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Isolation, structure determination and structure–activity relationship of anti-toxoplasma triterpenoids from *Quercus crispula* Blume outer bark

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Abstract

Toxoplasma gondii is an intracellular protozoan parasite of which infection can result in serious symptoms for fetuses or people who have weakened immune system. In our effort to discover novel anti-toxoplasma substances from tree barks, only outer bark extract from *Quercus crispula* Blume (mizunara) was revealed to show potent anti-toxoplasma activity. Isolation of the active principles was performed to identify three pentacyclic triterpenoids, namely 29-norlupane-3,20-dione, oleanolic acid acetate and ursolic acid acetate. These structures were determined by combining a series of spectral data, computational simulation and synthetic approach. All isolated compounds exhibited notable activities at IC_{50} of 6.8–24.4 μM and high selectivities against the parasite. The structure–activity relationship study conducted to probe key structure(s) indicated that the lack of free hydroxyl group at 3-position highly contributes to both the titer of activity and the selectivity. Moreover, skeleton and functionalities of E-rings were also suggested to affect to the activity. The present study demonstrated not only that the extract from *Q. crispula* Blume could be a promising source of toxoplasmacidal agent, but also that related extractive triterpenoids can be modified to furnish anti-toxoplasma activity.

Keywords: *Toxoplasma gondii*, Triterpenoids, *Quercus crispula*, Mizunara, Paraciticide

Introduction

Toxoplasma gondii is an intracellular protozoan parasite that can infect all warm-blood animals including humans. The infection to humans occurs transplacentally, or orally thorough infectious cat, contaminated soil, water or raw/undercooked meat. Although most people infected with Toxoplasma do not show symptoms, the parasite can cause hydrocephalus or stillbirth in the fetus and cause toxoplasmic encephalitis in people with weakened immune system [1, 2]. Regardless of its severity and worldwide distribution, toxoplasmosis has gained relatively little attention for its prevention or treatment mainly due to economic reasons. Therefore, this disease is designated as the neglected parasitic infections (NPIs) by Centers for Disease Control and Prevention in United States [3]. Currently, pyrimethamine and sulfadiazine are used as first-line medicines for treatment of toxoplasmosis, but their high rates of toxic side effects often prevent continuous administration [4].

The bark is one of the most abundant biomass and is currently used as heat sources, livestock bedding or feedstuffs after wood bark peeling. However, their sophisticated applications utilizing bark-specific characteristics have not yet been established. Focusing on the richness and the diversity of secondary metabolites in tree barks, we demonstrated the inhibitory activity of extracts from 8 species of northern tree barks on advanced glycation end-products (AGEs) formation and identified the active ingredients in previous work [5]. In this study, the search for toxoplasmacidal activity from bark extracts was conducted and the identification of active principles from

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Quercus crispula Blume outer bark was investigated by bioassay-guided isolation followed by extensive structure elucidation. Moreover, we report a structure–activity relationship study of isolated pentacyclic triterpenoids to gain a new insight into the development of toxoplasmacidal drugs.

Materials and methods

Measurement of anti-toxoplasma activity

In vitro growth assay of *T. gondii* was conducted according to the previously reported method [6]. Briefly, one thousand *T. gondii* RH strain parasites were inoculated into each well of a confluent human fibroblast culture in a 96-well plate. One day after infection, samples were applied with various concentrations. Three days after sample apply, culture supernatants were collected and the amount of MIC10 antigen was determined by the sandwich ELISA. The amount of MIC10 antigen was expressed as optical density (OD_{415}) value determined by a microplate reader (MTP-500, Corona Electric, Japan).

Cytotoxicity of each sample on human foreskin fibroblast was determined as follows. Human fibroblast was cultured in a 96-well plate as 50% confluent. Next day, samples were applied with various concentrations. After 2 days culture, cell viabilities were determined by formazan dye (Cell Counting Kit-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

Screening of anti-toxoplasma extract from tree barks

Barks of eight species, namely *Abies sachalinensis*, *Larix kaempferi*, *Betula platyphylla*, *Phellodendron amurense*, *Ulmus davidiana* var. *japonica*, *Aria alnifolia*, *Quercus crispula* Blume, and *Acer mono Maxim.*, were collected at Tomakomai Research Forest, Field Science Center for Northern Biosphere, Hokkaido University (42°40′N, 141°35′E) in May 2011. After harvest, outer and inner barks were separated manually and the methanolic extracts were prepared following the procedure described in reference 5. A total of 14 methanolic extracts (outer barks from *A. sachalinensis* and *B. platyphylla* and outer and inner barks of the remaining species) were dissolved in dimethyl sulfoxide (DMSO) at 10 μg/mL, and each sample was subjected to the assay. DMSO and nitazoxanide (1 and 2 μM) were used as negative and positive controls, respectively.

Isolation of anti-toxoplasma compounds from *Quercus crispula* Blume outer bark

Seven-hundred and seventy-seven grams of pulverized *Quercus crispula* Blume outer bark was treated with 60-fold amount of $CHCl_3$ (w/w) under mechanical stirring for 2.5 h. Filtration and extraction were repeated three times and combined filtrate was evaporated *in vacuo*, resulting in brown residue of 31.4 g. The half was subjected to flash silica gel column chromatography (Kanto Chemical, Silica Gel N60 spherical neutral) eluting with EtOAc:hexane = 1:9 to 1:7. Fractions were collected every 15 mL and were combined based on thin-layer chromatography (TLC) pattern. The fraction with an R_f value of ≈ 0.4 on TLC (EtOAc:hexane = 1:4) was evaporated and rechromatographed on silica gel column eluting with EtOAc:hexane = 1:10 to 1:7 to give 273.5 mg of crude fraction. In each step, active fractions were identified by the bioassay described above. One-hundred and forty-eight milligrams of crude fraction was then purified with reverse phase high-performance liquid chromatography (HPLC). Preparative HPLC was first performed with Mightysil RP-18 GP II $\phi 20 \times 250$ mm (Kanto Chemical, Tokyo, Japan) with eluting H_2O:MeOH = 7:93 (+0.1%AcOH) at 15.0 mL/min flow rate. UV detection was carried out at 210 nm. Among three major peaks detected, the peak with retention time (RT) of 12.15 min was recovered and evaporated to give 28.0 mg of pure compound (compound **1**). The peak at RT 13.25 min was likewise recovered as a two-component mixture (40.5 mg). The mixture was next separated into two fractions with the HPLC system equipped with PLC-6RcA recycle-valve system (GL Science, Tokyo, Japan). All conditions were same as 1st preparative HPLC, except the elution with H_2O:MeOH = 8:92 (+ 0.1% AcOH). After injection, the valve was switched to recycle position in 10 min and the recycle was repeated eight times. From resulting bimodal peak, only pure parts of two compounds were recovered as compound **2** (RT = 82.11 min) and compound **3** (RT = 83.59 min), respectively.

