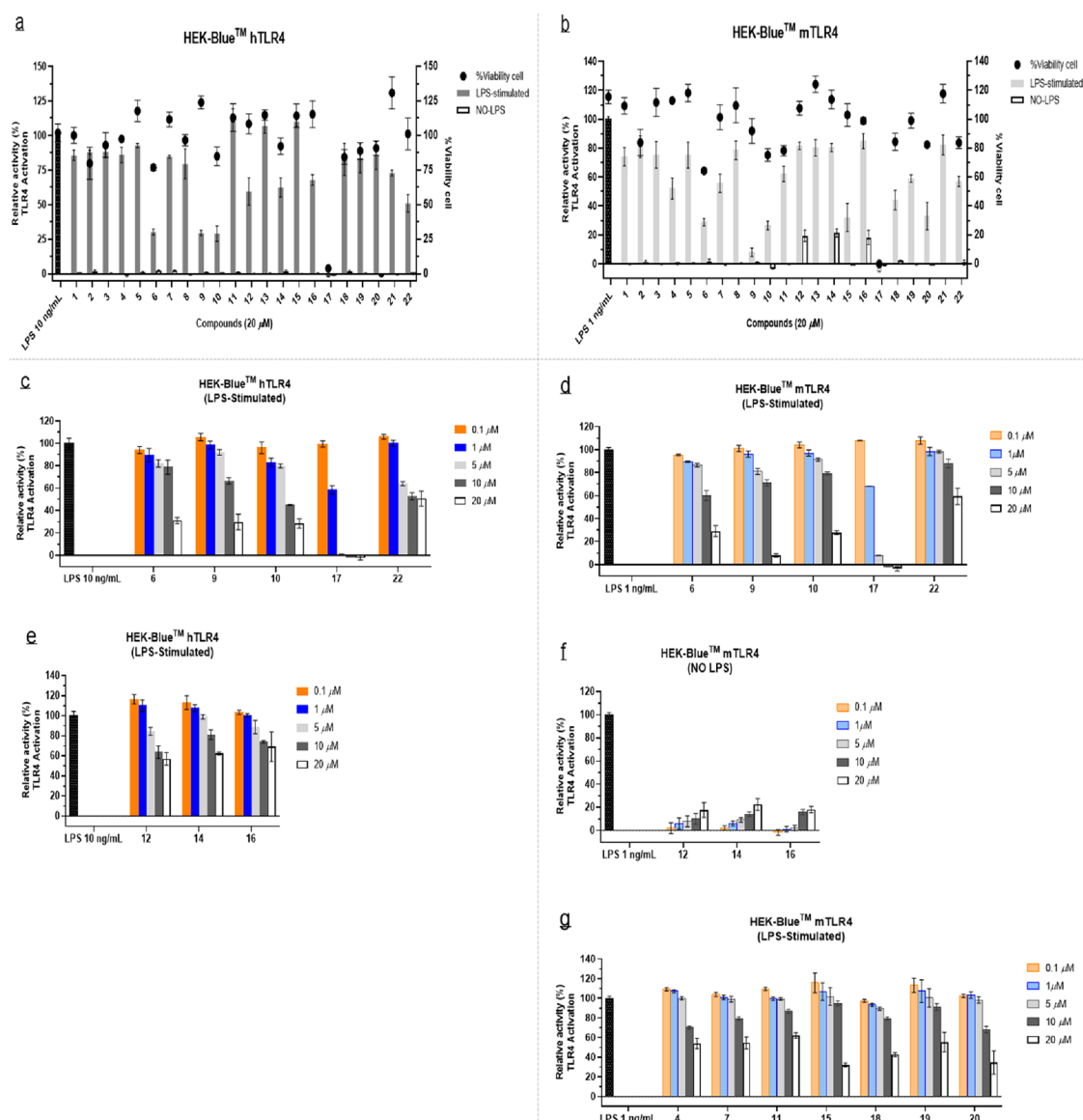


Figure 2. The SEAP assay and cytotoxicity of compounds 1–22 with the presence and absence of LPS against HEK-Blue™ hTLR4 (a) and HEK-Blue™ mTLR4 (b). The dose-dependent actions of the selected compounds with the antagonist (LPS-stimulated) on hTLR4 (c) and mTLR4 (d), switching effects with LPS-stimulated on hTLR4 (e) and NO LPS on mTLR4 (f), and partial antagonist (LPS-stimulated) on mTLR4 (g).

developing inflammation through I κ B kinase (IKK) of the NF- κ B signaling pathway, while IL-6 plays a positive role in inflammation through JAK/STAT signaling pathway.

