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Anticoccidial activity of the secondary metabolites in alpine plants frequently ingested by wild Japanese rock ptarmigans

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ABSTRACT

The Japanese rock ptarmigan (*Lagopus muta japonica*) is an herbivorous species of partridges that inhabits only alpine zones. Alpine plants are their main source of food. These alpine plants contain toxic compounds to deter herbivores from consuming them. A previous analysis of the alpine plants frequently consumed by Japanese rock ptarmigans revealed the presence of a unique mixture of secondary metabolites and a novel compound. Additionally, wild Japanese rock ptarmigans are often infected by two species of *Eimeria* parasites. When these parasites were experimentally administered to Svalbard rock ptarmigans (*Lagopus muta hyperborean*), which do not feed on alpine plants, the birds exhibited symptoms, such as diarrhea and depression, and in some cases, they died. Although little is known about the pathogenesis of these parasites in wild Japanese rock ptarmigans, it was hypothesized that compounds found in alpine plants, their main food source, may reduce the pathogenicity of *Eimeria* parasites. In the present study, we evaluated the anticoccidial activity of the compounds derived from alpine plants *in vitro* using *Eimeria tenella*, which infects chickens belonging to the same pheasant family, as an experimental model. Twenty-seven natural components were extracted from eight alpine plants. The natural components were added to *E. tenella* sporozoites and incubated for 24 h to evaluate their direct effect. Additionally, Madin-Darby bovine kidney cells were incubated with sporozoites and natural components for 24 h to evaluate the inhibitory effect of the components on sporozoite cell invasion. Six compounds from four alpine plants decreased sporozoite viability by up to 88.3%, and two compounds inhibited sporozoite invasion into the cells. Although further studies are needed to evaluate the effects of these components against *Eimeria* infections *in vivo*, our findings suggest that these alpine plants may reduce the degree of infection by decreasing the number of sporozoites in the intestinal tract.

1. Introduction

The Japanese rock ptarmigan (*Lagopus muta japonica*) is a sub-species of rock ptarmigan belonging to the order Galliformes and the family Phasianidae (Nakamura, 2007). They are endemic birds of Japan that are distributed only in the alpine zone of the Japanese Alps at elevations of 2500 m or higher (Nakamura, 2007). It is thought that Japanese rock ptarmigans have remained isolated on the main island of Japan ever since the Japanese archipelago was formed during the ice age (Nakamura, 2007). Subsequently, due to increasing temperatures, most of the birds migrated to alpine areas, resulting in their limited distribution to those areas (Nakamura, 2007). Thus, the Japanese rock ptarmigan is considered to be a relic of the ice age (Nakamura, 2007; Suzuki et al., 2013; Hotta et al., 2019). Its population has drastically decreased in recent years, dropping from approximately 3000 to 1700 birds between the 1980s and 2000s (Nakamura, 2007; Ueda et al., 2018; Hotta et al.,

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2019). Although the exact reasons for the decreasing remain unknown, complicated reasons including global warming can be a potential threat to its survival (Nakamura, 2007; Hotta et al., 2019) since this bird species has adapted to live in a cold environment. In 2012, the Japanese rock ptarmigan was added as an endangered species to the Fourth Red List of Threatened Species of the Ministry of the Environment of Japan (Wildlife Division of the Ministry of the Environment, 2012), and protection and propagation projects are now underway (Wildlife Division of the Ministry of the Environment, 2012; Kobayashi, 2020).

Unlike most other bird species, wild Japanese rock ptarmigans possess herbivorous characteristics, and primarily consume various alpine plants (Omachi Alpine Museum, 1964, 1992; Tateyama City, 2002; Kobayashi and Nakamura, 2011; Fujii et al., 2022). These alpine plants are also considered to be relics of the ice age (Ikeda, 2022). These plants contain various secondary metabolites, including toxic substances, such as alkaloids, cyanide compounds, and phenols (Tsuchida et al., 2017), which are harmful to other organisms. To detoxify these toxic components, Japanese rock ptarmigans maintain a particular intestinal microbiota balance (Tsuchida et al., 2017; Kobayashi et al., 2019). As part of the protection and propagation projects, our research group has analyzed the components of alpine plants that are frequently consumed by Japanese rock ptarmigans for the development of artificial foods suitable for the birds. In addition to the primary metabolites, which are essential for maintaining life activities, the composition of the secondary metabolites was analyzed in these alpine plants, and we found new compounds, such as bibenzyl, that have not been reported previously (Oka et al., 2020). Secondary metabolites are substances produced by an organism that are non-essential for their growth and development (Erb and Kliebenstein, 2020); however, they may be involved in ecological and assistive functions, including defense mechanisms, and can be strain-specific. In addition to the alkaloids, cyanide compounds, and phenols mentioned above, terpenoids and flavonoids are also groups of secondary metabolites. Interestingly, some secondary metabolites are potential drug candidates since they possess biological activities, such as anti-cancer, anti-inflammatory, and anti-microbial activities (Lima et al., 2021; Thawabteh et al., 2021; Shen et al., 2022).

A high prevalence of infection by *Eimeria* parasites, namely *Eimeria uekii* and *Eimeria raichoi*, is seen in wild Japanese rock ptarmigans (Matsubayashi et al., 2018a, 2018b). In general, *Eimeria* parasites are highly host-specific, and they are well-known pathogens that cause coccidiosis in livestock, especially in chickens that also belong to the family Phasianidae (Burrell et al., 2020). As part of their life cycle, *Eimeria* parasites infect chickens when the hosts orally ingest sporulated oocysts (Burrell et al., 2020). Subsequently, the sporozoites released from the oocysts invade into the mucosal epithelium of the gastrointestinal tract (Burrell et al., 2020). Thereafter, the sporozoites undergo asexual and sexual reproduction in the mucosa, and form oocysts (Burrell et al., 2020). The newly produced oocysts are then excreted in the feces (Burrell et al., 2020). In most cases, *Eimeria* parasites cause diarrhea, depression and weight loss, and some species of *Eimeria* can cause fatal bloody feces or diarrhea (Burrell et al., 2020; López-Osorio et al., 2020).