Computational prediction of ^{13}C chemical shift

All calculations were performed using the Spartan'16 (Wavefunction Inc., Irvine, CA, USA) [7]. The structures of 29-norlupan-3,20-dione and 29-nor-21α*H*-hopane-3,22-dione were built and the conformer distribution was first analyzed by MMFF in gas. After geometry optimization of all conformers by Hartree–Fock/3-21G, energies were evaluated using density functional (DF)/ωB97 X-D/6-31G* calculation. Geometry optimization and energy calculation were repeated using DF/ωB97 X-D/6-31G* and DF/ωB97 X-V/6-311 + G(2df, 2p), respectively. Finally, six conformers of 29-norlupan-3,20-dione and eight conformers of 29-nor-21α*H*-hopane-3,22-dione were obtained without discarding high-relative energy conformers (> 10 kJ/mol) through the process. NMR shift prediction was carried out using DF/ωB97 X-D/6-31G* calculation and the resulting shifts were calibrated based on Boltzman distribution in DF/ωB97 X-V/6-311 + G(2df, 2p) energy simulation.

Instrumental analyses of isolated and synthetic compounds

All ^{1}H, ^{13}C, ^{1}H–^{1}H COSY, HMBC, HSQC and NOESY NMR spectra of isolated compounds were measured with an AMX-500 (Bruker, Billeria, MA, USA) (500 MHz and 125 MHz). ^{1}H and ^{13}C NMR spectra of synthetic compounds were measured with a JNM-EX270 (JEOL, Tokyo, Japan) (270 MHz and 67.5 MHz). Chemical shifts are defined using tetramethylsilane as the internal standard and expressed in δ ppm. Mass spectra were acquired with FD techniques using a JMS-T100GCV (JEOL) or EI techniques by a JMS-SX102A (JEOL). All NMR spectra on AMX-500 were measured at the GC–MS and NMR Laboratory, Faculty of Agriculture, Hokkaido University. Optical rotations were determined on a P-2200 polarimeter (JASCO, Tokyo, Japan) in a $\phi 3.4 \times 50$ mm cell at 25 °C. A part of physicochemical data of isolated compounds is as follows: Compound **1**: $[\alpha]_D^{25}$ +13.9 (*c* 1.00, $CHCl_3$); HR-FI–MS *m/z* $[M]^+$: calcd. for $C_{29}H_{46}O_2$, 426.3498; found, 426.3510.; Compound **2**: $[\alpha]_D^{25}$ +48.1 (*c* 0.49, $CHCl_3$); HR-FD-MS *m/z* $[M]^+$: calcd. for $C_{32}H_{50}O_4$, 498.3709; found, 498.3729; Compound **3**: $[\alpha]_D^{25}$ +46.6 (*c* 0.99, $CHCl_3$); HR-FD-MS *m/z* $[M]^+$: calcd. for $C_{32}H_{50}O_4$, 498.3709; found, 498.3710. NMR peak lists are presented in Tables 1 and 2.

Gas chromatography/Mass spectrometry (GC–MS) analysis

1.0 mg of methanolic bark extract, 0.1 mg of isolated compounds **1**–**3**, commercial ursolic acid (Sigma-Aldrich, St. Louis, MO, USA) and oleanolic acid (TCI, Tokyo, Japan) were derivatized with 200 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide and diluted with 800 µL of $CHCl_3$. One microliter of each solution was analyzed by GC-2010/QP-2010 system (Shimadzu, Kyoto, Japan) under following conditions: column: BPX5 30 m, ϕ0.25 mm, *df* 1.0 µm (SGE analytical science, Melbourne, Australia); injection unit: 300 °C; column oven: 340 °C; carrier gas control: constant linear velocity (44.5 cm/sec); carrier gas: helium; interface: 250 °C; ion source: 250 °C. Chromatograms were obtained by monitoring *m/z* 426, the parent ion of 29-norlupan-3,20-dione, and *m/z* 320, the C–D–E ring fragment ($C_{19}H_{32}O_2Si$) of other triterpenoids. Retention times of authentic compounds were as follows: BisTMS-ursolic acid: 10.41 min; BisTMS-oleanolic acid: 11.30 min; 29-norlupan-3,20-dione: 12.28 min; TMS-oleanolic acid acetate: 12.53 min; TMS-ursolic acid acetate: 13.69 min.