ELISA results revealed that the antagonist SQs (6, 9, and 10) could significantly suppress IL-1 β production in PMA-treated THP-1 cells. These three antagonists strongly suppressed the secretion of TNF- α and completely inhibited LPS simulation driven IL-6 at concentration of 10 μ M, as shown in Fig. 3a. Similarly, in RAW 264.7 cells, antagonist SQs 6, 9, and 10 had strong inhibition on TNF- α expression, as well as reduced the IL-6 cytokine to the same level as negative control at 20 μ M in the presence of LPS 1 ng/mL. Additionally, the role of IL-1 signaling was investigated using IL-1 α due to the murine RAW 264.7 being naturally deficient in a CARD domain (ASC) of apoptosis-associated speck-like protein, preventing the activation of pro-caspase-1 and the production of IL-1 β ²⁸. The potential of SQ antagonists (6, 9, and 10) as immunosuppressive agents was also complemented by the decrement of IL-1 α level in murine TLR4 cells (Fig. 3b). In SQD antagonists, compounds 17 and 22 acted promising anti-inflammatory agents. Despite dysotican C (17) having cytotoxic effect in THP-1 and RAW 264.7 cell lines at 10 μ M (Figs. S4, S5), it might still be used as immunosuppressive agent at a lower concentration. The considerable immunosuppressive activity of 17 was proved by the ELISA results, as demonstrated in Fig. 4b. The data showed that production of the three proinflammatory cytokines used against human and murine macrophage cells reduced when treated by 5 μ M of 17, specifically decreased the level of IL-1 β , IL-6 in PMA-induced THP cells as well as TNF- α , IL-6 in RAW 264.7 cells. Furthermore, dysotican H (22) negatively modulated TLR4

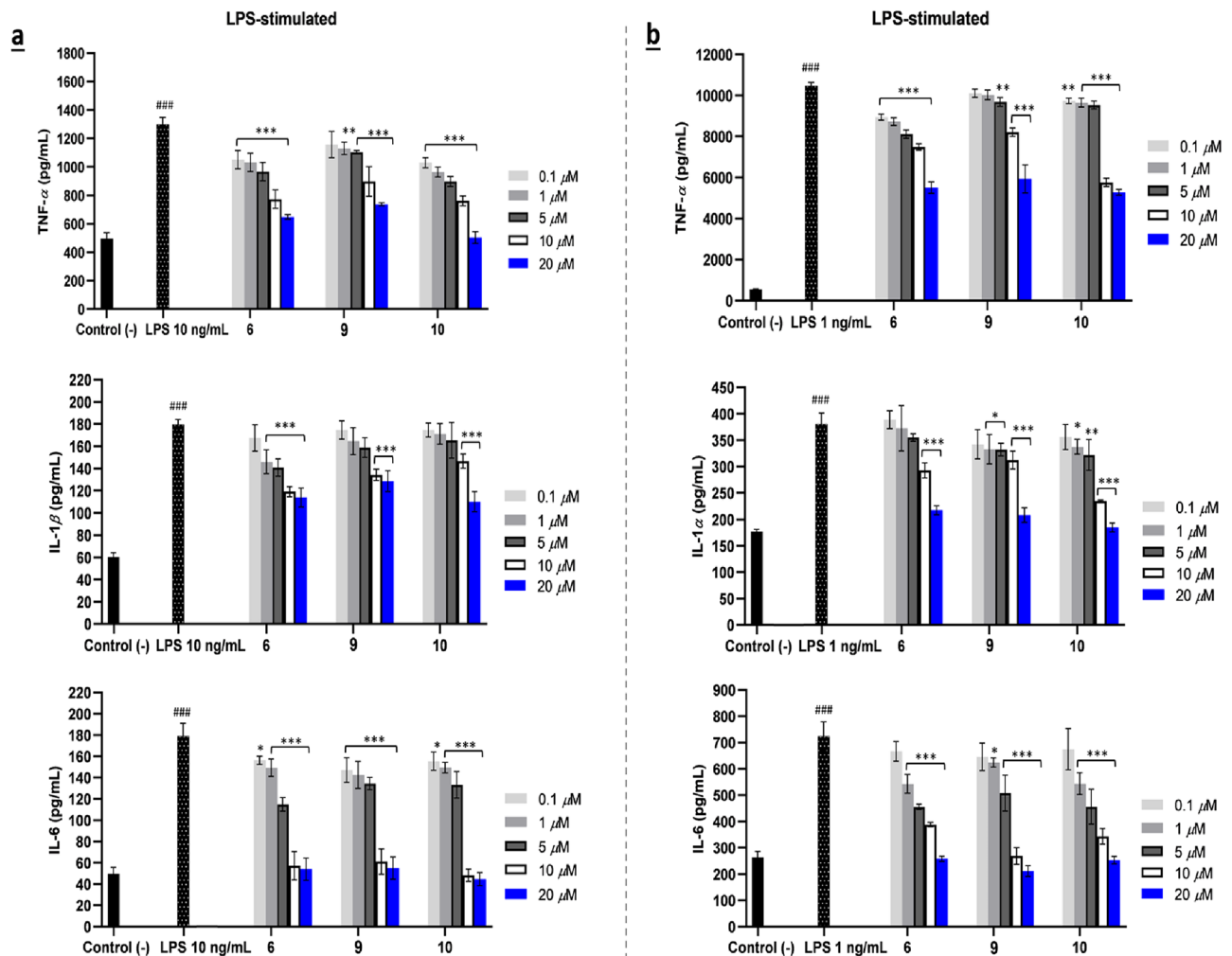


Figure 3. Proinflammatory cytokine productions of SQs (6, 9, and 10) as antagonist TLR4 on LPS-stimulated of TNF- α , IL-1 β , and IL-6 in THP-1 cells (a) and TNF- α , IL-1 α , and IL-6 in RAW 264.7 cells (b). The results are shown as means $\pm$ SD (n = 4). Significant results are indicated as * p < 0.05, ** p $\leq$ 0.01, *** p $\leq$ 0.001 compared to LPS and # p < 0.05, ## p $\leq$ 0.01, ### p $\leq$ 0.001 compared to negative control.