The pathogenicity of *Eimeria* species in rock ptarmigans has not been well documented, unlike for parasites of livestock. However, in a study using Svalbard rock ptarmigans (*Lagopus muta hyperborean*), a subspecies of rock ptarmigans that inhabit another region, birds that were experimentally infected with *Eimeria* species exhibited diarrhea and depression, and some birds died when orally inoculated with a high dose of oocysts (>10,000 oocysts) (Matsubayashi et al., 2023). Although the effects of *Eimeria* parasite infection in wild Japanese rock ptarmigans remain unknown, the birds appear to survive despite high infection rates. This led us to speculate that there may be some kind of factor in Japanese rock ptarmigans that reduces the virulence or degree of infection of *Eimeria* parasites. In the present study, we focused on the consumption of alpine plants that are highly palatable for Japanese rock ptarmigans as a candidate factor for the lower virulence or degree of infection of *Eimeria* parasites in wild Japanese rock ptarmigans when compared to Svalbard rock ptarmigans, which are normally fed artificial alternative bait. It was hypothesized that alpine plants favored by wild Japanese rock ptarmigans might contain unique secondary metabolites that possess anticoccidial activity.

The aim of this study was to screen for secondary metabolites from alpine plants that show anticoccidial activity by performing *in vitro* anticoccidial assays, and to better understand the symbiotic relationship between Japanese rock ptarmigans and *Eimeria* parasites. In the present study, instead of *E. uekii* and *E. raichoi,* which commonly infect wild Japanese rock ptarmigans, we used *E. tenella,* which is known to infect chickens, as a model to evaluate the anticoccidial activity of the plant extracts. First, natural components containing secondary metabolites were extracted from eight alpine plants and partially purified. Subsequently, the anticoccidial activities of these components were assessed using excysted sporozoites and cell cultures of the sporozoites *in vitro*.

2. Materials and methods

2.1. Preparation of parasites

The *E. tenella* OPU strain was used throughout the present study. Briefly, after inoculation of the oocysts into chicks, the feces were collected, and immature oocysts were purified by the sugar floatation centrifugation method (Sheather, 1923). These oocysts were sporulated in approximately 0.1% potassium dichromate solution (Nacalai Tesque, Kyoto, Japan) at 28 °C with shaking, and stored at 4 °C until use. The oocysts were treated with 5%–10% sodium hypochlorite (Nacalai Tesque) for 10 min at 4 °C, then washed five times with phosphate-buffered saline (PBS; pH 7.4). The excystation of sporozoites from oocysts was performed using a previously described method (Matsubayashi et al., 2019) with modifications. Briefly, an equal volume of 0.5–0.7-mm glass beads (As One, Osaka, Japan) was added to the oocysts (2×10^6 to 2×10^7). Sporocysts were released by vortexing to mechanically damage the oocyst walls. The beads were washed in PBS, and the sporocysts were collected. After centrifugation at 1800×*g* for 5 min at room temperature, the supernatant was removed, and the pellet was incubated at 41 °C for 1–2 h in excystation solution. The excystation solution contained Hank's buffered salt solution (Sigma-Aldrich, St. Louis, MO, USA) with 1% (w/v) taurodeoxycholic acid (Sigma-Aldrich) and 0.25% (w/v) trypsin (Sigma-Aldrich). The excysted sporozoites were centrifuged at 200×*g* for 2 min to remove large debris, and the supernatant containing sporozoites was collected. This process was repeated twice, then the supernatants were pooled and centrifuged at 1800×*g* for 5 min. The collected sporozoites were used for the experiments described below.

2.2. Extraction and component analysis of secondary metabolites from alpine plants

Alpine plants that grow in the same area where wild Japanese rock ptarmigans are found were examined (Table 1). The palatability of plants and plant parts for wild Japanese rock ptarmigans (highly or less palatable) was determined from previous studies (Omachi Alpine Museum, 1964, 1992; Kobayashi and Nakamura, 2011; Fujii et al., 2022) and field observations. The leaves and stems of *Empetrum nigrum* var. *japonicum*, the leaves of *Loiseleuria procumbens*, the leaves of *Vaccinium vitis-idaea*, the leaves of *V. ovalifolium*, the leaves of *Aconogonon weyrichii* var. *alpinum*, the flowers of *Oxytropis japonica* var. *japonica*, and the flowers of *Phyllodoce aleutica* were selected as being highly palatable for wild Japanese rock ptarmigans. The leaves and stems of *Phyllodoce aleutica* and the leaves of *Anemone narcissiflora* were selected as being less palatable for wild Japanese rock ptarmigans. Although the shape of the leaves of *P. aleutica* resemble those of *E. nigrum,* which is one of the highly palatable plants, the birds generally peck at only the flowers of *P. aleutica* (Kobayashi, unpublished data). In addition, although *A. narcissiflora* is one of the most common plants in the Japanese alpine

Table 1
The natural compounds derived from alpine plants[a].

Palatability	Plant (parts)	Secondary metabolite	Component No.
High	*Empetrum nigrum* var. *japonicum* (leaves & stems)	**α-Amyrin**, β-Amyrin	A-1
		4,2′-Dihydroxy-3,5-dimethoxydihydrostilbene	A-2
		Epicatechin	A-3
		Isoquercitrin	A-4
		Pinosylvin	A-5
	Empetrum nigrum var. *japonicum* (leaves & stems) & *Phyllodoce aleutica* (flowers)	Uvaol	B-1
		Uvaol, β-Amyrin	B-2
	Loiseleuria procumbens (leaves)	β-Amyrin	C-1
		8-Demethyleucalyptin	C-2
		Glucogallin, Maltose	C-3
		6-Methylated flavonoids	C-4
		Oleanolic acid	C-5
		Trilobatin	C-6
	Loiseleuria procumbens (flowers)	Phlorhizin	C-7
	Vaccinium ovalifolium (leaves)	Naringenin	D-1
		β-Sitosterol, methoxy flavonoids	D-2
		Ursolic acid	D-3
	Vaccinium vitis-idaea (leaves)	Arbutin	E−1
		Catechin	E−2
		Quercetin 3-O-sophoroside, Arbutin	E−3
	Oxytropis japonica var. *japonica* (flowers)	Gallocatechin	F-1
		β-Methylglucoside	F-2
	Aconogonon weyrichii var. *alpinum* (leaves)	Avicularin	G-1
Low	*Phyllodoce aleutica* (leaves & stems)	3-Methoxy-5-methylphenol	H-1
		5-Methylbenzene-1,3-diol	H-2
		Taxifolin 3-O-glucoside, Gallocatechin	H-3
	Anemone narcissiflora (leaves)	Cauloside G	I-1

Palatability: palatability of the plant for wild Japanese rock ptarmigans; Plants: alpine plants from which each compound was extracted, and the parts used.
[a] In mixed samples with two secondary metabolites, the primary compound is displayed in bold.

zone, Japanese rock ptarmigans rarely eat this species (Kobayashi and Nakamura, 2011). Hence, we classified these plants as being less palatable for the birds.