Synthesis of 28-*O*-*p*-toluenesulfonyl betulin (5)

To the solution of betulin (1.92 g, 4.34 mmol) in 18 mL of pyridine, *p*-toluenesulfonyl chloride (860 mg, 4.51 mmol) solution in 2.0 mL of pyridine was added dropwise at 0 °C. After 2 days stirring, 400 mg of *p*-toluenesulfonyl chloride was added portionwise. The reaction was allowed to stir for another day. Pyridine was azeotropically removed with toluene and the resulting mixture was subjected to flash silica gel column chromatography with EtOAc:toluene = 1:4 as an eluent. UV-absorbing fractions were collected and evaporated. The crude product was recrystallized with EtOAc–CH_2Cl_2 system to give 593.1 mg (23%) of betulin 28-tosylate as white solid. ^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.76, 0.77, 0.79, 0.90 and 0.96 (15H, s, H-23, 24, 25, 26 and 27), 1.63 (3H, s, H-30), 2.27 (1H, m, H-19), 2.46 (3H, s, $ArCH_3$), 3.16 (1H, m, H-3), 3.75 (1H, d, *J* = 9.5 Hz, H-28), 4.07 (1H, d, *J* = 9.2 Hz, H-28), 4.56 and 4.64 (2H, s, = CH_2), 7.36 (2H, d, *J* = 8.4 Hz, aromatic), 7.81 (2H, d, *J* = 8.4 Hz, aromatic); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.7 (C-27), 15.3 (C-24), 15.8 (C-26), 16.0 (C-25), 18.2 (C-6), 19.0 (C-30), 20.6 (C-11), 21.6 ($ArCH_3$), 25.0 (C-12), 26.5 (C-15), 27.3 (C-2), 27.9 (C-23), 29.1 (C-16), 29.2, 34.1 (C-7 and C-22), 37.1 (C-10), 37.6 (C-13), 38.6 (C-1), 38.8 (C-4), 40.7 (C-8), 42.6 (C-14), 46.7 (C-19), 47.6 (C-17), 48.7 (C-18), 50.2 (C-9), 55.2 (C-5), 69.3 (C-28), 78.9 (C-3), 110.0 (C-29), 128.0, 129.8, 132.9 and 144.7 (aromatic), 149.6 (C-20).

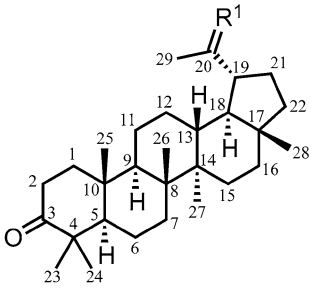
Synthesis of lupeol (6)

To a solution of tosylate **5** (568.0 mg, 0.928 mmol) in 25 mL of THF, 175.0 mg of $LiAlH_4$ and 48.1 mg of $LiBH_4$ was added. The mixture was allowed to stir at 50–65 °C for 3 days. The reaction was quenched with sat. NH_4Cl aq. and the resultant solution was washed with EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo.* The resulting crude product was subjected to flash silica gel column chromatography eluting with EtOAc:hexane = 1:4, followed by concentration of the corresponding fractions *in vacuo* to afford 109.0 mg of lupeol (28%).^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.76, 0.79, 0.83, 0.94, 0.96 and 1.03 (18H, s, H-23, 24, 25, 26, 27 and 28), 1.68 (3H, s, H-29), 2.37 (1H, dt, *J* = 10.9, 10.9, 5.6 Hz, H-19), 3.18 (1H, dd, *J* = 10.4, 5.5 Hz, H-3), 4.56 and 4.69 (2H, s, = CH_2); ^{13}C NMR ($CDCl_3$, 67.5 MHz) : δ 14.5 (C-27), 15.3 (C-24), 15.9 (C-26), 16.1 (C-25), 18.0 (C-28), 18.3 (C-6), 19.3 (C-30), 20.9 (C-11), 25.1 (C-12), 27.4 (C-15), 27.4 (C-2), 28.0 (C-23), 29.8 (C-21), 34.3 (C-7), 35.5 (C-16), 37.1 (C-10), 38.0 (C-13), 38.7 (C-1), 38.8 (C-4), 40.0 (C-22), 40.8 (C-8), 42.8 (C-14), 42.9 (C-17), 47.9 (C-19), 48.3 (C-18), 50.4 (C-9), 55.3 (C-5), 78.9 (C-3), 109.3 (C-29), 150.8 (C-20).

Synthesis of (3β)-hydroxy-29-norlupan-20-one (7)

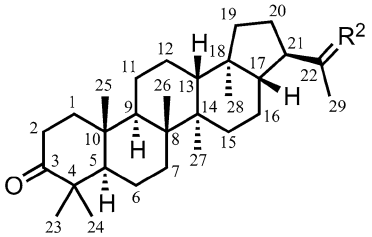
To a stirred solution of lupeol (62.5 mg, 0.146 mmol) in 4 mL of dioxane:H_2O (3:1, v/v) was added *N*-methylmorpholine *N*-oxide (17.1 mg, 0.146 mmol) and 25 µL of 2.5% OsO_4 solution. After 2 days stirring, 62.6 mg of $NaIO_4$

Table 1 ^{1}H and ^{13}C NMR peak lists of isolated compound 1 and predicted ^{13}C chemical shifts of 29-norlupane-3,20-dione and 29-nor-21α*H*-hopane-3,22-dione



R^1 = O: 29-Norlupan-3,20-dione (Cole et al. [8])

R^1 = CH_2: Lupenone (*e.g.* Regasa et al. [10])



R^2 = O: 29-Nor-21α*H*-hopane-3,22-dione (Minh et al. [9])

R^2 = CH_2: Moretenone (*e.g.* McLean et al. [11])