signaling, promoting the three cytokines at 10 μ M in a concentration dependent manner (Fig. 4a,b). These data clearly proved the potency of these SQ and SQD antagonists as negative regulators in human and murine cells, offering potential for anti-inflammatory drug discoveries. In particular, SQ dysotican A (10), and SQDs dysoticans C (17), H (22) represent compounds first isolated from this plant.

For compounds exhibiting divergent effects, namely 2,15-dihydroxycalamenene (12), aristegone A (14), and eudesm-4(15)-ene-1 β ,6 β -diol (16), their function as agonists for human TLR4 translated into a consequential agonistic impact on murine TLR4. The ELISA results (Fig. 5a) suggested a moderate reduction in IL-1 β and a strong inhibition of IL-6 levels by the presence of 10 ng/mL LPS along with stimulations of 12, 14, and 16 at 10 μ M in PMA-stimulated THP-1 cells. Simultaneously, the agonist effects were proved by an elevation in the level of IL-1 α and IL-6 in RAW 264.7 cells (Fig. 5b). Considering the SEAP data (Fig. 2a,b), it becomes apparent that these compounds had lower activity compared to SQ antagonists (6, 9, and 10) and showed moderate agonists mTLR4. The TNF- α cytokine is a major pathway to activate the NF- κ B signaling, as 12, 14, and 16 failed to suppress and recruit the TNF- α level, unlike other SQ antagonists and agonists with significant activities observed in HEK cells. In contrast, the expression of IL-1 α did not significantly contribute to the immunostimulant mTLR4 (SEAP), as their effects on activating TLR4 were relatively similar at concentration of 20 μ M (Figs. 2e,f, 5b).

The SQs 4 α ,10 α ,11-trihydroxy-1 β H,5 β H-guai-7(8)-ene (4), 10 β -hydroxy-4 α ,4 β -dimethyl-5 α H,7 α H-eudesm-3-one (7), dysotican B (11), and eudesm-4(15)-ene-1 β ,6 α -diol (15) obviously exhibited the immunosuppressive effects on mTLR4 in RAW 264.7 cells at 20 μ M across the three proinflammatory used (Fig. 6a). These results indicated that 4, 7, 11, and 15 were partial antagonist agents only on murine TLR4 cells. Based on observation, compound 15 showed a two-fold stronger decrease in LPS-stimulated TNF- α production compared to others at 20 μ M. When referred to the HEK-Blue mTLR4 cells (SEAP) (Fig. 2b), the TLR4 activation of this compound was almost the same as TNF- α secretion, including the effect and tendency. Therefore, it was confirmed that TNF- α could be the best-identified inducer in murine TLR4, triggering NF- κ B regulation. SQDs dysoticans D-F (18–20), were also assigned as antagonists due to their effects on decreasing LPS-stimulated expression of