Alpine plant samples were collected from Mt. Norikura and Mt. Kitadake. Each part of the plants was crushed and extracted with methanol. The leaves and stems were crushed together for *E. nigrum* and *P. aleutica*, because Japanese rock ptarmigans peck at them together (Kobayashi and Nakamura, 2011). The samples were fractionated by silica gel chromatography. The components were analyzed by thin-layer chromatography, and the chemical structures were determined by nuclear magnetic resonance (NMR) spectroscopy. The components are listed as A-1 to I-1 in Table 1.

To confirm the activity, commercially available ursolic acid (Tokyo Chemical Industry, Tokyo, Japan), α-amyrin (Extrasynthese, Lyon, France), β-amyrin (Extrasynthese), β-sitosterol (Tama Biochemical, Tokyo, Japan), and oleanolic acid (Tokyo Chemical Industry) were purchased and used. As a positive control, lasalocid (Sigma-Aldrich) was used. The resulting extracted components were dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque) to 10 mM as stock solutions. Lasalocid was also dissolved in DMSO (Nacalai Tesque) to 1 mM. All samples were stored at −20 °C until use.

2.3. In vitro viability assay for E. tenella sporozoites

Sporozoites were suspended at 5×10^6 sporozoites/ml in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque), which was free of phenol red and contained antibiotics (Antibiotic-Antimycotic Mixed Stock Solution, Nacalai Tesque). The medium containing sporozoites was dispensed at 99 μl/well into a 96-well plate (AGC Techno Glass Co., Ltd., Shizuoka, Japan), and supplemented with 1 μl of the natural components extracted from alpine plants or the commercially available pure compounds. A sporozoite viability assay was performed using the natural components extracted from the alpine plants (components A-1 to I-1) at the concentration of 100 μM and lasalocid at the concentration of 1 μM. As the blank and solvent controls, we used RPMI medium containing sporozoites without any compounds or with DMSO (Nacalai Tesque), respectively. Using the natural and commercial compounds showing antiparasitic activities, IC_{50} were analyzed in the ranging of 1–100 μM, and that of lasalocid was from 0.01 to 1 μM.

Subsequently, the samples were incubated for 24 h at 37 °C under 5% CO_2. Then, 10 μl of Cell Count Reagent SF (Nacalai Tesque) was added, and the samples were incubated for 3 h under the same conditions. Finally, 10 μl of 0.1 M hydrochloric acid (Nacalai Tesque) was added and mixed to stop the reaction. A spectrophotometer (Nivo Multimode Microplate Reader, PerkinElmer, Boston, MA, USA) was used to measure the absorbance at 450 nm and 620 nm, and the sporozoite viability was calculated using the following formula:

$$\text{Sporozoite viability (\%)} = \{(Abs_{450nm} - Abs_{620nm})_{compound} - (Abs_{450nm} - Abs_{620nm})_{blank}\} / \{(Abs_{450} - Abs_{620})_{DMSO} - (Abs_{450nm} - Abs_{620nm})_{blank}\} \times 100$$

Each experiment was repeated three to five times with a single well for each compound per experiment.

2.4. In vitro cytotoxicity assay of the natural components from alpine plants

Madin-Darby bovine kidney (MDBK) cells (NBL-1 strain, Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan) were maintained in Minimum Essential Media (MEM; Nacalai Tesque) with 10% fetal bovine serum (FBS; Cosmo Bio, Tokyo, Japan) and 100 U/ml penicillin/100 μg/ml streptomycin (Nacalai Tesque) in an incubator at 37 °C with 5% CO_2. When the cells reached approximately 70% confluence, they were detached using Accutase (Nacalai Tesque) and passaged.

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay was performed to test the effects of the natural components obtained from the alpine plants (components A-1 to I-1) and lasalocid on MDBK cell viability. MDBK cells were seeded at 2×10^4 cells/well in 96-well plates (AGC Techno Glass). After cultivation at 37 °C under 5% CO_2 for 24 h, each compound was added at the final concentrations of 0.1, 1, 10, 50, or 100 μM. DMSO (Nacalai Tesque) was used as a solvent control. After cultivation under the same conditions for 24 h, 10 μl of MTT (Nacalai Tesque) was added. Then, the cells were incubated for 2 h. Subsequently, 100 μl of 99% isopropyl alcohol (Nacalai Tesque) was added and vigorously mixed to completely dissolve the formazan. The absorbance at a wavelength of 562 nm was measured with a spectrophotometer (Nivo Multimode Microplate Reader, PerkinElmer). Cell viability was calculated according to the following formula:

$$\text{Cell viability (\%)} = Abs_{562nm\ compounds} / Abs_{562nm\ DMSO} \times 100$$

For each compound, the highest concentration at which no significant effect was seen on the cell viability (the maximum non-toxic concentration) was determined. Each experiment was repeated three times

with three technical replicates per compound per experiment.

