bands were collected and eluted from the silica gel to give bruceine A (**3**) (5.0 mg, Rf =0.36). Fr. I-2 was purified in the same manner to afford bruceantinol (**4**) (6.0 mg, Rf =0.38). Fr. II was further purified by HPLC (Capcell Pak C_{18}, 5 μm, 15 mm × 250 mm, Shiseido) with CH_3OH-H_2O (6.0:4.0 *v*/*v*) using a flow rate of 3 mL/min and detection with a UV spectrophotometer at 254 nm. The following quassinoids were obtained: dehydrobruceine B (**5**) (25.0 mg, t_R: 11.21 min), dehydrobrusatol (**6**) (28.0 mg, t_R: 14.40 min), and dehydrobruceine A (**7**) (10.3 mg, t_R: 18.32 min). Bruceine D (**8**, 94.5 mg) was recrystallized from Fr. III using $CHCl_3$. The supernatant was further purified by HPLC (Capcell Pak C_{18}, 5 μm, 15 mm × 250 mm, Shiseido) with the solvent CH_3OH-H_2O (6.0:4.0 *v*/*v*), using a flow rate of 3 mL/min and detection by UV spectrophotometry at 254 nm to give bruceoside A (**9**) (13.4 mg, t_R: 12.82 min) and yadanzioside G (**10**) (5.3 mg, t_R: 16.08 min).

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In vitro antitrypanosomal activities of quassinoid compounds from the fruits of a medicinal plant, *Brucea javanica*

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Abstract

The medicinal plant *Brucea javanica* (L.) Merr. (Simaroubaceae) is widely distributed throughout Asia where its bitter fruits have been used in traditional medicine for various ailments. Fifteen C-20 quassinoids were isolated from the fruits of *B. javanica* and examined for their *in vitro* antitrypanosomal activities against trypomastigotes of *Trypanosoma evansi*. Bruceine A, bruceantinol, bruceine C, brusatol, and bruceine B showed strong antitrypanosomal activities with IC_{50} values in the range of 2.9–17.8 nM, which compared well with the standard trypanocidal drugs diminazene aceturate ($IC_{50} = 8.8$ nM) and suramin ($IC_{50} = 43.2$ nM). However, dehydrobruceine A, dehydrobruceine B, and dehydrobrusatol were about 2100, 900, and 1200 times less active, respectively, than bruceine A, bruceine B, and brusatol. The relationship of the structure and antitrypanosomal activity of these quassinoid compounds suggested that the presence of a diosphenol moiety in ring A and the nature of the C-15 side chain are important for their activities against *T. evansi*. This is the first report on the antitrypanosomal activity of isolated quassinoids.
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Keywords: *Brucea javanica*; Quassinoids; *Trypanosoma evansi*; Antitrypanosomal; Medicinal plant

1. Introduction

Trypanosoma evansi is a flagellated, animal-pathogenic protozoan parasite. It infects a variety of large animals including equines, camels, cattle, buffaloes, goats, sheep, and pigs causing the trypanosomiasis condition known as surra. The disease has a wide geographical distribution because it is mechanically transmitted by blood-sucking insects of the genera *Tabanus*, *Stomoxys*, *Atylotus*, and *Lyperosia*. The disease causes great economical losses in areas of Africa, Asia, and South America, where thousands of animals die from *T. evansi* infections (Luckins, 1988; Brun et al., 1998; Giardina et al., 2003). Control of trypanosomiasis in livestock usually relies upon either curative or prophylactic treatment of the animals with trypanocidal drugs. Currently, the most commonly used drugs for the treatment of *T. evansi* infection are diminazene aceturate (berenil), suramin, and quinapyramine. However, it is well known that existing trypanocidal drugs have been associated with side effects, and the development of drug resistant trypanosomes has occurred in many regions (Kibona et al.,

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2006; Matovu et al., 2001; Anene et al., 2001). Therefore, research on new compounds for the treatment of surra, as well as sleeping sickness in man and nagana in cattle, is an urgent and important task (Lun et al., 1993).

In many parts of the world, extensive use is made of plants in traditional medicine. Antiparasitic plant-derived compounds have been used as leads to develop semi-synthetic or synthetic drugs with better efficacy and safety (Tagboto and Townson, 2001). *Brucea javanica* (L.) Merr., a plant species of the family Simaroubaceae, is distributed widely throughout Asia where the fruits are used in traditional medicine for various ailments including cancer, amoebic dysentery, and malaria in Indonesia (Subeki et al., 2007), Myanmar, Thailand, or China (Lin et al., 1990). Quassinoids are the bitter principles found in various species of the Simaroubaceae in the tropics (Yoshimura et al., 1984; Sakaki et al., 1984). Quassinoid compounds from *B. javanica* exhibited inhibitory activities on protozoan parasites such as *Plasmodium falciparum* (O'Neill et al., 1986, 1987), *Entamoeba histolytica*, *Giardia intestinalis*, and *Toxoplasma gondii* (Wright et al., 1993). Recently, we reported that quassinoids also had *in vitro* inhibitory activity against *Babesia gibsoni* (Subeki et al., 2007; Elkhateeb et al., 2008). This study comprises the first report on antitrypanosomal activity of quassinoid compounds *in vitro* against *Trypanosoma evansi* and the structure–activity relationship is discussed.

2. Materials and methods

2.1. Plant material

Fruits of *B. javanica* were purchased from the Bandar Jaya traditional market, Indonesia, in April 2005. The plant species was identified by Dr. Aris Winarso at the Herbal Medicinal Research and Education Centre, Lampung, Indonesia (Subeki et al., 2007). Other fruit samples of the same plant species were purchased from a local shop in Huaihua City, West Hunan province, China, in June 2007, and identified by Dr. Zhu Ming at Huaihua Red Cross Hospital, Huaihua City (Elkhateeb et al., 2008).

2.2. Extraction and isolation

In the present study we used 15 quassinoid compounds of the C-20 type (Fig. 1), which were isolated from *B. javanica* fruits and purified in our laboratories. From the ethyl acetate (EtOAc)-soluble fraction of the Indonesian plant material, bruceine A, bruceantinol, bruceine B, bruceine C, bruceine D, yadanziolide A, bruceantinol B, and bruceine J were isolated (Subeki et al., 2007). In addition to these compounds, brusatol, bruceantin, dehydrobruceine A, dehydrobruceine B, dehydrobrusatol, bruceoside A, and yadanzioside G were isolated from the Chinese plant material (Elkhateeb et al., 2008). In this study, we also purified bruceine D from the water-soluble fraction of the Indonesian *B. javanica* materials as follows. The dried powder of *B. javanica* fruits was extracted with methanol–H_2O and partitioned using EtOAc into water-soluble and EtOAc-soluble fractions. The water-soluble fraction was further extracted with ethanol and chromatographed on DIAION HP-20 and Sephadex LH-20 columns. The sample was finally purified over a silica gel column.

2.3. Identification

Optical rotations were measured with a Jasco DIP-370 digital polarimeter. Mass spectra were recorded on JEOL JMS-SX102A and JMS-AX500 spectrometers. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-EX 270 FT-NMR spectrometer and on a Bruker AMX-500 FT-NMR spectrometer. Column chromatography was conducted with silica gel 60 (Kanto Chemical, Japan).