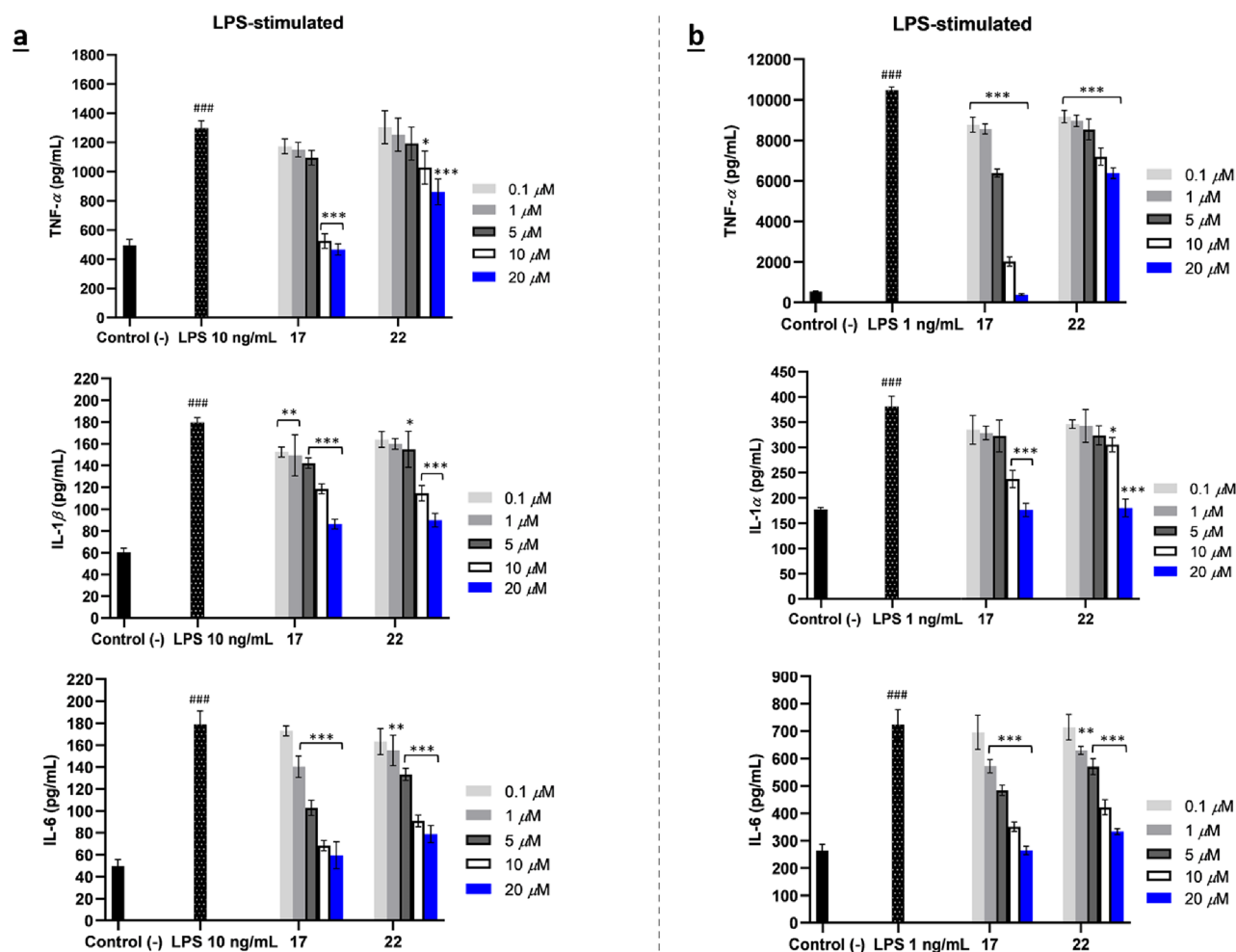


Figure 4. Proinflammatory cytokine productions of SQDs (17 and 22) as antagonist TLR4 on LPS-stimulated of TNF- α , IL-1 β , and IL-6 in THP-1 cells (a) and TNF- α , IL-1 α , and IL-6 in RAW 264.7 cells (b). The results are shown as means $\pm$ SD ($n = 4$). Significant results are indicated as * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to LPS and # $p < 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ compared to negative control.

cytokines applied in this study (Fig. 6b). Regarding the SEAP (Fig. 2g) and ELISA data (Fig. 6a,b), SQs 7, 11, and 15 as well as SQDs 18–20 were partial antagonists exclusively in murine TLR4. This led to an agreement that interactions between hMD-2 and mMD-2 binding regions to the same compound as a ligand were different.

Structure-activity relationships of SQs and SQDs in their activity on TLR4 receptor

A series of guaiane compounds 1–4 suggested the impacts of several functional groups on their immunomodulatory TLR4 activities. Among these compounds, 4 was the only guaiane variant with activity as immunosuppressive agent against murine cells. These implied that an attachment of hydroperoxide at C-1 of 1 had no effects on its activity since 2 and 3 without hydroperoxide group displayed no activity. The inactive effects of 1–3 compared to 4 also confirmed that hydroxyls at C-4 and C-10 were not crucial on their activity as immunomodulator agents. According to the conclusion from SAR analysis, the $\Delta^{7,8}$ in 4 plays a dominant role to its activity within the guaiane series of 1–4. This assignment was deduced by the presence of 1-hydroperoxide in 1, 4-OH in 3 and 4, 7-OH in 1 and 4, as well as $\Delta^{6,7}$ in 2 and 3. Meanwhile, two series of aromadendrane compounds indicated that the substitution of 14-methyl and hydroxyl at C-10 in 6 enhanced the immunosuppressive effect on the TLR4 receptor. Conversely, the antagonist activity was rendered inactive when these functional groups were replaced by 14-methylene sp^2 .

The existence of 15-aldehyde in a cadinene compound is a key moiety in the structural composition 9, acting as an antagonist TLR4 agent. This was in contrast to its analog 8 that was inactive due to the occurrence of 15-methyl. Based on the biological activity of compounds 10 and 17, hydroxyl at C-3 was responsible for the immunosuppressive effect of 10, while the dimerization strongly increased cytotoxicity against TLR4-based cells. In addition, the epimerization of methyl at C-10 and isopropyl at C-7 in 10 and 17 might be affecting their immunomodulatory activity and cytotoxicity. Similar cadinanes, compounds 11 and 12 by the double bond movement showed that $\Delta^{6,7}$ is favorable for partial antagonist against murine TLR4 cells of 11. On the other hand, $\Delta^{1,6}$ is responsible for the switching effect depending on the origin of TLR4 in 12. The hydroxyl at C-2 in the tris-*nor*-cadinane of 13 showed contrasting effects depending on the origin of the receptor (human or mouse)