2.5. In vitro cell invasion inhibition assay for E. tenella sporozoites

In this assay, we used the natural components from alpine plants (components A-1 to I-1) as well as lasalocid as a positive control. MDBK cells were seeded onto 16-well chamber slides (Thermo Fisher Scientific, Waltham, MA, USA) or 8-well chamber slides (Matsunami Glass Ind., Ltd., Osaka, Japan). The cells were cultured at 37 °C under 5% CO_2 until they reached confluence. The purified sporozoites were suspended in MEM at a concentration of 2.5×10^4 sporozoites/ml (for the 16-well chamber slides) or 3.5×10^4 sporozoites/ml (for the 8-well chamber slides). After the culture medium was removed, the sporozoites and components at the maximum non-toxic concentrations determined above were simultaneously added to the MDBK cells. Subsequently, the cells were incubated at 37 °C under 5% CO_2 for 2 h. Then, uninfected sporozoites were removed by changing the media. After incubation for an additional 22 h, the slides were fixed in methanol and stored at −20 °C. The parasites were then blocked with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich) for 10 min at room temperature. Next, rabbit anti-*E. tenella* oocyst polyclonal antibody (1:1000) (Matsubayashi et al., 2014) was applied to the cells, and the slides were incubated for 1 h at room temperature in the dark. Afterwards, the slides were washed three times with PBS containing 1% BSA. They were subsequently incubated with AlexaFluor 488 goat anti-rabbit IgG (H + L) (1:1000, Thermo Fisher Scientific) for 1 h under the same conditions. After washing three times with PBS containing 1% BSA, they were sealed with 7% polyvinyl alcohol (Kishida Chemical Co., Ltd., Osaka, Japan) and kept at 4 °C. The number of parasites present in MDBK cells was counted under a fluorescent microscope (BX50, Olympus, Tokyo, Japan). The invasion rate of sporozoites was calculated according to the following formula:

Invasion rate (%) = the number of parasites within cells in a 15-mm^2 area in the presence of test compounds/the number of parasites within cells in a 15 mm^2 area in the presence of DMSO × 100.

Each experiment was repeated at least three times with two technical replicates per compound per experiment.

2.6. Statistical analysis

Outliers were tested using Thompson's test ($p < 0.05$). The student's t-test was used without outliers to determine the differences in the sporozoite viability and the invasion rate ($p < 0.01$). Welch's *t*-test was used to determine the differences in cell viability ($p < 0.05$).

3. Results

3.1. Natural components from alpine plants

A total of 27 natural components were obtained from 10 parts of eight alpine plants. The secondary metabolites identified in each of the components are listed in Table 1.

3.2. Effect of the natural components against E. tenella sporozoite viability

The results showed a significant reduction of sporozoite viability with the following components at 100 μM: A-1 (30.4% viability), B-1 (86.6% viability), B-2 (11.7% viability), C-5 (39.1% viability), D-2 (12.8% viability), and D-3 (21.1% viability; $p < 0.01$; Fig. 1). As a control, the sporozoite viability was 21.9% with 1 μM lasalocid. Next, the half maximal inhibitory concentration (IC_{50}) values of the effective components (components A-1, B-2, C-5, D-2, and D-3) and lasalocid against sporozoites were determined, although B-1 could not be tested since the viability was higher than 50%. The IC_{50} values were 65.51 μM for A-1, 51.66 μM for B-2, 21.19 μM for C-5, 10.24 μM for D-2, 47.81 μM for D-3, and 0.03 μM for lasalocid (Fig. 2). The main compounds in the components were α-amyrin and β-amyrin for A-1, uvaol and β-amyrin for B-2, uvaol for B-1, oleanolic acid for C-5, β-sitosterol and methoxy flavonoids for D-2, and ursolic acid for D-3.

Next, we conducted a confirmation assay for the active compounds found in A-1, B-2, C-5, D-2, and D-3 using commercially available pure products. Sporozoite viability was significantly reduced to 18.5% by oleanolic acid, and to 37.3% by ursolic acid ($p < 0.01$, Fig. 3A). No significant effect was observed from the other compounds. The IC_{50} value of oleanolic acid and ursolic acid was 19.25 and 20.14 μM, respectively (Fig. 3B).

3.3. Cell toxicity and inhibitory effect against sporozoite cell invasion of the components derived from alpine plants

In this assay, the toxicity of the natural components from alpine plants (components A-1 to I-1) and lasalocid on MDBK cells was determined, and the inhibitory effect of these components on sporozoite cell invasion was examined. Concentrations higher than 10 μM of C-4 or lasalocid, concentrations higher than 50 μM of B-1, C-2, D-2 or D-3, and 100 μM of A-1 or C-5 significantly reduced the viability of MDBK cells (Table 2). Based on these results, we determined the final concentration of each of the components to use for the cell invasion assay using sporozoites and MDBK cells (Table 2).

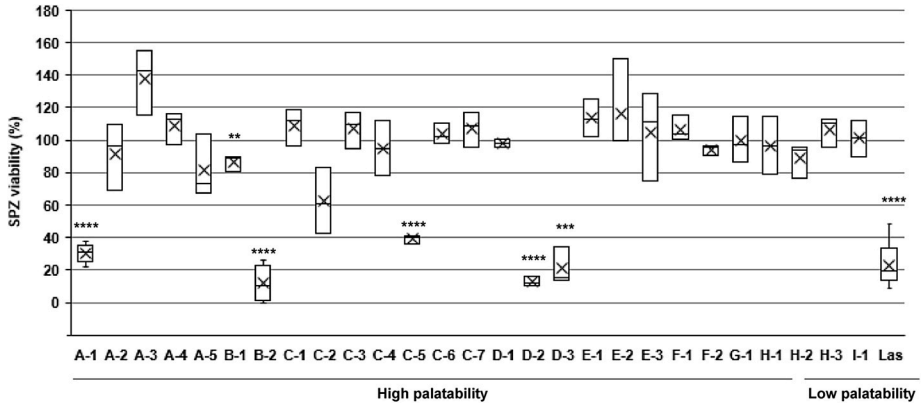

Fig. 1. Direct effects of the natural components derived from alpine plants on *E. tenella* sporozoites. The viability of sporozoites treated with each natural component derived from alpine plants or lasalocid (positive control) with the viability of the DMSO-treated group set as 100%. The final concentration was 100 μM for the natural components, and 1 μM for lasalocid. SPZ: sporozoite; Las: lasalocid. Outliers were tested using Thompson's test ($p < 0.05$), and the student's t-test was utilized to compare the data with the DMSO-treated group as a control (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