Compound 1		Calculated ^{13}C shifts ($\Delta\delta_C$ between 1)			
^{1}H	^{13}C	Position	29-Norlupan-3,20-dione	Position	29-Nor-21α*H*-hopane-3,22-dione
1.82–1.90 m and *	39.5	1	40.4 (− 0.9)	1	39.9 (− 0.4)
2.45 m	34.1	2	33.0 (1.1)	2	33.1 (1.0)
–	218.2	3	214.9 (3.3)	3	215.0 (3.2)
–	47.3	4	47.2 (0.1)	4	47.2 (0.1)
*	54.8	5	53.3 (1.5)	5	52.6 (2.2)
*	19.7	6	20.8 (− 1.1)	6	20.9 (− 1.2)
*	33.5	7	33.8 (–0.3)	7	31.7 (1.8)
–	40.8	8	40.8 (0.0)	8	42.0 (− 1.2)
*	49.6	9	50.0 (− 0.4)	9	49.5 (0.1)
–	36.9	10	36.7 (0.2)	10	36.9 (0.0)
*	21.5	11	22.3 (− 0.8)	11	21.5 (0.0)
2.02–2.10 m and *	27.2	12	26.9 (0.3)	12	23.8 (3.4)
1.58–1.69 m	37.1	13	36.8 (0.3)	13	47.2 (− 10.1)
–	42.7	14	43.3 (− 0.6)	14	42.3 (0.4)
1.58–1.69 m and *	27.3	15	28.1 (− 0.8)	15	32.3 (− 5.0)
*	34.9	16	34.5 (0.4)	16	21.9 (13.0)
–	43.0	17	42.4 (0.6)	18	44.4 (− 1.4)
1.84 t	49.5	18	47.9 (1.6)	17	52.3 (− 2.8)
2.59 dt	52.6	19	51.9 (0.7)	21	52.5 (0.1)
–	212.9	20	209.5 (3.4)	22	209.5 (3.4)
2.02–2.10 m and *	27.7	21	27.9 (− 0.2)	20	26.6 (1.1)
*	39.8	22	39.7 (0.1)	19	39.8 (0.0)
1.03 s	21.0	23	21.1 (− 0.1)	23	21.1 (− 0.1)
1.07 s	26.7	24	26.5 (0.2)	24	26.5 (0.2)
0.92 s	16.0	25	18.7 (− 2.7)	25	17.7 (− 1.7)
1.06 s	15.7	26	17.2 (− 1.5)	26	17.7 (− 2.0)
0.98 s	14.4	27	15.3 (− 0.9)	27	18.8 (− 4.4)
0.79 s	18.0	28	18.5 (− 0.5)	28	15.9 (2.1)
2.16 s	29.2	29	28.3 (0.9)	29	29.6 (− 0.4)

* 1.03–1.54 ppm

(0.293 mmol) was added. When the reaction completed, sat. Na_2SO_3 aq. was added to the mixture followed by extraction with Et_2O. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo.* The resulting crude product was purified by flash silica gel column chromatography eluting with EtOAc:hexane = 2:5 to give 43.9 mg of (3β)-hydroxy-30-norlupan-20-one (71%). ^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.76, 0.77, 0.83, 0.97 and 1.02 (18H, s, H-23, 24, 25, 26, 27 and 28), 1.82 (1H, t, *J* = 11.3 Hz, H-18), 2.05 (1H, m, H-21), 2.15 (3H, s, H-29), 2.58 (1H, dt, *J* = 11.2, 11.2, 5.7 Hz, H-19), 3.19 (1H, dd, *J* = 10.7, 5.0 Hz, H-3); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.4 (C-27), 15.4 (C-24), 15.9 (C-26), 16.0 (C-25), 17.9 (C-28), 18.3 (C-6), 20.9 (C-11), 27.1 (C-12), 27.3 (C-2 and C-15), 27.6 (C-21), 28.0 (C-23), 29.1 (C-29), 34.2 (C-7), 34.9 (C-16), 37.0 (C-13), 37.1 (C-10), 38.6 (C-1), 38.8 (C-4), 39.8 (C-22), 40.7 (C-8), 42.6 (C-17), 43.0 (C-14), 49.6 (C-18), 50.2 (C-9), 52.6 (C-19), 55.2 (C-5), 78.8 (C-3), 212.9 (C-20).

Synthesis of 29-norlupan-3,20-dione

To a stirred solution of **7** (43.9 mg, 0.103 mmol) in 7 mL of CH_2Cl_2 was added 87.4 mg of Dess-Martin periodinane (0.206 mmol). After 1 h, the mixture was partitioned between sat. $NaHCO_3$ aq. solution and $CHCl_3$, and the organic layer was washed with brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude product was purified with flash silica gel column chromatography eluting with EtOAc:hexane = 1:2 to give 36.9 mg of 29-norlupan-3,20-dione (84%). $[\alpha]_D^{25}$ +20.4 (*c* 1.23, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.79, 0.92, 0.98, 1.03, 1.06 and 1.07 (18H, s, H-23, 24, 25, 26, 27 and 28), 1.84 (1H, t, *J* = 11.5 Hz, H-18), 1.84–2.15 (4H, m, H-1b, H-12 and H-21b), 2.15 (3H, s, H-29), 2.37–2.49 (2H, m, H-2), 2.59 (1H, dt, *J* = 11.2, 11.2, 5.8 Hz, H-19); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.4 (C-27), 15.7 (C-26), 15.9 (C-25), 18.0 (C-28), 19.6 (C-6), 21.0 (C-23), 21.4 (C-11), 26.7(C-24), 27.2 (C-12), 27.3 (C-15), 27.6 (C-21), 29.2 (C-29), 33.4 (C-7), 34.1 (C-2), 34.9 (C-16), 36.8(C-10), 37.1 (C-13), 39.5 (C-1), 39.8 (C-22), 40.6 (C-8), 42.7 (C-14), 43.0 (C-17), 47.2 (C-4), 49.5 (C-18), 49.6 (C-9), 52.5 (C-19), 54.8 (C-5), 212.7 (C-20), 218.1 (C-3).

Synthesis of oleanolic acid acetate

To a solution of 106.4 mg of oleanolic acid (0.219 mmol) in 4.0 mL of pyridine, 413 μL of acetic anhydride and 80.7 mg of *N*,*N*-dimethyl-4-aminopyridine were added. After 2 h stirring, the mixture was partitioned between 0.5 M HCl aq. and EtOAc. The organic layer was washed with sat. $NaHCO_3$ aq. and brine. After drying over anhydrous Na_2SO_4 and subsequent evaporation, the crude product was purified by flash silica gel column chromatography eluting with EtOAc:hexane = 1:4 to give 101.8 mg of oleanolic acid acetate (93%).

Synthesis of ursolic acid acetate

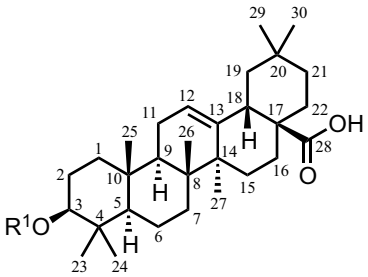
Following the same procedure described above, 17.5 mg of ursolic acid acetate was prepared from 15.6 mg of ursolic acid in quantitative yield. Flash silica gel column chromatography was performed with MeOH:$CHCl_3$ = 5:95.