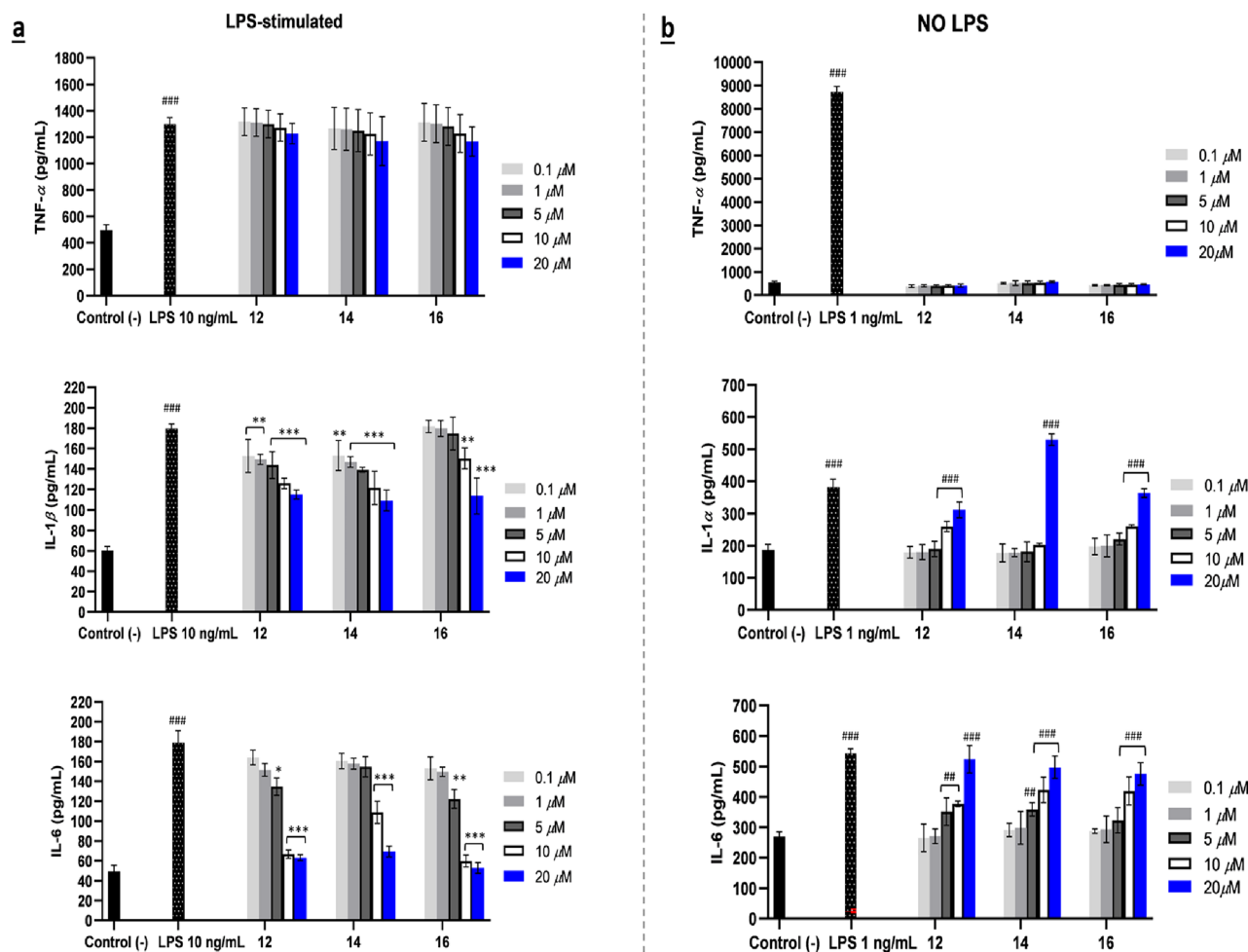


Figure 5. Proinflammatory cytokine productions of SQs (12, 14, and 16) with switching effects on LPS-stimulated of TNF-α, IL-1β, and IL-6 in THP-1 cells (a) and NO LPS of TNF-α, IL-1α, and IL-6 in RAW 264.7 cells (b). The results are shown as means ± SD (n = 4). Significant results are indicated as * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to LPS and # $p < 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ compared to negative control.

and become inactive by the hydroxyl shift to C-3 in 14. The epimerization of hydroxyl at C-6 in 2 eudesmanes, compounds 15 and 16, extremely altered their immunomodulatory activity. Compound 15 with α -hydroxyl acted partial antagonist effect on mTLR4, while β -hydroxyl in 16 displayed switching effects in human and murine cells. Consequently, 2 symmetrical dimers 21 and 22 demonstrated that the formation of hydroxyl at C-7'/7'' and ketonic at C-8'/8'' groups decreased the antagonist activity of 21. The epimerization of 14-methyl might facilitate the immune regulating effects of these dimers. The SAR of related SQs and SQDs was presented in Fig. 7.