2.4. Parasite and culture medium

Trypanosoma evansi (H3 strain, isolated from deer in Thailand) were kindly supplied by Dr. Onuma, Graduate School of Veterinary Medicine, Hokkaido University, Japan. Trypomastigotes of the parasite were maintained in HMI-9 medium (Hirumi and Hirumi, 1994) supplemented with 20% heat-inactivated horse serum (Sigma), 0.01 mg/ml bovine holo-transferrin (Sigma), 0.01 mM bathocuproine disulfonic acid (Sigma), 1.5 mM L-cysteine (Kanto), 0.16 mM thymidine (Wako Chemicals, Japan), 2 mM 2-mercaptoethanol (Sigma), 1 mM pyruvate (Kanto), 2 mM L-glutamine (Wako), 60 mM HEPES (Sigma), 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in a 5% CO_2–air mixture. Subculturing was performed every 3 days by approximately 50-fold dilution of the cultures.

2.5. *In vitro* test for antitrypanosomal activity

In vitro antitrypanosomal tests were performed in a 96-well microtiter plate using the 15 quassinoids

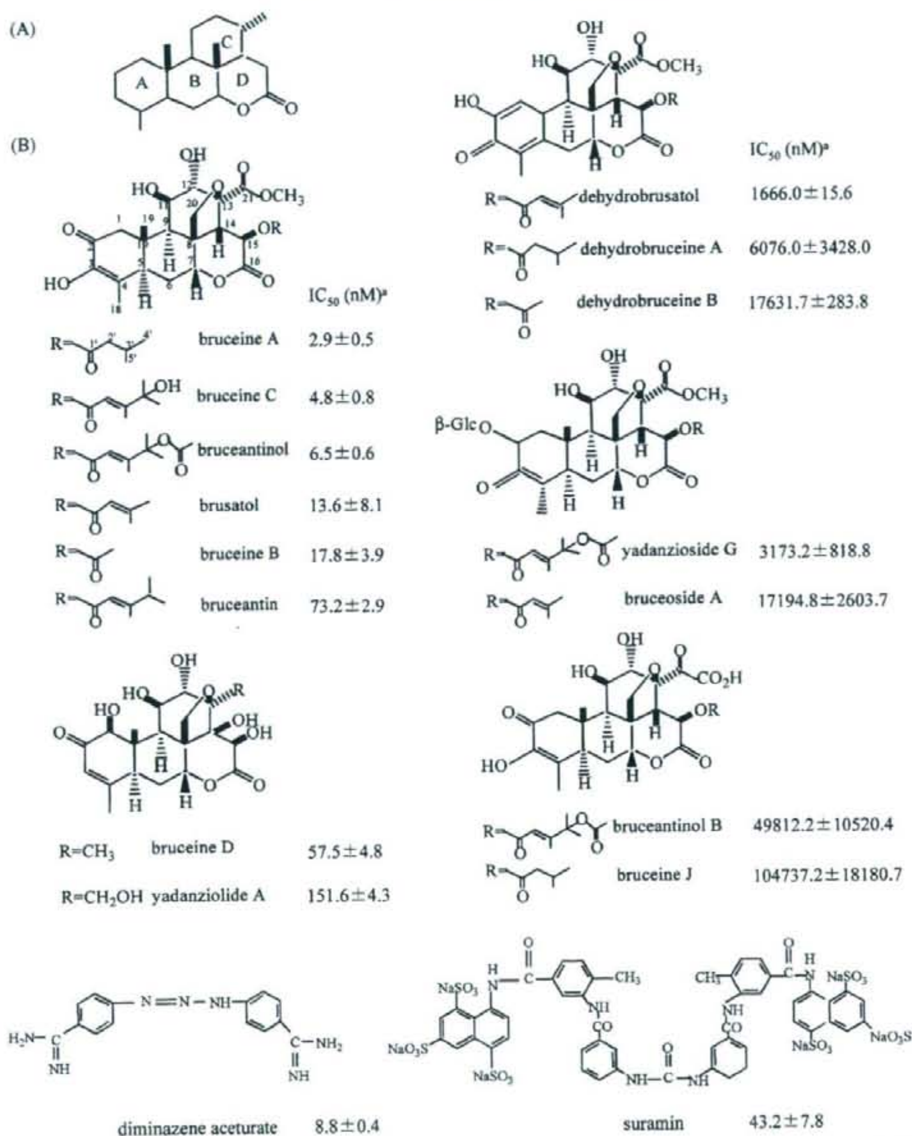

Fig. 1. Chemical structures and *in vitro* antitrypanosomal activities of quassinoids isolated from *B. javanica* fruits. (A) Skeleton of C-20 quassinoid, indicating rings A to D. (B) Chemical structures and IC_{50} values of quassinoids, diminazene aceturate, and suramin against *T. evansi*. [a]The IC_{50} value is the mean ± standard deviation of two to three independent experiments.

described above and two standard trypanocidal drugs, diminazene aceturate (Sigma) and suramin (Sigma). Bruceine D and diminazene aceturate were dissolved in distilled water and other quassinoids and suramin were dissolved in dimethyl sulfoxide (DMSO). Two-fold serial dilutions of these compounds were prepared in HMI-9 medium in the presence or absence of 0.5% DMSO. Trypomastigotes of *T. evansi* were incubated in each well at a concentration of 5×10^4 cell/ml in 200 μl of medium in the presence of two-fold serial dilutions of each compound. The plates were incubated at 37 °C in 5% CO_2 in air for 72 h and the number of

motile parasites was counted using a Neubauer hemocytometer. To determine the 50% inhibitory concentration (IC_{50}) on parasite growth for each compound, triplicate assays of the compounds at each concentration were prepared. The IC_{50} value was calculated by computerized probit analysis. All tests were performed independently two to three times.

3. Results

3.1. Isolation of quassinoids

From 4.5 kg dried powder of *B. javanica* fruits, we isolated and purified 28 mg bruceine D in the water-soluble fraction. The structure was determined by comparing the ^1H and ^{13}C NMR, mass spectral data, and optical rotation values with that of reported data (Lee et al., 1979; Yoshimura et al., 1985).

3.2. Antitrypanosomal activities of quassinoids against *T. evansi*

Among the 15 C-20 type quassinoids compounds tested, bruceine A, bruceantinol, and bruceine C showed higher antitrypanosomal activities, with IC_{50} values in the range of 2.9–6.5 nM, than the standard trypanocidal drug diminazene aceturate with an IC_{50} value of 8.8 nM (Fig. 1). Brusatol, bruceine B, and bruceantin also showed sufficient antitrypanosomal activity with IC_{50} values in the range of 13.6–73.2 nM, as compared to the other standard drug suramin with an IC_{50} value of 43.2 nM. However, quassinoids such as dehydrobruceine A, dehydrobruceine B, and dehydrobrusatol were approximately 2100, 990 and, 1200 times less active, respectively, than the activities observed for bruceine A, bruceine B, and brusatol. Glycosylation at *O*-C-2 in ring A markedly reduced the antitrypanosomal activity. Glycosides such as bruceoside A and yadanzioside G were approximately 5900 and 1100 times less potent, respectively, than bruceine A. Bruceine D, the only water-soluble quassinoid isolated in this study, showed promising trypanocidal activity with an IC_{50} value of 57.5 nM. Yadanziolide A was three times less active than bruceine D (Fig. 1). Bruceantinol B was 7700 times less active than bruceantinol.