Results and discussion

Isolation of anti-toxoplasma compounds from *Quercus crispula* Blume outer bark

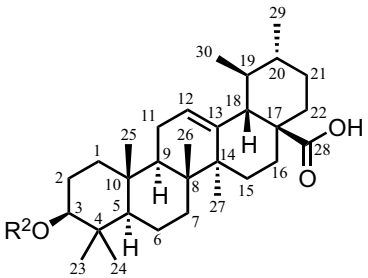
To explore novel anti-toxoplasma natural products, the activities of methanolic bark extracts from northern tree species were evaluated. The anti-toxoplasma assay was performed based on ELISA quantifying microneme 10 (MIC10) protein [6]. MIC10 protein is secreted by toxoplasma when invading a host cell and has key actions in invasion and proliferation of the parasite. Therefore, the quantity of MIC10 can reflect the growth of *T. gondii*. Among tested 14 bark extracts, only an outer bark extract from *Quercus crispula* Blume (mizunara) showed complete inhibition at 10 μg/mL whereas others did not exhibited any activity (data not shown). To identify the active compound(s) from Mizunara outer bark extract, next a bioassay-guided isolation was undertaken. The bark extract was prepared with $CHCl_3$, which can extract equal titer of active ingredients to previously used methanol. The extract was subjected to flash silica gel column chromatography with eluting EtOAc:hexane = 1:9 to 1:7 and then EtOAc:hexane = 1:10 to 1:7. Obtained active fraction was purified with reverse phase HPLC. As shown in Fig. 1a, three major peaks were detected and a following bioassay revealed that they all show anti-toxoplasma activities, whereas other regions showed no activity. The first peak at RT 9.62 min was identified as the mixture of pentadecanoic acid and bis-unsaturated C18 fatty acid by EI mass spectrometry (MS) coupled with NIST library search. Although this mixture showed same activity as fractions later discussed, further investigation was not made due to its low yield (3.4%) and the possibility as contaminants. The second peak at RT 12.15 was isolated as pure compound **1**. The third peak at RT 13.25 was also fractionated, but the fraction was still a binary mixture. As they could not be separated under any conditions tested (e.g., use of acetonitrile, normal phase or HILIC mode), recycle HPLC separation was applied. The third fraction was subjected to ODS HPLC and the eluate was recycled 8 times in the system, which resulted in a successful isolation of two active compounds **2** and **3** (Fig. 1b).

Table 2 ^{1}H and ^{13}C NMR peak lists and signal assignments of isolated compounds 2 and 3



R^1 = Ac: Oleanolic acid acetate (**2**)

R^1 = H: Oleanolic acid (**8**)



R^2 = Ac: Ursolic acid acetate (**3**)

R^2 = H: Ursolic acid (**9**)

Position	Compound 2		Compound 3	
	^{1}H	^{13}C	^{1}H	^{13}C
1	* and ***	38.1	† and †††	38.3
2	*** and 1.87–1.90 m	23.5	‡ and ‡‡	23.6
3	4.49 m	80.9	4.50 dd (*J* = 9.8, 6.3 Hz)	80.9
4	–	37.7	–	37.7
5	0.83–0.88 m	55.3	0.82–0.91 m	55.2
6	** and ***	18.2	†† and †††	18.2
7	**	32.6	†† and †††	32.8
8	–	39.3	–	39.5
9	***	47.6	†††	47.5
10	–	37.0	–	36.9
11	*** and 1.87–1.90 m	23.4	‡ and ‡‡	23.3
12	5.28 t (*J* = 3.5 Hz)	122.6	5.24 t (*J* = 3.5 Hz)	125.7
13	–	143.6	–	138.0
14	–	41.6	–	41.9
15	* and ***	27.7	† and ‡‡	28.0
16	** and 1.99 dt (*J* = 13.6, 13.6, 3.9 Hz)	22.9	‡‡ and 2.01 dt (*J* = 13.5, 13.5, 4.0 Hz)	24.1
17	–	46.5	–	48.0
18	2.82 dd (*J* = 13.6, 4.1 Hz)	41.0	2.18 d (*J* = 11.2 Hz)	52.5
19	* and ***	45.9	††	39.0
20	–	30.7	††	38.8
21	**	33.8	†† and †††	30.6
22	***	32.4	‡	36.7
23	0.85 s	16.7	0.85 s	16.7
24	0.87 s	28.0	0.87 s	28.1
25	0.94 s	15.4	0.96 s	15.5
26	0.76 s	17.1	0.77 s	17.0
27	1.13 s	25.9	1.07 s	23.6
28	–	182.7	–	183.5
29	0.93 s	23.6	0.86 d (*J* = 5.6 Hz)	17.1
30	0.91 s	33.1	0.95 d (*J* = 6.3 Hz)	21.2
acetyl CH_3	2.05 s	21.3	2.05 s	21.3
acetyl C=O	–	171.0	–	171.1

* 1.00–1.13 ppm; ** 1.17–1.48 ppm; *** 1.52–1.81 ppm

†: 1.03–1.19 ppm; ††: 1.25–1.39 ppm; †††: 1.46–1.58 ppm; ‡: 1.60–1.73 ppm; ‡‡: 1.83–1.92 ppm