Conclusions

In conclusion, this study evaluated the immunomodulatory activity of 16 SQs and six SQDs previously isolated from *D. parasiticum* (Osbeck). Kosterm stem bark on TLR4 receptor of human and murine cells. Based on the results, SQs 6, 9, and 10 and SQDs 17 and 22 were classified as antagonist TLR4 on both cells. Among these compounds, 10-hydroxyl-15-oxo- α -cadinol (9) was discovered to be the most potent anti-inflammatory agent without negative effects on cell viability. On the other hand, several compounds, including SQs 4, 7, 11, and 15 as well as SQDs 18, 19, and 22, regulated partial antagonist exclusively on murine TLR4 cells. A switching effect was observed during the transition from human to murine TLR4/MD-2/CD14 system with SQs 12, 14, and 16 moving from antagonistic hTLR4 to agonistic mTLR4. Furthermore, a brief structure-activity relationship presented the impacts of double bond position, attachment of hydroxyl, aldehyde, and methyl groups, as well as position of hydroxyl and epimerization, which are crucial to the immune regulating system on TLR4 receptor.

Materials and methods

Extract preparation and isolation of SQs and SQDs

D. parasiticum (Osbeck) Kosterm. was collected from Bogor Herbarium Botanical Garden (BHBG), West Java Province, Indonesia (latitude -6.597629, longitude 106.799568, elevation 277 m), where it is widely cultivated. Identification and authentication of the plant were conducted at the BHBG by Mr. Harto, an expert in the BHBG,

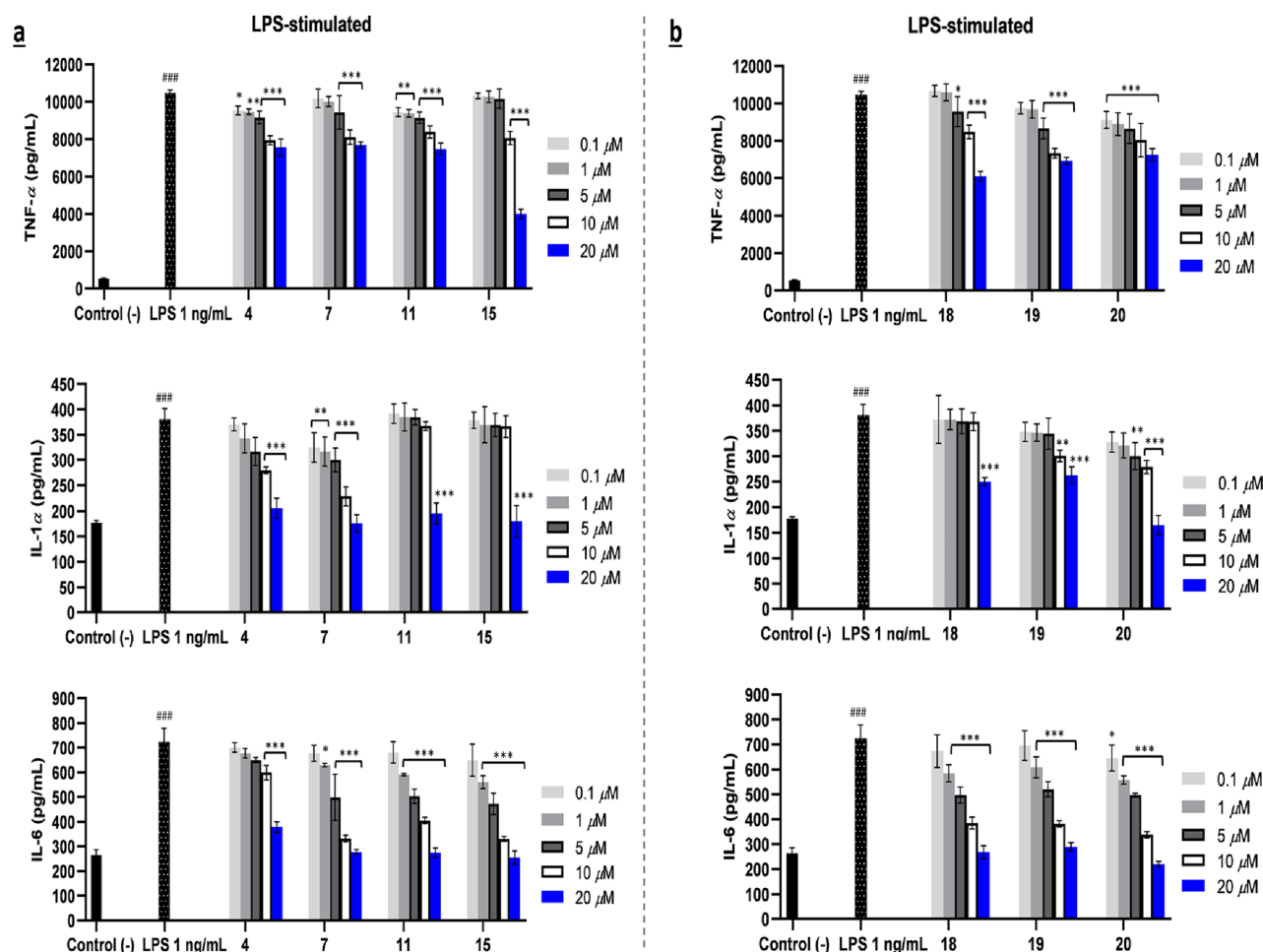


Figure 6. Proinflammatory cytokine productions (TNF- α , IL-1 α , and IL-6) in RAW 264.7 cells as partial antagonist mTLR4 of SQs (4, 7, 11, and 15) (a) and SQDs (18–20) (b). The results are shown as means $\pm$ SD ($n=4$). Significant results are indicated as * $p<0.05$, ** $p\leq 0.01$, *** $p\leq 0.001$ compared to LPS and # $p<0.05$, ## $p\leq 0.01$, ### $p\leq 0.001$ compared to negative control.