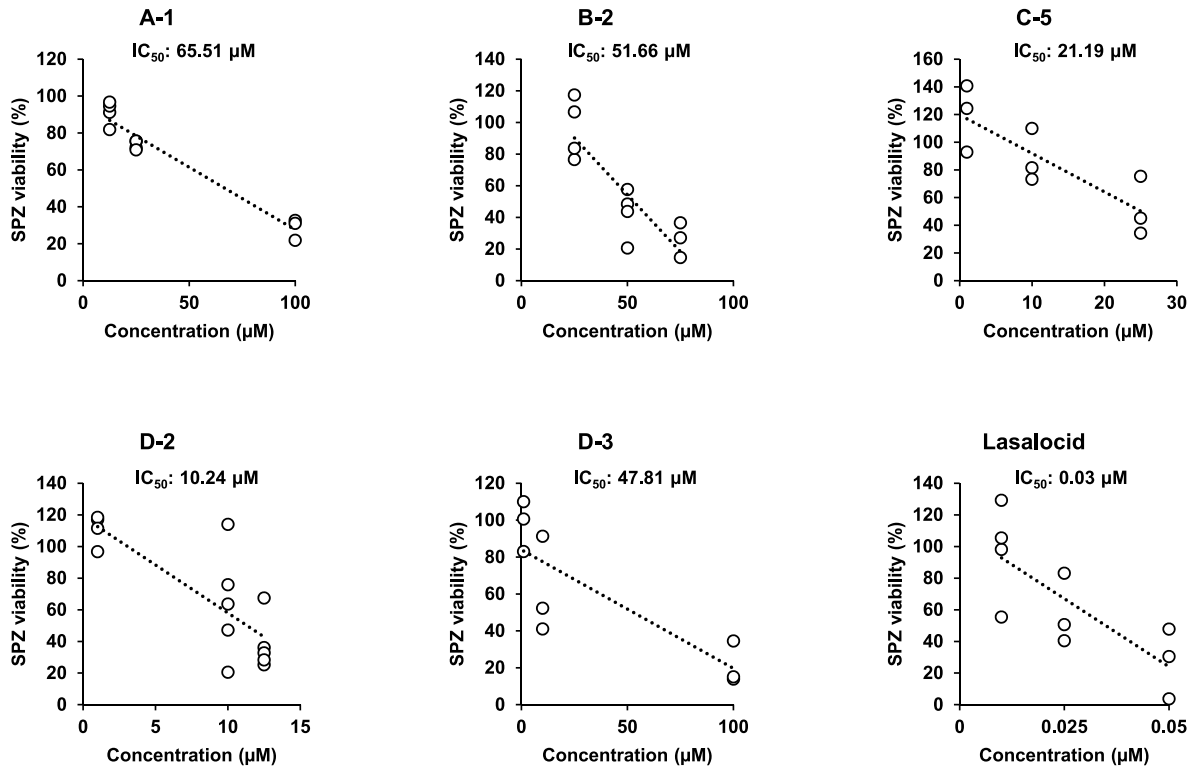

Fig. 2. The efficacy of the natural components against *E. tenella* sporozoites. The viability of sporozoites was determined at various concentrations of the compounds that showed effectiveness at 100 μM. The half maximal inhibitory concentration (IC_{50}) value was determined from the approximate curves obtained from these results. SPZ: sporozoite.

We evaluated the effects of the natural components on sporozoite invasion into MDBK cells. The invasion rate decreased significantly to 78.9% with B-1, 87.2% with C-7, and 39.3% with lasalocid ($p < 0.01$; Fig. 4). No significant decrease was observed with the other components. The main compounds in the components were uvaol for B-1, and phlorhizin for C-7 (Table 1).

4. Discussion

We analyzed the components extracted from 10 parts of eight alpine plants that are highly palatable for Japanese rock ptarmigans. In total, 27 extracts of natural components were obtained, and they contained 28 secondary metabolites. In the *in vitro* assays using sporozoites of *Eimeria* parasites, six of the natural components significantly reduced the sporozoite viability. These components were derived from four alpine plants that are highly palatable for Japanese rock ptarmigans, *i.e., E. nigrum* var. *japonicum*, *P. aleutica*, *L. procumbens,* and *V. ovalifolium*. Nonetheless, the diet of Japanese rock ptarmigans changes depending on the season and the vegetation in their habitat. Evergreens, such as *E. nigrum* var. *japonicum* and *L. procumbens*, are the main food source of Japanese rock ptarmigans from spring to autumn in various populations (Omachi Alpine Museum 1992; Kobayashi and Nakamura, 2011; Fujii et al., 2022). The flowers of *P. aleutica* and *V. ovalifolium* are also an important food source during the summer (Kobayashi and Nakamura, 2011; Fujii et al., 2022). During the period from June to September, *Eimeria* oocysts are very commonly detected in the feces of wild Japanese rock ptarmigans (Matsubayashi et al., 2018b). It is interesting that the period when wild rock ptarmigans consume these plants coincides with the period when the rate of *Eimeria* parasite infection is high. These findings suggest that anticoccidial compounds present in plants might reduce the degree of *Eimeria* parasite infections (pathogenicity) in wild rock ptarmigans.

In the present study, we found that the main components possessing anti-parasitic effects contained five secondary metabolites, *i.e.,* α-amyrin, uvaol, oleanolic acid, ursolic acid, and β-sitosterol (in components A-1, B-1, B-2, C-5, D-2, and D-3). Although β-amyrin was a subcomponent of A-1, which contained both α-amyrin and β-amyrin, and B-2, which contained both uvaol and β-amyrin, C-1, which contained only β-amyrin, showed no significant effect against the sporozoites. Additionally, the commercially available pure α-amyrin and β-amyrin did not significantly reduce the sporozoite viability. Compared to the activity of B-2, B-1, which contained uvaol, reduced sporozoite viability to a lesser degree. Therefore, β-amyrin may have a synergistic effect with α-amyrin or uvaol, although clear evidence has yet to be obtained. Similarly, although D-2, which contained β-sitosterol and methoxy flavonoids, showed a highly anti-parasitic effect, the commercially available pure β-sitosterol did not. These findings also indicated a possible synergistic effect between other minor sub-components.