4. Discussion

As far as can be determined, this study is the first report on the antitrypanosomal activity of isolated quassinoids. The relationship between the structure and activity of these quassinoids suggested that the presence of a diosphenol moiety in ring A and the nature of the C-15 side chain are important for the antitrypanosomal activities of these C-20 quassinoids. Phillipson and O'Neill (1986) classified a series of 26 quassinoids into eight different structure types. According to their structural features, quassinoids such as bruceine A, bruceantinol, bruceine C, brusatol, bruceine B, and bruceantin have a common diosphenol moiety in ring A. All of these quassinoids except bruceantin showed strong antitrypanosomal activity with IC_{50} values in the range of 2.9–17.8 nM. The IC_{50} of bruceantin was 73.2 nM, although this was only slightly larger than that of the standard drug suramin, which had an IC_{50} value of 43.2 nM. The differences in the antitrypanosomal activities may be due to differences in the C-15 side chains (Fig. 1). However, dehydrobruceine A, dehydrobruceine B, and dehydrobrusatol, which have an α-hydroxydienone moiety in ring A, did not exhibit significant trypanocidal activities with IC_{50} values about 1000–2000 times higher than the related compounds of bruceine A, bruceine B, and brusatol.

A comparison of the *in vitro* antiprotozoal activities of quassinoid compounds against different protozoan species is shown in Table 1. Quassinoids with a diosphenol moiety in ring A appeared to show greater selectivity against *T. evansi*. Although bruceantin was the most active quassinoid against *E. histolytica*, *T. gondii*, and *P. falciparum*, bruceine A and brusatol were the most active against *T. evansi* and *B. gibsoni*, respectively. As quassinoids are potent inhibitors of protein synthesis in *P. falciparum*, most likely due to effects upon the ribosome rather than upon nucleic acid metabolism (Kirby et al., 1989), this selectivity may be due to differences in protein synthesis systems between different parasite species (Edlind, 1989). Although at present, target molecules of quassinoids on typanosomes are unknown, synthesis of many proteins may be inhibited with different degrees.

Plants, microorganisms and marine organisms are potential sources of new drugs since they contain a countless quantity of natural products with a great variety of structures and pharmacological activities (Newman et al., 2003). The diversity of natural products with antiprotozoal activities has been reported and there are publications reporting the activity of purified natural products against trypanosomes responsible for sleeping sickness in humans and nagana in domesticated animals. Hoet et al. (2004) grouped these compounds according to their structures in 5 categories: alkaloids, phenolic

Table 1
In vitro antiprotozoal activity of quassinoids isolated from *B. javanica* fruit.

Quassinoids	IC_{50} (nM)				
	Entamoeba histolytica[a]	*Toxoplasma gondii*[b]	*Plasmodium falciparum*[c]	*Babesia gibsoni*[e]	*Trypanosoma evansi*
Bruceine A	222.0	Not tested	21.0	7.7	2.9
Bruceantinol	Not tested	Not tested	3.3[d]	19.8	6.5
Bruceine B	638.0	75.0	23.0	1860.4	17.8
Bruceine C	495.0	842.0	9.0	189.7	4.8
Bruceine D	941.0	7560.0	37.0	2031.6	57.5
Bruceantin	35.0	11.5	1.5	24.4[f]	73.2
Brusatol	62.0	179.0	6.0	1.4[f]	13.6
Yadanziolide A	Not tested	Not tested	Not tested	505.8	151.6
Dehydrobruceine A	Not tested	Not tested	88.5	59.6[f]	6076.0
Dehydrobruceine B	Not tested	Not tested	Not tested	644.7[f]	17631.7
Dehydrobrusatol	Not tested	Not tested	Not tested	20.3[f]	1666.0

[a] Data from Wright et al. (1988).
[b] Data from Wright et al. (1993).
[c] Data from O'Neill et al. (1987).
[d] Data from O'Neill et al. (1986).
[e] Data from Subeki et al. (2007).
[f] Data from Elkhateeb et al. (2008).

derivatives, quinones, terpenes and other metabolites, and compared their activities based on each IC_{50} value and selectivity index (SI) *in vitro*. In the review, only several compounds had an IC_{50} value in the nanomolar range and relative selectivity ($SI \geq 20$). Among the active compounds, sinefungin, a natural nucleoside produced by *Streptomyces* species, had the lowest IC_{50} value of 0.4 nM against *T. brucei rhodesiense* bloodstream trypanosomes with a very high SI ($SI > 10^6$), but it was found to be very nephrotoxic in goats. In the present study, quassinoids compounds, a group of degraded triterpenes, from a medicinal plant, *B. javanica*, were examined for their *in vitro* antitrypanosomal activities. Among quassinoids isolated, bruceine A had the highest activity against *T. evansi* bloodstream trypanosomes with an IC_{50} of 2.9 nM and the SI calculated from a result (IC_{50} = 187.7 nM on KB cells) by Anderson et al. (1991) was 64.7. Similarly, the SI values on KB cells calculated were 64.4, 7.8, 49.0, 0.2 and 14.4 for bruceine B, C, D, bruceantin, and brusatol, respectively.

Antitrypanosomal activities of quassinoid compounds discovered in this study may not parallel their cytotoxicity and side effects. Among quassinoid compounds, bruceantin has been studied extensively to assess its toxicity in Phase I trials. Hypotension, nausea, and vomiting were common side effects at higher doses (3.6 or 4.5 mg/m^2/day for 5 days), but hematological toxicity was moderate to insignificant and manifested mainly as thrombocytopenia and fever in patients (Bedikian et al., 1979). Recent study showed that oral administration of bruceine A at a dose of 6.4 mg/kg/day for 5 days resulted in no clinical findings in a dog with normal ranges of hematological and biochemical values in the blood (Nakao et al., in press). Diminazene aceturate, suramin, and quinapyramine have long been used for the treatment of human African trypanosomiasis or animal trypanosomiasis, including nagana in cattle and surra in a variety of domesticated animals. These drugs also cause various side effects such as damage to the liver and kidney in animals and humans (Homeida et al., 1981; Peregrine and Mamman, 1993; Gutteridge, 1985). Thus, further work should be carried out to evaluate potent trypanocidal drugs for the treatment of different animal species. To reduce adverse side-effects and improve their activities and specificities, structural modifications should be considered to discover more specific compounds. Alternatively, different drug delivery systems, such as using liposomal formulations (Lian and Ho, 2001; Date et al., 2007), can also be explored.

In conclusion, bruceines A, B, C, and D, and other related compounds are promising, new candidates for the treatment of trypanosomiasis. However, further studies, including elucidation of their mechanisms of actions, cytotoxicities, and activities against other trypanosome species, are necessary. Evaluation of their *in vivo* effects in animal models is also required. The content of each bruceine in the plants appears to be

dependent upon climatic, seasonal and geographic factors (Pavanand et al., 1986). To isolate a large amount of quassinoids from *B. javanica* or other plant sources, the development of a simple and sensitive method for the detection of quassinoids from crude plant extracts is necessary and preliminary studies are underway.