and a voucher specimen (III. F.79) was deposited for the plant species. Collection and experimental research on this plant comply with relevant institutional, national, and international guidelines and legislation.

The dried stem bark was powdered and extracted with MeOH. Subsequently, 16 SQs and 6 SQDs were isolated from the *n*-hexane and ethyl acetate extracts by chromatography over silica gel, followed by purification on reverse phase ODS, as described in previous publications^{22–24}.

SEAP NF- κ B-dependent reporter assay

HEK-Blue cells (hTLR4 and mTLR4, InvivoGen) were cultured in accordance with the instructions of the manufacturer. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). A 96-well multiwell plate was seeded with a total of 2×10^4 cells/well in medium supplemented with 1% penicillin-streptomycin. The plate was incubated at 37 °C, 5% CO₂, and 95% humidity overnight. Subsequently, the supernatants were replaced by fresh DMEM high glucose (supplemented with 0.1% FBS and 1% penicillin-streptomycin). Samples were added at different concentrations (0.1; 1; 5; 10; and 20 μ M) and exposed to 10 ng/mL and 1 ng/mL of LPS from *E. coli* (Fujifilm Wako Pure Chemical Co., Ltd) for hTLR4 and mTLR4, respectively, up to 24 h. Additionally, supernatants containing SEAP were gathered and combined with *para*-nitrophenyl phosphate (pNPP) at room temperature for 4 h. A microplate reader was used to measure the optical density (OD) at 405 nm.

Sandwich ELISA assay

THP-1 cells (JCRB0112) were purchased from the JCRB Cell Bank (Ibaraki, Osaka, Japan) and cultured in Roswell Park Memorial Institute (RPMI) 1640 (+ 10% FBS and + 1% penicillin-streptomycin). The cells were used at a density of 1×10^5 cells/well and differentiated into macrophages by adding 0.3 μ L/mL phorbol 12-myristate 13-acetate (PMA) for 24 h. After incubation, the medium was replaced by new RPMI (+ 1%