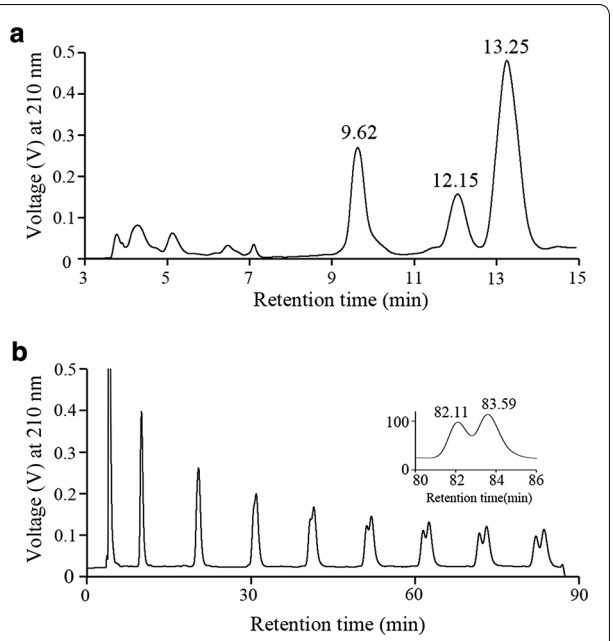
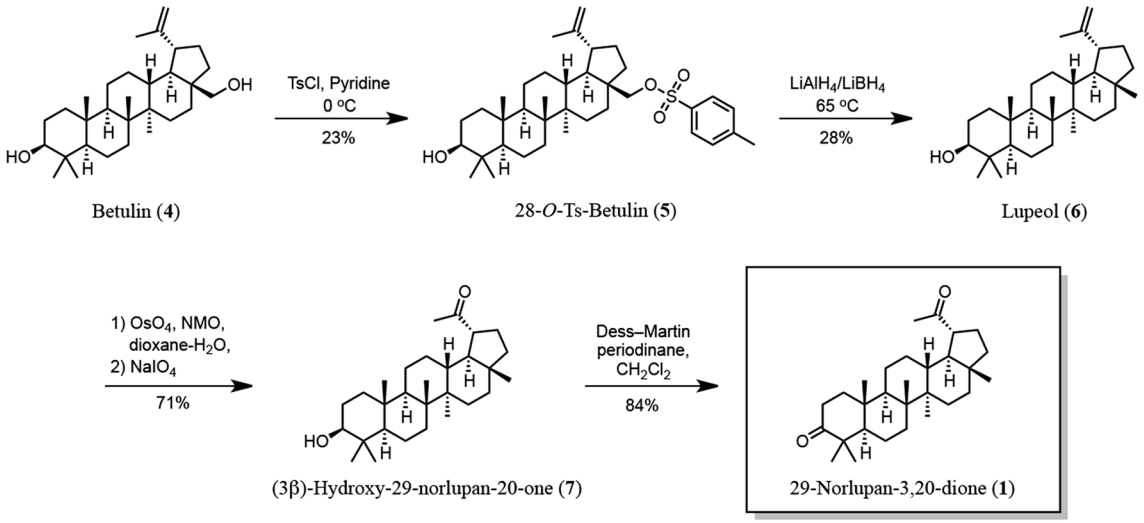


Fig. 1 HPLC chromatogram of **a** active fraction obtained from *Quercus crispula* Blume outer bark extract; **b** recycle-valve separation of two-component mixture (peak at 13.25 min in **a**)

Structure elucidation of isolated compounds 1, 2 and 3

The structures of isolated compounds were elucidated on the basis of NMR, specific rotation, MS analyses and the comparison with reported data. Two partial structures of compound **1** were successfully deduced from ^{1}H, ^{13}C, COSY, HSQC and HMBC NMR spectra, and the molecular formula was determined as $C_{29}H_{46}O_2$ from MS. Although these results suggested two candidate structures, 29-norlupane-3,20-dione [8] and 29-nor-21α*H*-hopane-3,22-dione [9], it could not be concluded because both NMR data were highly consistent with compound **1** ($\Delta\delta_C < 0.2$ ppm). However, this coincidence in two reported structures is unlikely to occur given the differences between lupenone [10] and moretenone [11], the structurally scrutinized analogs of the above two compounds. Lupenone and moretenone have the same ring system, substituent position and steric configuration as 29-norlupane-3,20-dione and 29-nor-21α*H*-hopane-3,22-dione, respectively (Table 1). Meanwhile, their chemical shifts in ^{13}C NMR are clearly distinguishable especially in D- and E-ring ($\Delta\delta_C = 2.5–14.7$ ppm). These differences are significant enough to exclude a coincidence in chemical shifts of 29-norlupane-3,20-dione and 29-nor-21α*H*-hopane-3,22-dione. Thus, we next synthesized 29-norlupane-3,20-dione as a reference compound from structurally reliable lup-20(29)-ene-3β,28-diol, also known as betulin (**4**). As depicted in Scheme 1, a hydroxyl group at C-28 was first tosylated and reduced to give lupeol (**6**). The double bond at C-20(29) was converted to carbonyl group under $OsO_4/NaIO_4$ conditions to give compound **7** and the 3-OH group was oxidized to ketone to form 29-norlupane-3,20-dione. The spectral data of synthesized 29-norlupane-3,20-dione were highly consistent with those of isolated compound **1** ($\Delta\delta_C < 0.2$ ppm) showing the same positive specific rotation. Thus, the identity between compound **1** and 29-norlupane-3,20-dione was demonstrated. On the other hand, 29-nor-21α*H*-hopane-3,22-dione clearly conflicts with compound **1** in consideration of NMR chemical shift difference between the following synthetic intermediates. In the synthesis, the conversion of C-20(29) double bond to



Scheme 1 Synthesis of 29-norlupan-3,20-dione

carbonyl group (**6** to **7**) resulted in lower field shift at C-19 by 4.5 ppm, with affecting the shifts of surrounding C-18 and C-21 ($\Delta\delta_C$: +4.5 and −2.2 ppm, respectively). Meanwhile, if the same conversion was made for moretenone (giving Minh's 29-nor-21α*H*-hopane-3,22-dione), the signal of C-21 is considered to shift by around 10.7 ppm *higher* field without affecting C-20 ($\Delta\delta_C$: ±0 ppm). Despite being in distant locations, C-15, C-16 and C-28 are also considered to shift by 0.8–6.9 ppm. Thus, the possibility of compound **1** to be 29-nor-21α*H*-hopane-3,22-dione can be excluded. The results of computational prediction for ^{13}C NMR also supported this prospect as indicated in Table 1. The density functional theory-based calculations coupled with Boltzman weight calibration were applied for both compounds and the results were compared with experimental spectrum [7]. The predicted ^{13}C chemical shifts of 29-norlupane-3,20-dione showed satisfactory matches ($\Delta\delta_C < 1.6$ ppm except C-3, C-20 and C-25) with compound **1**. In contrast, many indispensable differences were observed in the case of 29-nor-21α*H*-hopane-3,22-dione, such in C-12, C-13, C-15, C-16, and C-27 ($\Delta\delta_C = 3.4$–13.0 ppm) even after the reassignment of shift values to minimize deviations. From these observations, here we concluded that compound **1** is 29-norlupane-3,20-dione not 29-nor-21α*H*-hopane-3,22-dione. The spectral data reported by Minh et al. indicate that their isolated compound is 29-norlupane-3,20-dione [9].