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Original Article

Leishmaniasis in Sindh, Pakistan: outbreak and review of the literature

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Abstract ***Background*** Cutaneous leishmaniasis (CL) is endemic in Pakistan and is widely spreading day by day. Earlier, we proposed that leishmaniasis is endemic in Jacobabad, Dadu and Larkana districts of Sindh Province. It was pointed out that the disease is dramatically spreading in the country and warned that if the preventive measures were not taken at right time there would be a serious public health problem in the country. Since then, we have regular watch on the disease.

Objective Fresh clinical observations of CL patients who visited our department from 2004 to 2005.

Patients and methods This study was conducted in the Department of Dermatology, Chandka Medical College Larkana. A total of 1640 cases of CL were seen during this period.

Results Among the 1640 patients, 470 were residents of different cities of Balochistan Province; and 1170 were the residents of different cities of Sindh Province like Jacobabad, Ratodero, Shahdadkot, Qambar, Warah, Mehar, Dadu, Dokri and Larkana. All the patients were aged between 3 months and 60 years. Seven hundred eighty were males and 860 were females. Duration of the disease ranged from 1 to 12 months. Most of the patients had single lesions but multiple lesions were also seen on the exposed (mainly) and unexposed parts of the body. Clinically, the lesions were classified as dry ulcerative, wet ulcerative, dry papular, nodular, and crusted lesions. Diagnosis was made on the basis of smear test and clinical presentation of the skin lesions. All the cases were treated with the meglumine antimoniate 600 mg/day (adults) and 15 mg/kg/day (children) either intramuscularly for 20 consecutive days or intralesionally for 10 alternate days.

Conclusion Cutaneous leishmaniasis is endemic in the Sindh Province. The molecular characterization and other studies are needed for further evaluation of this disease.

Key words
Leishmaniasis, outbreak, epidemiology, Pakistan.

Introduction

Leishmaniases are a group of diseases caused by several species of the genus *Leishmania*. Each species tends to occupy a particular zoo-geographical zone. These parasites can affect several mammalian species including humans. Human leishmaniasis is caused by at least 20 different species and subspecies of the genus *Leishmania*. In general, leishmaniasis is a zoonotic disease and the parasite is transmitted to man from a reservoir mammalian host by a sandfly vector during a bite. The type of infection of leishmaniasis gives wide range of clinical changes that divide the disease into following

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subcategories: A) cutaneous leishmaniasis (CL); B) diffuse cutaneous leishmaniasis (DCL); C) mucocutaneous leishmaniasis (MCL); and D) visceral leishmaniasis (VL). CL can be classified largely into two forms: an Old World form mainly caused by *L. tropica* complex[1]; and a New World form caused by *L. (Viannia) braziliensis* and *L. mexicana* complexes.[2-5]

Leishmaniasis is endemic in 88 countries in 5 continents with a total of 350 million population at risk. The estimated annual number of new cases of VL and CL are about 5 lacks and 15 lacks, respectively.[6,7] About one hundred thousand deaths due to VL were estimated among 2.8 lacks people in the endemic area of southern Sudan between 1984 and 1994[8] and an epidemic of CL is ongoing in Afghanistan and other surrounding states with hundreds of thousands of cases over there.[9]

Leishmaniasis was reported from Pakistan in 1960 for the first time. Initially it was limited to the northern mountainous region, but now it is widely spreading all over the country. In this study, we present our recent observations and the current status and up-to-date information of leishmaniasis and its epidemiology in Pakistan, by surveying the literatures reported to date in Pakistan. The vector sandflies and factors suspected for the spread of disease in country will also be discussed.

Patients and methods

These data were collected from the outpatient clinic of the Department of Dermatology, Chandka Medical College (CMC) Hospital, Larkana, Sindh, Pakistan. A total of 1640 cases were seen in our department from September, 2004 to December, 2005. All the patients examined at our department were either referred by local doctors working in rural health centers in villages and/or visited on their own. These patients were divided into two groups: (1) those that had a positive history of travel to the Balochistan Province before the appearance of lesion(s) and/or to have come from Balochistan Province; and (2) those that belonged to the newly reported endemic areas of CL in the Sindh Province. All the cases were diagnosed on the basis of clinical findings and smear test for Leishman-Donovan bodies. All the cases were treated with the meglumine antimoniate 600 mg/day (adults) and 15 mg/kg/day (children) as intramuscular for 20 consecutive days or intralesionally for 10 alternate days.

Results

A total of 1640 cases were seen during the 15 months period, which is a quite high number in comparison to previously reported cases from these areas. Among them 470 either had a positive history of travel to the Balochistan Province or were residents of that province. They were likely to have been infected from the previously recognized endemic areas. 1170 were the residents of different cities of Sindh Province like Jacobabad, Ratodero, Shahdadkot, Qambar, Warah, Mehar, Dadu, Dokri and Larkana. Seven hundred and eighty patients were males and 860 were females. Age of the patients ranged from 3 months to 60 years. There was no significant difference between the male and female ratio. Duration of the disease ranged from 1 to 12 months. Most of the cases were seen during winter season; however, rare cases were seen in summer. All the patients had lesions mostly on the exposed parts of the body such as face, neck, ears, hands, arms, feet and legs (**Figures 1-3**); and rarely the lesions were also seen on the other unexposed parts of the body including the back and abdomen. No cases of MCL or VL were seen in this study.

Figure 1 A 16-year-young girl showing the dry ulcerative lesion on cheek.

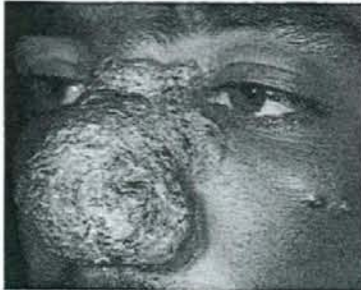

Figure 2 A 25-year-old man has wet ulcerative lesion on the nose since 2 months.

Figure 3 Papulo-nodular lesions are visible on the leg of male patient.

The two types of sandflies from the villages of parasitologically positive cases were identified as *Phlebotomus sergenti* and *P. papatasi*.

Discussion

Epidemiology of leishmaniasis in Pakistan

Pakistan is a tropical country situated in the north-west of South Asia, sharing borders with China, Afghanistan, Iran and India. Incidentally, all these neighboring states are also endemic for the leishmaniasis.[9,10,11]

As regards epidemiology of the disease, Ahmed *et al.*[12] were the first who reported 30 cases of VL in the Baltistan, northern areas from 1957 to 1960. In another study, Ahmad and Burney[13] observed the increasing incidence of leishmaniasis in northern areas of Pakistan. Baltistan is a part of the northern areas of Pakistan, lying between latitude 35° to 35.5°N and longitude 75.5° to 76.5°E, and comprises of many valleys having altitude of 6000 to 10000 feet above sea level with majority of the population is rural Later, Ahmad and Burney[13] presented their studies in detail and all their reported 30 cases of kala azar (VL) were admitted to the combined military hospital, Skardu, during the period of April 1957 to march 1960. Among 30 patients 13 were children below 10 years, 10 were 10-15 years, and 7 were 15-35 years old. They also conducted a survey from June 26 to July 3, 1960 in nine villages of the Baltistan and discovered 60 cases of VL.[14]

In 1974, Burney *et al.*[15] discovered new foci in Kharmang Valley and 25 cases of VL from the villages were recorded. In 1975, they recorded only two cases from Parkuta village in Kharmang valley. In 1979, they reported high seropositivity of antileishmania antibodies using the complement fixation test (CFT) and immunofluorescence assay (IFA), in the children age groups 6-10 and 11-15. They also concluded that there was no animal reservoir of infection, and that disease transmission was from man to man via sandflies.