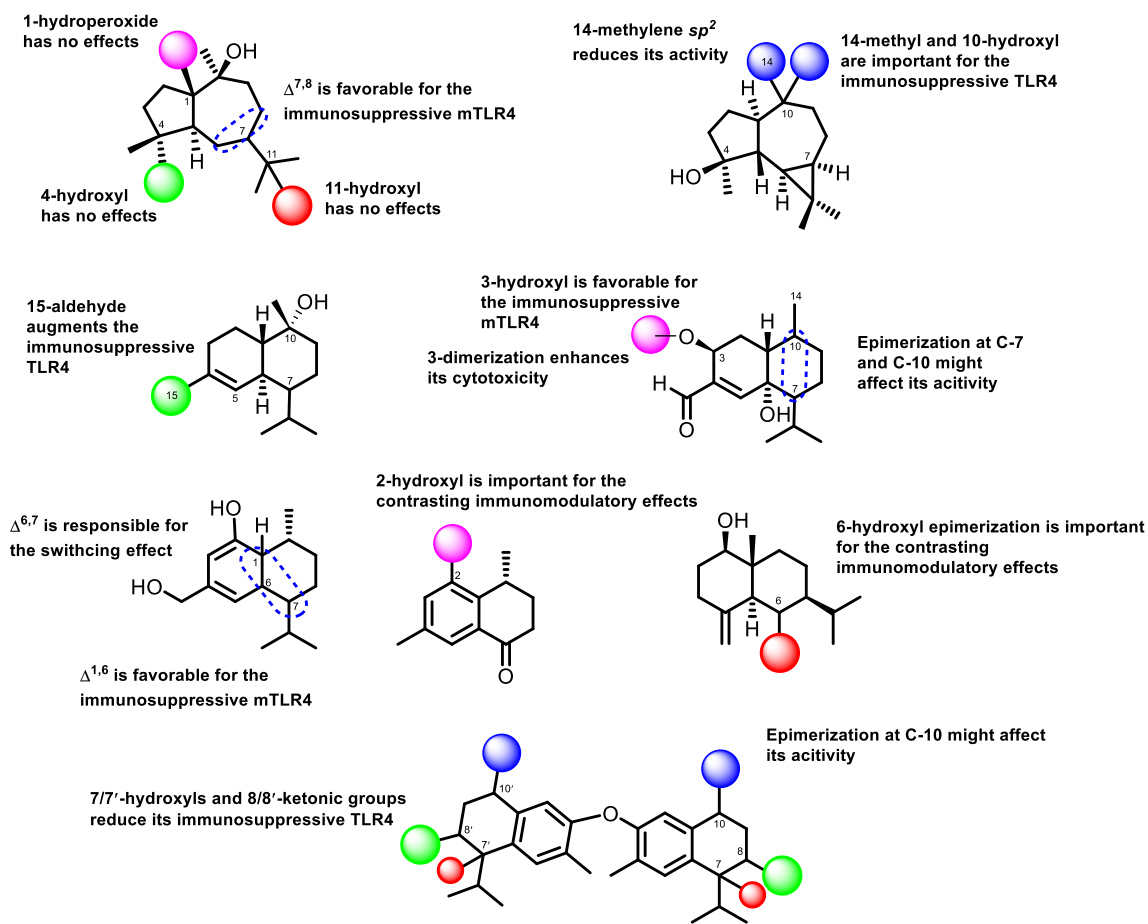


Figure 7. Structure–activity relationship (SAR) of SQs and SQDs.

FBS, + 1% penicillin–streptomycin, and + 10 mM HEPES) and incubated for 24 h. The cells were pretreated with all compound variants (0.1–20 μ M) for 1 h, followed by exposure to LPS (10 ng/mL) up to 24 h for LPS-stimulated design.

RAW 264.7 cells (EC91062702-F0) were purchased from the Kyorin Pharmaceutical Group Factories Sumitomo Pharma Co., Ltd, Japan and cultured in DMEM high glucose, supplemented with 10% FBS and 1% penicillin–streptomycin. Cell scraper was used to detach cells, and the cell concentration was estimated by Trypan Blue (Sigma-Aldrich). Furthermore, a density of 1×10^4 cells/well was seeded into 96-well plates in fresh DMEM high glucose (1% FBS, + 1% penicillin–streptomycin, and + 10 mM HEPES). Following overnight incubation (37 $^{\circ}$ C, 5% CO₂, 95% humidity), the cells were treated with increasing concentrations of samples dissolved in EtOH and diluted in DMEM. After 1 h, cells were stimulated with 10 ng/mL of LPS for up to 24 h.

For the determination of cytokine production, supernatants of THP-1 and RAW 264.7 were collected and tested using sandwich enzyme-linked immunosorbent assay (ELISA). The proinflammatory cytokines of TNF- α (LOT: 218819-004, Invitrogen), IL-1 β (LOT: 252921-005, Invitrogen), and IL-6 (LOT: 23638-011, Invitrogen) from human uncoated ELISA kit were adopted for THP-1 supernatant. Meanwhile, the RAW 264.7 supernatant was analyzed using murine uncoated ELISA kit of TNF- α (LOT: 306473-002, Invitrogen), IL-1 α (LOT: B352593, Biolegend), and IL-6 (309364-001, Invitrogen). The assay was conducted according to the specification of the manufacturer, including the coating of capture antibody, blocking using ELISPOT, adding the collected supernatant, and preparation of the standard of protein of interest. The measurement of targeted proinflammatory cytokines was performed by adding the detection antibody, enzyme of horseradish peroxidase (HRP), and substrate of tetramethylbenzidine (TMB). Additionally, the OD was observed on a microplate reader at 450 nm.

Viability assay

Cell viability was assessed after supernatant collection. The monolayers of remaining cells (HEK-Blue hTLR4, HEK-Blue mTLR4, THP-1, and RAW 264.7) in the 96-well plates were added with 90 μ L/well of new medium and 10 μ L/well of cell counting kit 8 (CCK8). Additionally, the formazan concentration was determined by measuring the OD at 450 nm on a microplate reader.

Statistical analysis

All experiments were performed independently in 4 replicates. The results were normalized with control cells which were not exposed to any drug. The GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) was used to generate the statistical analyses. In addition, each data was visualized as the mean $\pm$ standard

deviation (SD). The one-way analysis of variance (ANOVA) and Dunnett's or Bonferroni multiple comparison post-tests were adopted for significant comparison between treatment groups. A p -value < 0.05 was considered statistically significant ($*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ compared to LPS and $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ compared to negative control).

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

Received: 16 February 2024; Accepted: 24 June 2024

Published online: 06 July 2024

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Acknowledgements

The authors are grateful to the Directorate of Ministry of Research, Technology/National Research and Innovation Agency, Indonesia for rendering financial support with contract No: 1207/UN6.3.1/PT.00/ 2021 under the PMDSU and PKPI program 2022, as well as Universitas Padjadjaran, for the Post-Doctoral Grant, No: 2292/UN6.3.1/PT.00/2023 provided by Unang Supratman.

Author contributions

Conceptualization, A.A.N.; methodology, software, validation, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review and editing, A.A.N., T.M., E.H., U.S., X.H.; visualization, supervision, project administration, K.K., A.S., Y.M., K.F., U.S. All authors have read and agreed to the published version of the manuscript.

Funding

Open access funding provided by University of Padjadjaran.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-65829-0>.

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