The structures of compounds **2** and **3** were identified as acetates of oleanolic acid [12, 13] and ursolic acid [14], respectively. The chemical shifts of ^{1}H and ^{13}C NMR spectra were all in good agreement with the reported values (Table 2). The relative configuration of each was determined based on NOESY, and the absolute configuration was established from the positive specific rotation to be identical with reported one. In addition, authentic samples corresponding to **2** and **3** were prepared from commercially available oleanolic acid (**8**) and ursolic acid (**9**). The agreements between the spectral data of the isolated compounds and those of the synthetic compounds confirmed the accuracy of structure elucidation. The qualitative GC–MS analysis of the methanolic extract was performed to verify the possibility of two acetates being artifacts arisen from isolation procedures (Fig. 2). Interestingly, both acetates (**2** and **3**) and alcohols (**8** and **9**) could be observed in original extract. The result evidenced that **2** and **3** were naturally occurring in the bark. To date there has been no report on the biological activity of 29-norlupane-3,20-dione and the anti-toxoplasma activity of oleanolic acid acetate and ursolic acid acetate. The present study first unveiled novel activity of these compounds.

Anti-toxoplasma activities of isolated compounds and structure–activity relationship study

The anti-toxoplasma activities of isolated compounds **1**–**3** were determined as IC_{50} using the in vitro growth assay of *T. gondii* [6] as shown in Table 3. Cytotoxicity against human foreskin fibroblast (HFF) cells was tested and the selective toxicity to parasites was shown as selectivity index (SI), the ratio of IC_{50} for the host divided by IC_{50} for *T. gondii*. Interestingly, isolated compounds **1**–**3** showed IC_{50} of 6.8–24.4 μM against the parasite, whereas tenfold higher concentrations were required against the host cell (68.0–375.9 μM). The isolated compounds exhibited superior activities over maslinic acid, a known anti-toxoplasma triterpenoid whose IC_{50} and SI were 53.81 ± 1.4 μM and 4.4, respectively [15]. The results demonstrated that the isolated compounds could be specific anti-toxoplasma agents or promising lead compounds for developing novel drugs.

To identify key structure(s) for activity and selectivity, the next structure–activity relationship study was conducted using analogs of the isolated compounds. Results are presented in Table 3 as IC_{50} values. Betulin (**4**) exhibited potent activity with IC_{50} of 18.3 μM, but was more toxic to host cells (SI = 0.3). Oleanolic acid (**8**) and ursolic acid (**9**) showed similar activity to their acetates (**2** and **3**) but selective toxicities against *T. gondii* were impaired (SI = 1.8 and 1.2). Synthetic (3β)-hydroxy-30-norlupan-20-one (**7**) as an analog of 29-norlupane-3,20-dione did not show inhibitory activity against *T. gondii* and host cells even at 1.0 mM. From the comparison between **1** and **3** and their analogs, it could be concluded that the presence of C-3 hydroxyl group decreases the selectivity against *T. gondii* whereas acetylation or oxidation to carbonyl enables lowering toxicity against host cells. Capping or oxidizing free hydroxyl group at C-3 would elevate the anti-toxoplasma activity itself, as clearly seen in the comparison between **1** and **7**, or **2** and **8**. The inferior activity and selectivity of maslinic acid follow these trends. On the other hand, ursolic acid (**9**) exceptionally retains high anti-toxoplasma activity regardless of its free 3-hydroxyl group, while the selective toxicity of **9** was comparable to **8**. Non-selective toxicities of **9** and maslinic acid suggest that inhibitory activity against protease might correlate with non-selective toxicities of these oleananes [15]. Functionalities around E-ring seem to have a crucial impact on activity, as observed in the case of betulin (**4**) that shows potent activity despite its structural analogy to inactive compound **7**. Although stringent comparison is difficult with the present compounds, high polar functionality, such as carboxyl or hydroxyl group on E-ring, might contribute to