In the eighth decade, the cases of VL were reported from the district of Chilas in the northern areas. Later, cases were also

reported from the sub-Himalayan region of Azad Jammu and Kashmir (AJK), and the neighbouring areas of North-West Frontier Province (NWFP) and Punjab Province.[16] Rab and Evans[17] reported the existence of *L. infantum* in the Himalayas region. They reviewed the record of 10 years and revealed the 239 cases of VL from the Rawalpindi, Islamabad, Gilgit (northern areas) and Muzaffarabad (AJK) hospitals. They observed that 52% were under the age of 2 years, while 86% of all cases were below 5 years old. They also randomly skin tested with leishmanin in 1938 individuals, tested for anti-leishmania antibodies in 580 samples by enzyme-linked immunosorbent assay (ELISA) and direct agglutination test (DAT), and another 1403 as blood samples on filter paper, were tested by DAT alone. Parasites were isolated from 15 patients, 11 from bone marrow, 3 from normal skin and one from spleen. The parasites were typed as *L. infantum* zymodeme LON-49 (=MON-1).

In order to know the role of dogs in the epidemiology of human visceral leishmaniasis, a serological study was conducted in the domestic dogs in the rural communities in the districts of Chilas, Abbotabad, Bagh, Poonch and Muzaffarabad (AJK).[18] A total number of 244 dogs were examined for the evidence of anti-leishmania antibodies. Deoxyribonucleic acid (DNA) probing by ^{32}P-labelled Lmet 2 cDNA probe showed high sensitivity with aspirates obtained from the popliteal lymph nodes of dogs but not with skin snips. Parasites isolated from dogs in these foci were identified as *L. infantum* by isoenzyme characterization. Hence, they confirmed the role of dogs as the reservoir of visceral leishmaniasis in these endemic foci in northern areas of Pakistan.

In Pakistan, both VL and CL are endemic; and two types of CL, zoonotic CL and anthroponotic CL are reported endemic in different parts of the country.[19,20,21] In one study, the isolated parasites from the cutaneous lesions of 13 patients were typed as *L. tropica*; and it was concluded that anthroponotic cutaneous leishmaniasis is caused by *L. tropica* in Pakistan.[22] Later, the VL was reported in the 10 children between 2 to 10 years at DHQ Hospital, Timergara district Dir, NWFP.[23]

Slowly and gradually the disease has been spreading to the other parts of the country like Balochistan Province and the affected cases have had the both type of leishmaniasis i.e. the CL as well as VL. Nagi and Nasimullah[24] reported the presence of several cases of VL in Balochistan Province. Further more, Yasinzai *et al.*[25] studied on various aspects of CL and VL in the Balochistan Province. It was observed that the disease was affecting equally the adults and children. However in another study, the VL was more frequently seen in the children.[26] The mean age of these children was 2.9 years. They concluded that Pakistani children with VL tended to be younger than the affected children from Africa and were less likely to have lymphadenopathy. Hassan *et al.*[27] reported 38 cases of VL from the Rawalpindi Medical College Hospital. The majority of these patients (84.2%) came from Azad Kashmir, especially from areas around Poonch, and the others were from the areas around Muzaffarabad and 2 patients belonged to Gilgit agency, 4 cases came from villages around Murree, Rawalpindi and Abbotabad. They proposed that VL was endemic in these areas.

In the last decade of last century, the disease rapidly spread to the central and south parts of the country, the Central Punjab and Sindh provinces. CL is also reported as endemic in the central part of Punjab. Mujtaba and Khalid[28] reported 305 cases of cutaneous leishmaniasis from the Nishtar Medical

College, Multan during 1995 to 1997. They observed only dry type of lesions in their patients and suspected for the presence of *L. tropica* in the region. Later, 173 cases of CL were reported from the central part of same city.[29] Although, the dry and wet type of ulcerative lesions are the common features of the CL, unusual clinical variants like acute paronychial, chancriform, annular, palmoplantar, zosteriform and erysipeloid type lesions are also reported in the country.[30] No case of visceral leishmaniasis is reported yet from the central part of the Punjab and Sindh provinces.

In 1996, we observed the frequent number of cases in the interior part of Sindh Province. We noticed that most of the CL patients visiting our department had never visited any previously reported endemic areas of leishmaniasis before the appearance of lesion on the body. This prompted us to conduct the careful study regarding the disease. The aim was to investigate whether this disease is really becoming endemic in this interior region of Sindh Province. For this purpose we divided our patients in two groups; 1) those who had traveled or belonged to the endemic areas of leishmaniasis before the appearance of first lesion; 2) those who had neither traveled nor belonged to the endemic areas of leishmaniasis. A total of 1210 cases were seen in our department from 1996 to 2001. We observed that 450 patients had the positive history of travel to the endemic areas of CL in the country. They were likely to have been infected from the previously recognized endemic areas. 750 patients never traveled to the endemic areas before or after the appearance of the lesion, and they were residing in the central part of the Sindh Province i.e. Larkana, Dadu and Jacobabad districts.[31] On the basis of these findings we proposed that three districts of Sindh province i.e. Jacobabad, Dadu and Larkana were endemic for cutaneous leishmaniasis. Since then, hundreds of patients are visiting our department every year. The present study was conducted on the similar pattern. A total of 1640 cases were seen during the 15 months period, which is quite high as compared to previously reported cases. Among them 470 were residents of different cities of Balochistan Province; and 1170 were the residents of different cities of Sindh province like Jacobabad, Ratodero, Shahdadkot, Qambar, Warah, Mehar, Dadu, Dokri and Larkana. Every year the number of cases is increasing dramatically.

In order to see the nature of the disease in these new endemic areas, we took a survey of many CL related villages. It appears that leishmaniasis entered in the Sindh Province from the Balochistan Province through the routes of mountainous belt continuing from the borders of Balochistan and Sindh province, under specific conditions. Further studies are required in this regard. The number of patients residing in Sono Khan Chandio Village was much higher than the number of patients from other cities of Sindh Province.[32,33,34] The possible factors responsible for the outbreak and spread of disease from north to south of the country may be considered as: I) flow of canals and rivers from north to south, II) increased population, III) refugees influx, IV) heavy vehicles (trucks) used for the domestic transportation of fruits, luggage and other purposes, V) military activities, VI) stoppage of anti-insecticidal spray, once a regular practice for agricultural purposes, VII) environmental modifications like construction of water dams can change the temperature and humidity of the soil and vegetation, which may result in changes of the composition and density of sandfly species as well as changes in rodent species.

The incidence of CL in other cities of Sindh Province like Nawabshah, Khairpur,

Shikarpur and Sukkur districts is also on rise (personal communication with Dr. Aziz Memon). Similarly, Malir and Landhi areas of the coastal city of Karachi are being suspected as endemic for cutaneous leishmaniasis (personal communication, Dr. S. Sharaf Ali Shah).