α-Amyrin and β-amyrin are found in some plants, including edible ones, such as *Olea europaea* and *Solanum lycopersicum* (Viet et al., 2021). It has been reported that these metabolites possess anti-protozoan activities. For example, α-amyrin and β-amyrin extracted from *Eugenia pyriformis*, which is a popular food plant in Brazil, affected the viability of extracellular forms of parasites, including *Leishmania amazonensis* promastigotes and *Trypanosoma cruzi* trypomastigotes and epimastigotes, *in vitro* (de Souza et al., 2020). However, only α-amyrin weakly inactivated the *T. cruzi* trypomastigotes and *T. brucei* bloodstream forms (Hoet et al., 2007; Pardo-Rodriguez et al., 2023), and β-amyrin showed slight inhibitory effects against the *L. donovani* promastigotes and *T. brucei* bloodstream forms *in vitro* (Hoet et al., 2007; Nor Azman et al., 2018). Thus, it was speculated that the activity of α-amyrin or β-amyrin alone against parasites might not be so higher than that of the two compounds together, although there is no explanation for that yet.

Here, two natural components containing uvaol showed anticoccidial effects, *i.e.,* B-1, which contained uvaol, and B-2, which contained uvaol and β-amyrin. Uvaol has been detected in some house and edible plants, such as *Plumeria rubra* and *O. europaea,* and in traditional

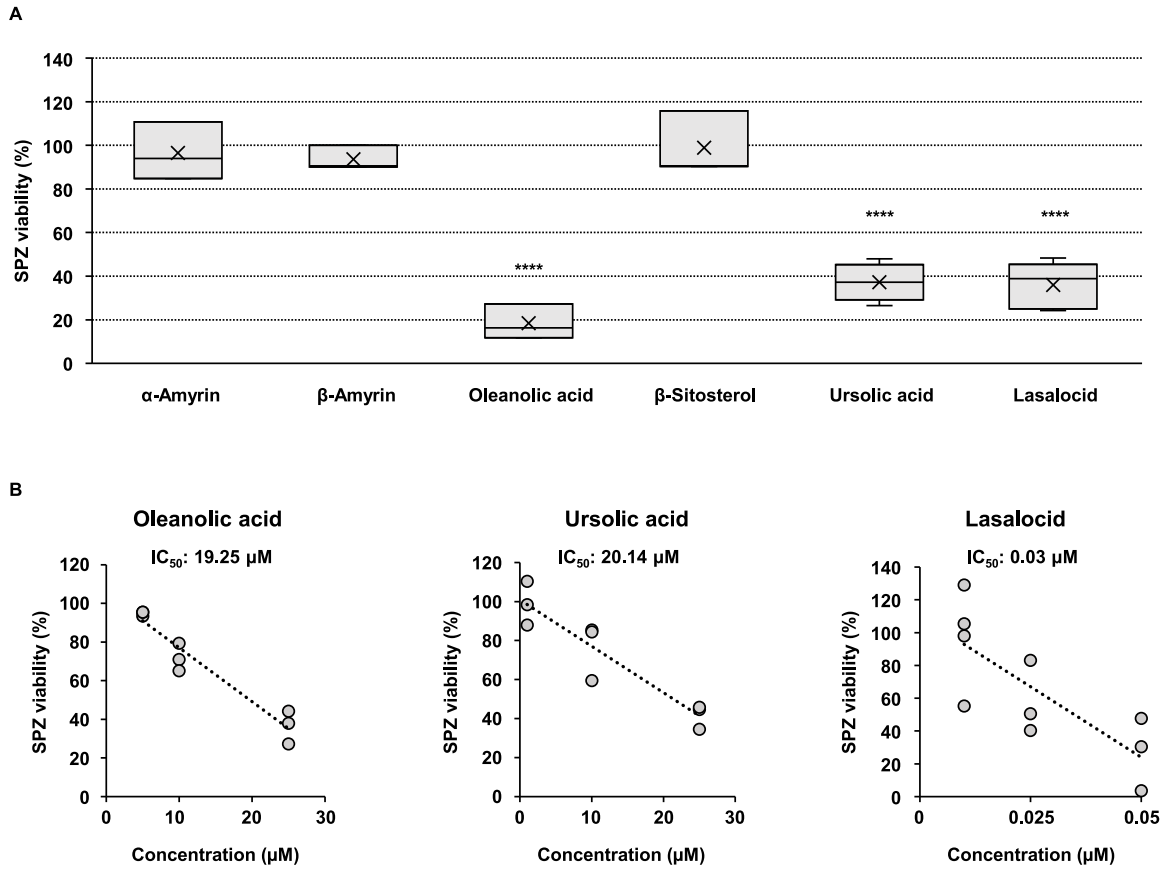

Fig. 3. Confirmation of the active compounds using commercially available compounds and their efficacy. (A) The viability of sporozoites treated with each commercially available compound or lasalocid (positive control) was compared to the viability of the DMSO-treated group, which was set as 100%. The final concentration was 100 μM for the synthetic compounds, and 1 μM for lasalocid. SPZ: sporozoite, Las: lasalocid. The student's t-test was used for the comparisons (****$p < 0.0001$) without outliers, as tested using Thompson's test ($p < 0.05$). (B) The viability of sporozoites was determined at each concentration of the synthetic compounds that showed effectiveness at 100 μM. The half maximal inhibitory concentration (IC_{50}) value was determined by approximating the curves obtained from the results.

medicinal plants, such as *Strychnos spinosa*, which is used for African trypanosomiasis (Hoet et al., 2007; Ghorai and Bag, 2021). In some reports, uvaol showed anti-parasitical effects against extracellular forms of *Trypanosoma* and *Leishmania* parasites *in vitro* (Hoet et al., 2007; Filho et al., 2009; Lafi et al., 2023). Although the mechanisms for these anti-parasitical effects have not yet been demonstrated, several biological functions of uvaol have been reported. For example, uvaol showed anti-cancer activities through apoptosis or cell cycle arrest (Martín et al., 2009; Allouche et al., 2011; Bonel-Pérez et al., 2020). The induction of apoptosis was thought to be mediated by the regulation of the expression of proteins involved in mitochondrial apoptosis, such as B-cell leukemia/lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) (Bonel-Pérez et al., 2020). Additionally, cell cycle arrest can be caused by uvaol through the downregulation of the phosphatidylinositol 3-kinase/protein kinase B signaling pathway in hepatocarcinoma and breast cancer cells (Bonel-Pérez et al., 2020). Thus, uvaol can regulate a variety of enzymes and proteins necessary for the survival of mammalian cells. However, the precise mechanisms for its antiparasitic activities, including anti-*E. tenella* activities, remain to be elucidated, and further studies are needed.