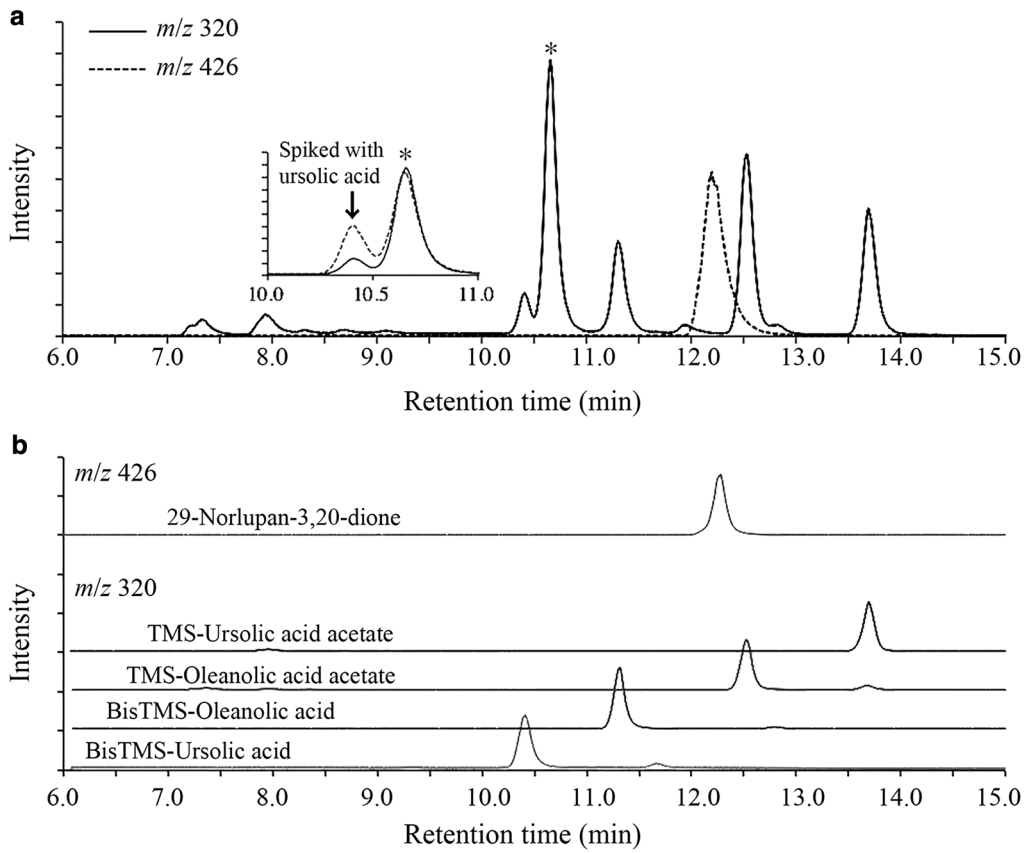
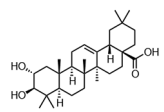


Fig. 2 Qualitative GC–MS chromatogram of **a** TMS-derivatized methanolic extract from *Q. crispula* Blume outer bark; **b** TMS-derivatized authentic compounds. *Unidentified triterpenoid-like compound

Table 3 Anti-toxoplasma activities of isolated compounds 1–3 and their analogs

Compounds	*T. gondii* inhibition (IC_{50})	HFF cytotoxicity[a] (IC_{50})	Selectivity index[c]
Isolated			15.4
29-Norlupan -3,20-dione (**l**)	24.4 ± 1.5	375.9 ± 95.9	10.0
Oleanolic acid acetate (**2**)	6.8 ± 0.8	68.0 ± 2.2	12.1
Ursolic acid acetate (**3**)	7.9 ± 0.4	95.6 ± 6.8	
Analogs			
Betulin (**4**)	18.3 ± 0.4	4.7 ± 6.8	0.3
(3β)-Hydroxy-29 -norlupan-20-one (**7**)	> 1000	> 1000	–
Oleanolic acid (**8**)	54.8 ± 1.1	96.4 ± 4.8	1.8
Ursolic acid (**9**)	5.9 ± 0.1	6.4 ± 0.1	1.1
 Maslinic acid [15]	53.81 ± 1.4	236.61 ± 6.4[b]	4.4

Results are given as the mean ± SD. ($n = 3$)

[a] Human foreskin fibroblasts

[b] Vero cells were used instead of HFF

[c] Selectivity Index = IC_{50} of host cells/IC_{50} of *T. gondii* inhibition

notable activities of **2**, **3**, **4** and **8**. Blockage of gliding motility of *T. gondii* tachyzoites was observed by treating with maslinic acid. One of the action mechanisms of maslinic acid may exert on the tachyzoites in their interaction with the host cell via protease. Maslinic acid and other protease inhibitors cause cytopathological effects on intra- and extracellular forms of the parasite, too [15]. Compound **8** is 2-deoxy analog of maslinic acid, thus it might have the same molecular target as maslinic acid. The isolated compounds **1**, **2**, and **3** may have different mode of action due to their higher selective indexes in comparison with maslinic acid and **8**. The findings from this structure–activity relationship study would be highly beneficial for the development of new anti-toxoplasma drugs with high efficacy and high selectivity. In addition, derivatization of betulin, the most abundant extract in *Betula platyphylla* outer bark, could quantitatively provide such useful drug candidates.

Conclusion

The authors discovered three potent anti-toxoplasma agents, namely 29-norlupane-3,20-dione (**1**), oleanolic acid acetate (**2**) and ursolic acid acetate (**3**) from the outer bark extract of mizunara. Anti-toxoplasma activity and selective toxicity of these compounds against *T. gondii* were outstanding compared to other pentacyclic triterpenoids. The structure–activity relationship study has shown that the presence of the carbonyl group on E-ring and modification of 3-hydroxy group is important for potent activity expression and selectivity. The results opened the door for the novel utilization of analogous triterpenoids obtained from natural resources like wood barks.

Abbreviations

Ac: acetyl; COSY: correlation spectroscopy; DF: density functional; DMSO: dimethyl sulfoxide; EI: electron ionization; ELISA: enzyme-linked immunosorbent assay; EtOAc: ethyl acetate; FD: field desorption; FI: field ionization; GC: gas chromatography; HFF: human foreskin fibroblast; HILIC: hydrophilic interaction chromatography; HMBC: heteronuclear multiple bond coherence; HPLC: high-performance liquid chromatography; HR: high resolution; HSQC: heteronuclear single quantum coherence; IC_{50}: half maximal inhibitory concentration; MIC10: microneme 10; MMFF: Merck molecular force field; MS: mass spectrometry; NIST: National Institute of Standards and Technology; NMO: *N*-methylmorpholine *N*-oxide; NMR: nuclear magnetic resonance; NOESY: nuclear Overhauser effect spectroscopy; ODS: octadecylsilyl; R_f: retardation factor; RT: retention time; SI: selectivity index; THF: tetrahydrofuran; tlc: thin-layer chromatography; TMS: trimethylsilyl; Ts: *p*-toluenesulfonyl.

Authors' contributions

KS, SM and MU conceived and planned the experiments. ME, KS and MI carried out the experiment. KS wrote the manuscript with support from MI and MU. KS and MU supervised the project. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. The spectral data of NMR are available from the corresponding author on reasonable request.

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