Vector sandflies

Burney *et al.*[14] captured the sandflies from the houses of the VL and CL patients in Baltistan which were identified as *Phlebotomus chinensis*, *P. major*, *P. kandelakii* and *P. burneyi*. They considered *P. burneyi* as a new species of sandfly. *P. papatasii* is the commonest vector in the Gilgit area. At that time, authors could not incriminate the species of *Phlebotomus* responsible for the transmission of disease in the area. In order to know the reservoir host in the Baltistan area, a number of studies were carried out. Although the number of dogs were rare in the houses of the kala azar patients, the bone marrow smear from dogs were checked for the *Leishmania* that was negative. Squirrels, gerbils and lizards were not available in the region. Even not a single monkey or donkey was present in the area. Forty-eight sera from goats, sheeps, cows, horses and yaks collected from affected houses did not reveal complement-fixing antibodies against *Leishmania*. Rodents were captured from the kala azar houses, they were dissected and their smears were examined for L.D. bodies and cultured on Novy-MacNeal-Nicolle (NNN) medium but the results were negative. On the basis of their findings they concluded that the disease might be transmitted from man to man through any of the species of *Phlebotomus* present in the area.

Anthroponotic cutaneous leishmaniasis (ACL) caused by *L. tropica* is a major epidemic in Kabul city of our neighboring state of Afghanistan. Hewitt *et al.*[9] conducted a study among children in 2 high-rise apartment blocks in the city in Kabul and suggested that most transmission of ACL took place in homes. ACL is also common and endemic in the southern city of Kandhar, the western city of Herat and central provinces of Kabul and Parwan.[35,36,37] Rowland *et al.*[38] conducted the study in the Afghan refugee camp at Timargara, in the district Dir, North West Frontier Province of Pakistan (NWFP). They examined the lesions parasitologically and the amastigotes were detected by microscope in only 36% of lesions, and, 48% of slide negative cases showed positive cultures; however the same cases negative to both microscopy and culture were positive by PCR. They concluded that *L. tropica* was existing type of *Leishmania* and *P. sergenti* was a known vector in the area.

In order to see the behavior, characteristics and various aspects of the disease different studies like molecular characterization of *Leishmania* isolates from patients, reservoir host/s (animals) and vector sandflies, and the molecular type of *Leishmania* from tissues are needed.

Regarding the reservoir host and the existing type of sandflies in the new endemic areas of Sindh Province, studies are under way. At present, we have found two types of sandflies in the villages of parasitologically positive cases that are *P. sergenti* and *P. papatasi*.

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Authors Declaration

Authors are requested to send a letter of undertaking signed by all authors along with the submitted manuscript that:

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FULL PAPER *Parasitology*

Evaluation of Efficacy of Bruceine A, a Natural Quassinoid Compound Extracted from a Medicinal Plant, *Brucea javanica*, for Canine Babesiosis

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ABSTRACT. Bruceine A, a natural quassinoid compound extracted from the dried fruits of *Brucea javanica* (L.) Merr., was evaluated for its antibabesial activity *in vitro* and *in vivo*. Bruceine A inhibited the *in vitro* growth of *Babesia gibsoni* in canine erythrocytes at lower concentration compared with the standard antibabesial drug diminazene aceturate and killed the parasites within 24 hr at a concentration of 25 *n*M. Oral administration of bruceine A at a dosage of 6.4 mg/kg/day for 5 days resulted in no clinical findings in a dog with normal ranges of hematological and biochemical values in the blood. Three dogs were infected with *B. gibsoni* and two of them were treated with bruceine A at a dosage of 6.4 mg/kg/day for 6 days from day 5 post-infection. An untreated dog developed typical acute babesiosis symptoms including severe anemia, high fever, and complete loss of appetite and movement. However, the two bruceine A-treated dogs maintained their healthy conditions throughout the experimental period of 4 weeks although complete elimination of parasites from the peripheral blood was not achieved and decreases in the packed cell volume and the erythrocyte and platelet counts were observed. Since natural quassinoid compounds have been used as traditional medicines for the treatment of various ailments including cancer and malaria, the present results suggest that bruceine A or other related compounds are potential candidates for the treatment of canine babesiosis.

KEY WORDS: *Babesia gibsoni*, *Brucea javanica*, Bruceine A, Chemotherapy, Medicinal plant.

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Canine babesiosis is a tick-borne disease caused by the intraerythrocytic apicomplexan parasites, *Babesia gibsoni* and *B. canis*. Clinical signs of *B. gibsoni* infection are anemia, fever, thrombocytopenia, splenomegaly, lymphadenopathy, and lethargy [19]. During the acute phase of infection, infected dogs develop severe anemia and occasionally die if adequate treatment is not provided. However, most dogs that recovered from the acute phase become carriers of the parasites and may suffer from disease relapses for the rest of their life. *B. gibsoni* infection is endemic in many regions in Asia, Africa, Europe, Australia, Brazil, and North America [4, 10]. In Japan, *B. gibsoni* infection has long been problematic especially in western regions, but recently the distribution appears to be expanding to the eastern parts of Japan [21].

Diminazene aceturate and imidocarb dipropionate are the major drugs for the treatment of *B. gibsoni* infection [16], but these drugs are unable to eliminate the parasites completely from infected dogs [30]. These drugs also have some disadvantages. The toxicity of diminazene aceturate to kidney, brain, and liver can result in serious side-effects such as weakness, irritability, paralysis, lack of responsiveness to stimuli, and fatal hemorrhage in the central nervous system [20, 26]. Due to these side-effects, the practical use of diminazene aceturate is not approved by the Food and Drug Administration (FDA) in the U.S.A. [5], and this drug was recently withdrawn from the market in Japan. The limited use of imidocarb dipropionate may be due to its high cost and systemic side-effects such as acute hepatic and renal failure especially in debilitated animals [1]. Therefore, an alternative chemotherapeutic agent with better activity and fewer side-effects is needed urgently. One possible source of such affordable treatment lies in the use of medicinal plants.

In the preceding study [24], Indonesian medicinal plants were screened for antibabesial activity *in vitro* and active quassinoid compounds were extracted from the fruit of *Brucea javanica* (L.) Merr., a plant species of the family Simaroubaceae. This plant contained a number of quassinoids as the bitter principles. Among these quassinoids, bruceine A, bruceantinol, and bruceine C showed sufficient antibabesial activities. The 50% growth-inhibitory concentration (IC_{50}) values were 4 *ng*/m*l* (7.7 *n*M), 12 *ng*/m*l* (19.8 *n*M), and 107 *ng*/m*l* (189.7 *n*M), respectively, which compared well with the standard drug, diminazene aceturate, having an IC_{50} value of 103 *ng*/m*l* (172.6 *n*M).

The present study was examined on the effect of bruceine A against *B. gibsoni in vitro* in more detail and evaluated the efficacy of bruceine A for dogs at an early stage of infection with *B. gibsoni* by investigating their clinical signs, the level of parasitemia, and hematological and biochemical values in

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the blood. The amount of parasite DNA in the peripheral blood was also monitored using a real-time polymerase chain reaction (PCR) method as a new assessment of antibabesial chemotherapeutics.