The natural component C-5 contained oleanolic acid as the main secondary metabolite. Oleanolic acid is found in edible plants, such as *O. europaea* and *Argania spinosa*, as well as in medicinal plants, such as *Arrabidaea triplinervia* and *S. spinosa* (Leite et al., 2006; Hoet et al., 2007; Castellano et al., 2022). Although the mechanisms underlying the anti-parasitic effects have not yet been fully clarified, oleanolic acid derived from plants has been demonstrated to reduce the survival of *T. cruzi* trypomastigotes, *T. brucei* bloodstream trypomastigotes, and *L. tropica* promastigotes (Leite et al., 2006; Hoet et al., 2007; Lafi et al., 2023). In addition to its antiparasitic effects, oleanolic acid has also been reported to have activities against multiple types of cancer cells (Tang et al., 2022; Nistor et al., 2023). For example, oleanolic acid induced apoptosis in the cancer cells such as lung adenocarcinoma cells and breast cancer cells through a reduction in the expression of the anti-apoptotic protein Bcl-2, and an increase in the expression of the pro-apoptotic protein Bax (Tang et al., 2022). Furthermore, in colon cancer cells, oleanolic acid inhibited aldo-keto reductase family 1, member B10, which is an oxidoreductase (Tang et al., 2022). This could induce the inhibition of downstream pathways, including mitogen-activated protein kinase kinase and extracellular regulated protein kinase, which ultimately leads to apoptosis (Tang et al., 2022). It is possible that oleanolic acid also activates the apoptotic process in *Eimeria* parasites via the inhibition or activation of analogous proteins.

The natural component D-3, which contained ursolic acid, and the commercially available pure ursolic acid appeared to inhibit sporozoites in our assays. Ursolic acid is found in edible plants and herbal plants, including *Malus domestica, Origanum vulgare,* and *Rosmarinus officinalis* (Namdeo et al., 2023). It has anti-parasitical effects against *Leishmania*, *Trypanosoma*, and *Acanthamoeba* parasites (Leite et al., 2006; Hoet et al., 2007; Filho et al., 2009; Yamamoto et al., 2015; Sifaoui et al., 2017). Yamamoto et al. demonstrated that ursolic acid extracted from *Petiveria alliaceae* reduced the survival of the promastigotes of *L. amazonensis* through changes in the plasma membrane structure via the translocation of phosphatidylserine, which is generally localized in the inner side of

Table 2
The cell viability and maximum non-toxic concentrations of the natural components derived from alpine plants.

Component No.	Average cell viability (%)					Maximum non-toxic concentration (μM)
	Concentration of secondary metabolites (μM)					
	0.1	1	10	50	100	
A-1	84.1	86.3	94.1	107.9	34.9**	50
A-2	98.7	105.9	107.0	103.4	107.3	100
A-3	103.1	91.0	92.3	109.0	107.9	100
A-4	98.0	110.3	104.3	109.0	104.3	100
A-5	103.3	102.2	102.7	109.7	105.7	100
B-1	95.6	99.9	110.1	55.9**	36.2**	10
B-2	96.8	87.3	88.2	98.2	93.3	100
C-1	78.1	82.6	88.6	87.7	82.4	100
C-2	97.8	92.5	98.7	85.2*	80.8*	10
C-3	91.0	95.1	95.1	106.4	107.7	100
C-4	108.9	92.3	56.9**	41.1**	19.9**	1
C-5	99.9	99.2	101.8	105.0	16.2**	50
C-6	89.1	90.7	91.4	92.9	93.6	100
C-7	87.9	90.9	98.2	95.8	107.4	100
D-1	92.2	97.3	99.3	109.1	106.6	100
D-2	99.6	97.2	71.6	56.0**	39.9**	10
D-3	92.9	86.4	105.3	−13.1**	−14.2**	10
E−1	83.1	86.4	87.1	108.3	102.5	100
E−2	101.1	102.5	109.8	104.9	114.0	100
E−3	93.2	100.5	94.0	98.9	104.1	100
F-1	80.8	81.4	93.9	92.6	85.2	100
F-2	99.2	102.1	104.6	102.0	104.9	100
G-1	94.6	96.9	88.5	103.5	89.5	100
H-1	106.3	109.2	105.9	112.6	111.8	100
H-2	92.0	95.3	94.8	93.4	93.3	100
H-3	96.9	92.9	95.5	98.3	92.0	100
I-1	100.7	103.3	103.7	97.0	89.4	100
Las	107.1	109.9	54.3**	1.1**	0.4**	1

The concentrations used in the assay are shown in bold. Welch's *t*-test was used to determine the differences in the cell viability (*$p < 0.05$, **$p < 0.01$).

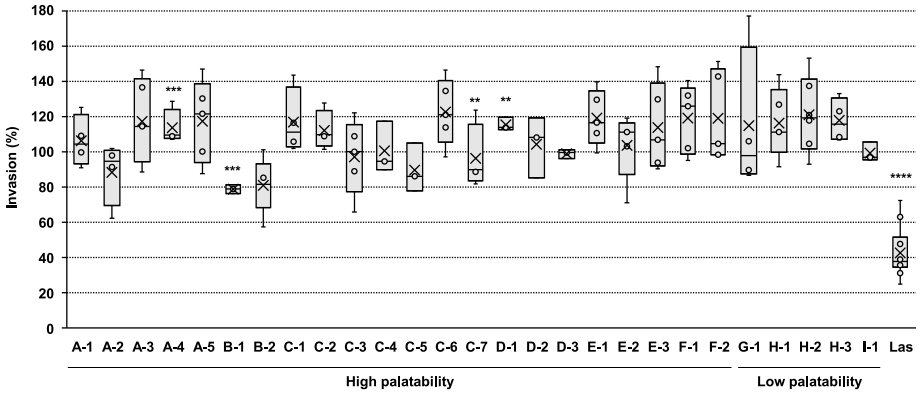

Fig. 4. The inhibitory effects of the natural components derived from alpine plants on sporozoite cell invasion. The invasion rate of sporozoites treated with each natural component derived from alpine plants or lasalocid (positive control) with the viability of the DMSO-treated group set as 100%. Each compound was used at its maximum non-toxic concentration. Las: lasalocid. The student's t-test was utilized to compare the data with the DMSO-treated group as a control (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Outliers were identified and removed using Thompson's test ($p < 0.05$).

the lipid bilayer of the plasma membrane, to the cell surface, and the disruption of mitochondria by reducing their membrane potential (Yamamoto et al., 2015). Sifaoui et al. reported that ursolic acid induced cell death in *Acanthamoeba castellanii* trophozoites, which was accompanied by plasma membrane damage and changes to the mitochondrial membrane potential (Sifaoui et al., 2017). In the present study, we could not clarify the mechanisms by which ursolic acid inactivated *Eimeria* parasites, but based on these findings, it is conceivable that ursolic acid might affect the cell membrane of the parasites, resulting in reduced viability.

The natural component D-2, which contained β-sitosterol, is found in herbal plants and oil-rich plants, including *Urtica dioica*, *Serenoa repens*, and *Arachis hypogaea* (Babu and Jayaraman, 2020). It showed anti-parasitical effects against *Leishmania* promastigotes and *Trypanosoma brucei* bloodstream trypomastigotes (Nweze et al., 2011; Pramanik et al., 2020). In a previous report, β-sitosterol reduced the survival of *L. donovani* promastigotes due to the competitive inhibition of trypanothione reductase, which is one of the redox-maintaining enzymes, through binding around the active site (Pramanik et al., 2020). When trypanothione reductase was inhibited and reactive oxygen species were produced, an intracellular redox imbalance, such as a decrease in the mitochondrial membrane potential, could be induced, resulting in lethal changes to the promastigotes (Pramanik et al., 2020). Although trypanothione reductase is present in only kinetoplastid parasites, *e.g.*, *Leishmania* and *Trypanosoma* parasites, glutathione reductase in *Eimeria* parasites is known to be similar to trypanothione reductase and to maintain redox homeostasis (Krauth-Siegel and Inhoff, 2003; Reid et al., 2014; Pramanik et al., 2020). Glutathione reductase has been reported

to have binding affinity to and inhibitory activity on β-sitosterol *in silico* (Jannat et al., 2022). Therefore, it was inferred that β-sitosterol may exert anticoccidial effects by disrupting the redox balance through the inhibition of glutathione reductase.

In general, α-amyrin, β-amyrin, oleanolic acid, and ursolic acid have similar structures, and α-amyrin and β-amyrin as well as oleanolic acid and ursolic acid are known to be isomers (Voronov et al., 2023). When comparing the structural formulas, both α-amyrin and β-amyrin have a methyl group at the same position, and both ursolic acid and oleanolic acid have a carboxyl group at the C-28 position. Previously, a carboxyl group at the C-28 position was reported to be important for anti-leishmanial and anti-trypanosomal effects (Filho et al., 2009; Isah et al., 2016), which is in agreement with the results of the present study. Among the effective compounds, α-amyrin, β-amyrin, uvaol, oleanolic acid, and ursolic acid are classified as pentacyclic triterpenoids, which have a 30-carbon skeleton with five interconnected rings (Voronov et al., 2023). Isah et al. have categorized the potency of the anti-parasitical effects of pentacyclic triterpenes into three steps according to the IC_{50} values, *i.e.,* high, moderate, and low/no potency (Isah et al., 2016). Based on these criteria, component C-5 has high potency against *Eimeria* parasites while components A-1, B-2, and D-3 have low/no potency. Although it is necessary to evaluate by the *in vivo* experiments, it may be possible that the ingestion of alpine plants that contain these compounds could partially reduce the number of sporozoites in the intestinal tract and lighten the severity of *Eimeria* infections rather than completely eradicate the parasites.

Finally, we evaluated the 27 natural components for inhibitory effects on sporozoite cell invasion. Two natural components, uvaol and phlorhizin, showed inhibitory effects on sporozoite cell invasion, although the effects were weak. Phlorhizin was first detected in apple bark (*Malus domestica*) (Tian et al., 2021), and is known to be an inhibitor of the Na-glucose co-transport system (Kutner et al., 1987; Silfen et al., 1988). The compound has been reported to block the *Plasmodium*-induced permeation pathway on infected erythrocyte membranes, resulting in the inhibition of their development (Kutner et al., 1987; Silfen et al., 1988). Uvaol and phlorhizin might affect the pathways involved in sporozoite invasion of *Eimeria* parasites into host cells.

5. Conclusions

In the present study, multiple secondary metabolites derived from alpine plants exhibited anticoccidial activity. Rather than inhibiting sporozoite invasion into cells, the compounds had a direct effect on sporozoite viability. Although further studies are needed to evaluate the effects of these compounds against *Eimeria* infections *in vivo*, our results suggest that these alpine plants may contribute to reducing the degree of infection with *Eimeria* species by decreasing the number of sporozoites in the intestinal tract.

Declarations of interests

None.

Ethics statement

We performed experiments, including the animal studies, in accordance with the guidelines and policies for animal studies of Osaka Metropolitan University, and these experiments were approved by the Animal Care and Use Committee of Osaka Metropolitan University (Approval numbers: 15003, 21022, and 22073) or Okayama University (Approval number: OKU-2021611).

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CRediT authorship contribution statement

Asako Haraguchi: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Jyunki Nagasawa:** Methodology, Investigation, Formal analysis, Data curation. **Kouji Kuramochi:** Writing – original draft, Resources, Investigation, Formal analysis. **Sayaka Tsuchida:** Writing – review & editing, Methodology, Funding acquisition, Data curation. **Atsushi Kobayashi:** Writing – original draft, Resources, Methodology, Funding acquisition, Formal analysis, Data curation. **Toshimitsu Hatabu:** Writing – review & editing, Resources, Methodology, Formal analysis. **Kazumi Sasai:** Writing – review & editing, Funding acquisition, Formal analysis. **Hiromi Ikadai:** Writing – review & editing, Resources, Formal analysis, Data curation. **Kazunari Ushida:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition. **Makoto Matsubayashi:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

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