MATERIALS AND METHODS

Preparation of bruceine A: The dried fruits of *Brucea javanica* were purchased from Bandar Jaya traditional market, Lampung, Indonesia, in April 2005. The plant species was identified by Mr. Aris Winarso at the Herbal Medicine Research and Education Centre, Lampung, Indonesia. Extraction and purification of bruceine A was described in the previous paper [24]. Briefly, air dried fruits (1 kg) were boiled with 5 *l* of water for 30 min twice. The boiling water was filtered and extracted with ethyl acetate (EtOAc) to give aqueous and EtOAc fractions. The EtOAc fraction was filtered, evaporated, and chromatographed on a silica gel column with chloroform, methanol (MeOH)-chloroform (3:97, 2*l*), MeOH-chloroform (1:4, 2*l*), MeOH-chloroform (7:3, 2*l*), and MeOH, successively. Each fraction was tested for its antibabesial activity against *B. gibsoni*. The active fraction was then chromatographed on a silica gel column with hexane-EtOAc (1:1) to give ten fractions. Bruceine A was detected in the fifth fraction and crystallized using MeOH. The structure was determined by means of NMR and mass spectra.

Parasites: The strain of *B. gibsoni* used was originally isolated from a naturally infected dog in Nagasaki Pref. in 1973 and has been maintained in dogs at Hokkaido University since then. The parasites were also maintained in 24-well plates (Corning, Corning, NY, U.S.A.) at 37°C with a gas flow mixture composed of 5% CO_2, 5% O_2, and 90% N_2. Approximately 60% of the culture supernatant was replaced daily with an equal volume of fresh medium. Every 7 days, a half volume of erythrocyte suspension was replaced with the same volume of uninfected erythrocyte suspension.

Preparation of canine erythrocyte suspension: Canine erythrocytes were prepared by the method of Yamasaki *et al.* [34] with some modifications. Venous blood was collected from the jugular vein of healthy adult dogs. The erythrocytes were washed three times in a modified Vega y Martinez phosphate-buffered saline solution (mVYM) [29] by centrifugation at 1,200 × g for 5 min at 4°C. After two additional washes with RPMI-1640 (Gibco, Invitrogen Co., CA, U.S.A.) medium, the cells were resuspended in RPMI-1640 supplemented with 0.1 mg/m*l* sodium pyruvate (Wako, Osaka, Japan), 0.3 mg/m*l* L-glutamine (Wako, Osaka, Japan), 2 mg/m*l* sodium bicarbonate (Wako, Osaka, Japan), 100 units/m*l* penicillin G potassium (Meiji Seika, Tokyo, Japan), and 20% (v/v) heat-inactivated dog serum to yield a packed cell volume (PCV) of 5%.

Assay for in vitro antibabesial activity of bruceine A: Bruceine A and diminazene aceturate (Hoechst AG, Germany) were dissolved in DMSO and kept in the dark at 4°C until use. The stock solution was diluted with culture medium containing 0.1% DMSO. *B. gibsoni*-infected erythrocytes (approximately 1% parasitemia) were obtained by diluting the cultures (5.0–6.5% parasitemia) with non-infected erythrocyte suspension. To each well of a 24-well culture plate a volume of 500 *µl* infected erythrocyte suspension was added. After settling, a 50 *µl* aliquot of the supernatant was removed and 50 *µl* of bruceine A solution was added to give final concentrations of 0, 6.3, 12.5, 25, 50, and 100 *n*M. The plate was incubated at 37°C with a gas flow mixture composed of 5% CO_2, 5% O_2, and 90% N_2 for 7 days. Approximately 60% of the supernatant was removed and replaced every 24 hr with fresh medium containing bruceine A at the appropriate concentration. Diminazene aceturate was used at concentrations of 0, 50, 100, 400, and 1,600 *n*M. Every 24 hr, 10 *µl* of the erythrocyte suspension was sampled from each well and mixed with 90 *µl* of phosphate buffered saline (PBS). The mixture was centrifuged and attached to a slide using a cytocentrifuge (Shandon Cytospin 2; Shandon, Cheshire, England) for 5 min at 450 rpm. The slide was fixed with methanol and stained with Giemsa solution (pH 7.4). The parasitemia level was determined by counting the number of parasitized erythrocytes in 2,000 erythrocytes. When the parasitemia level was less than 1%, an additional 3,000 erythrocytes were examined. The experiment was carried out in triplicate. Statistical analysis was carried out using Student's *t*-test.

Assay for antibabesial activity of bruceine A in dogs: Four beagle dogs (10-month-old, male) were used in this study. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University. One dog (dog A) was used to examine the side-effects of bruceine A and received a gelatin capsule containing bruceine A powder orally at dosages of 0.4, 0.8, 1.6, 3.2, and 6.4 mg/kg at 24 hr intervals. Since no acute toxicity was observed at any dosage, the same dog was administered bruceine A at an oral dosage of 6.4 mg/kg for 5 days at 24 hr intervals. Thus, dog A received bruceine A at a total dosage of 44.4 mg/kg. Clinical findings, body weight, and body temperature were monitored. Peripheral blood (5 m*l*) was collected and subjected to hematological and biochemical examinations. Packed cell volume (PCV), red blood cell counts, and platelet counts were measured using an automatic cell counter (Celltac-α, MEK-6258; Nihon Kohden, Tokyo, Japan). Serum biochemical parameters such as alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (CRE), gamma-glutamyl transpeptidase (GGT), glucose (GLU), and total cholesterol (TCHO) were measured using an analyzer (DRI-CHEM 7000V; Fuji Mechanical Industry Co., Ltd., Tokyo, Japan), and serum levels of electrolytes (Na^+, K^+, and Cl^-) were measured with an ion-selective electrode (Dri-Chem Slide Na-K-Cl; Dri-Chem 800 V; Fuji Film Co., Ltd., Tokyo, Japan).

Three dogs (dogs B, C, and D) were inoculated intravenously with 1.2×10^9 *B. gibsoni*-parasitized erythrocytes in a volume of 9 m*l*, which were harvested from a dog chroni-

cally infected with *B. gibsoni*. Bruceine A in a gelatin capsule was administered to two dogs (dogs B and C) at an oral dosage of 6.4 mg/kg/day for 6 days (a total dosage of 38.4 mg/kg) starting from day 5 post-infection. Dog D was served as the control and administered a gelatin capsule containing glucose powder. Clinical findings and body temperature were monitored for the experimental period of 28 days. Peripheral blood (500 μl) was collected daily and a 200 μl volume of each blood sample was subjected to hematological, biochemical, and microscopic examinations as described above. The rest of the blood sample was stored at −20°C until use for real-time PCR assays. Hematological examinations were conducted daily, while serum biochemical examinations were performed on days 0, 4, 11, 18, and 25 post-infection. Blood smears were made from EDTA-anticoagulated peripheral blood samples and stained with Giemsa solution. The level of parasitemia was determined by counting 10,000 erythrocytes.

Quantification of B. gibsoni p18 gene in the blood by real-time PCR: The copy number of the *B. gibsoni* p18 gene in the peripheral blood was estimated using the real-time PCR method by Matsuu *et al.* [18]. DNA was extracted from 200 μl of EDTA-anticoagulated whole blood samples using a commercial kit (QIAgen DNA Blood Mini Kit; Qiagen, Tokyo, Japan). Real-time PCR assays were performed with a reaction mixture (25 μl) containing each primer (500 *n*M) and template DNA extract (0.5 μl) using an Applied Biosystems 7300 real-time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) and Power SYBR Green PCR Master Mix (Applied Biosystems, Tokyo, Japan). The mixture was incubated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The relative copy number of the p18 gene in the blood samples was estimated from a standard curve created by plotting the log initial copy number of input plasmid, which contains a 182-base pair fragment of the p18 target gene, against the threshold cycle (Ct) value. Each sample was measured in triplicate and the results were expressed as the copy number per 100 erythrocytes (mean ± SD).

RESULTS

In vitro antibabesial activity of bruceine A: The kinetics of parasitemia in the canine erythrocytes with *B. gibsoni* in culture for 7 days is shown in Fig. 1. While previous report showed that bruceine A had an IC_{50} value of 4 *ng/ml* (7.7 *n*M) against the parasites at day 3 of the culture [24], a similar result was obtained at day 3 in this study, in which the level of parasitemia for the untreated culture and the culture treated with 6.3 *n*M bruceine A was 3 and 1.6%, respectively (Fig. 1-A, inset). Thus, bruceine A at 6.3 *n*M inhibited parasite growth by 47% at day 3 of the culture. Although parasitemia reached 12% at day 7 in the drug-free culture, it remained around 3% in the culture in the presence of 6.3 *n*M bruceine A, indicating that complete growth inhibition was not obtained at this concentration of the drug. On the other hand, parasitemia levels rapidly decreased within one day with 25 *n*M bruceine A, (Fig. 1-A, inset) and some morphological abnormalities of the parasites including a pyknosis-like and comma-shaped changes were observed (data not shown). Similar results of rapid decrease in parasitemia and morphological changes of the parasites were observed in the presence of 50 and 100 *n*M bruceine A (data not shown). However, no such rapid reduction in parasitemia was observed with the standard antibabesial drug, diminazene aceturate, even at the highest concentration of 1,600 *n*M (Fig. 1-B).

In vivo effect of bruceine A on B. gibsoni-infected dogs: One dog (dog A) was used to the examination for the side-effects of bruceine A and received this compound orally in a gelatin capsule at a dosage of 6.4 mg/kg/day for 5 days. No serious clinical findings were found in this dog. Hematological and serum biochemical values including PCV, red blood cell counts, platelet counts, ALP, ALT, AST, BUN, CRE, GGT, GLU, TCHO, and electrolytes (Na^+, K^+, and Cl^-) were all within respective reference range (data not shown).

Three dogs were then infected with *B. gibsoni*. Two of them (dogs B and C) were administered 6.4 mg/kg/day bruceine A orally for 6 days from day 5 post-infection, and the other one (dog D) was kept untreated. The kinetics of parasitemia and body temperature in these dogs is shown in Fig. 2. In the untreated dog D a gradual rise in parasitemia was observed and the value reached a peak of 2.3% on day 13 (Fig. 2, Dog D). This dog showed severe pallor in the mucous membranes from day 12 and its body temperature began to rise from day 8 and rose to 40.4°C on day 14 (Fig. 2, Dog D). The dog exhibited complete loss of appetite and movement on day 13 and reached the humane endpoint (*f* in Fig 2, Dog D) on day 14 before receiving a subcutaneous infusion to prevent lasting harm. Bruceine A-treated dogs (dogs B and C) also showed a gradual increase in parasitemia levels until day 11, but there were no obvious peaks and the levels were kept below 1% during the experimental period (Fig. 2, Dogs B and C). Dog B did not show any clinical signs. Dog C showed pallor in the mucous membranes from day 13 for 4 days and developed high fever sporadically on days 14 and 15. Anorexia and depression were, however, not observed in these bruceine A-treated dogs.

The kinetics of PCV and platelet counts is shown in Fig. 3. In dog B the PCV level decreased gradually but it was maintained at over 28% (Fig. 3, Dog B). The PCV in dog C decreased markedly and reached nadir values of 18% on day 15, and 15% (the humane endpoint in this experiment) in dog D on day 14, respectively (Fig. 3, Dogs C and D). However, in dog C the level recovered and was maintained at around 30 to 40%. The decrease in erythrocyte counts was associated with the decrease in PCV values in each dog (data not shown). A drastic decrease in platelet counts was observed in these dogs. Although the respective dogs had about 300,000/μl platelets in the blood before infection, they contained less than 10,000/μl on day 14 (Fig. 3, Dogs B, C, and D). Dog B maintained platelet counts at a lower

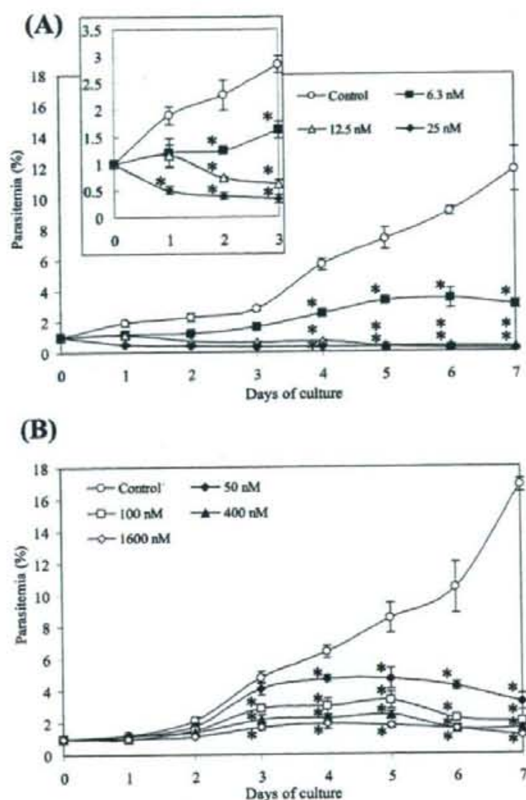

Fig. 1. Growth of *Babesia gibsoni* in the canine erythrocytes *in vitro* in the presence of bruceine A (A) and diminazene aceturate (B). Every 24 hr, each culture suspension was sampled and parasitemia was determined. Data show the means ± SD of two separate experiments. Insert indicates an expanded view of the growth curves for the initial 3 days in the presence of bruceine A. The asterisk (*) indicates statistically significant difference at $p<0.01$ from the control values by Student's *t*-test.

level of less than 55,000/μl during the experimental period. In dog C, the platelet counts increased to a normal level (240,000/μl) on day 21 but then decreased again.

With respect to serum biochemical values and serum levels of electrolytes, only the ALT value (136 U/*l*) in dog B on day 4 exceeded the reference value (17–78 U/*l*), whereas all other parameters were within each reference range (data not shown).

Detection of B. gibsoni p18 gene in the blood: The kinetics of the p18 gene copy number and parasitemia in the dogs is shown in Fig. 4. Parasite DNA was detected in the peripheral blood from day 1 and the p18 gene copy number reached a first peak on day 11, showing 4.3, 8.0, and 7.0 copies/100 erythrocytes in dogs B, C, and D, respectively (Fig. 4). The copy number decreased subsequently in all three dogs but then gradually increased again in dogs B and C.

DISCUSSION

This study showed that bruceine A had potent antibabesial activities against *B. gibsoni in vitro* and *in vivo*. The *in vitro* results indicated that a rapid decrease in parasitemia was induced by bruceine A at a concentration of 25 *n*M, suggesting that the parasites were killed at this concentration. The oral administration of bruceine A to dogs at an early