Studies on Antitrypanosomal Activity of Medicinal Plants

（薬用植物の抗トリパノソーマ活性に関する研究）

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CONTENTS

LIST OF ABBREVIATIONS

AcOH:	Acetic acid
AUC:	Area under curve
DMSO:	Dimethyl sulfoxide
EtOAc:	Ethyl acetate
EtOH:	Ethanol
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMI 9:	Hirumi-9 medium
HPLC:	High performance liquid chromatography
IC_{50}:	50% inhibitory concentration
MeOH:	Methanol
μM:	Micromolar
MRM:	Multiple reactions monitoring
MRC-5:	Human lung fibroblast cell line
MS:	Mass spectrometry
m/z:	Mass to charge
nM:	Nanomolar
nm:	Nanometer
NMR:	Nuclear magnetic resonance
SI:	Selectivity index
UPLC:	Ultra performance liquid chromatography

GENERAL INTRODUCTION

1. Morphology, biology, life cycle, pathogenesis and distribution in Asia of *Trypanosoma evansi*

Trypanosoma evansi is a typically monomorphic protozoan and has only long-slender trypomastigote form (Fig. 1). The size is between 15-36 μm in length (Stevens and Brisse, 2004). Phylogenetic analysis of the 18S rDNA of *Trypanosoma* species indicates that *T. evansi* is not differentiated from *Trypanoaosma brucei brucei*, *Trypanosoma b. rhodesiense*, *Trypanosoma b. gambiense* or *Trypanosoma equiperdum* (Fig. 2). Such a result supports the hypothesis that *T. evansi* has recently evolved from *T. b. brucei* (Stevens and Brisse, 2004). However, *T. evansi* is distinguished from *T. b. brucei* by its distinctive kinetoplast DNA (kDNA), which shows a lack of minicircle sequence heterogeneity and an absence of maxicircles. The characteristics of kDNA also support the distinction of *T. evansi* into two strain groups, typical type and a camel type (Borst *et al.*, 1987).

Trypanosoma evansi is found worldwide except North America. It is mechanically transmitted by biting arthropods, especially horse flies (*Tabanus* spp.) and stable flies (*Stomoxys* spp.), from one infected host to another. However, the role of vectors may vary in different regions. In Africa, the tsetse fly (*Glossina* spp.), like other blood-sucking flies, can act as a mechanical vector in areas where both *T. evansi* and tsetse flies occur. In South and Central America, *T. evansi* can be transmitted by vampire bats (*Desmodus rotundus*), which serve as both vectors and reservoir hosts

(Stevens and Brisse, 2004).

Trypanosoma evansi infects a wide range of domestic animals (e.g. camels, equines, bovines, goats and dogs) and wild animals with varying degrees of pathogenicity. In China, *T. evansi* is an important pathogen of draught buffalo. In both sub-Saharan and North Africa, beyond the tsetse belt, it is a major pathogen of camels. This association has been suggested as a possible route for the parasite's transportation beyond Africa and its evolution from *T. brucei* (Stevens and Brisse, 2004). The first human case of *T. evansi* infection was reported in India (Joshi et al., 2005).

The parasite causes a severe wasting disease, surra, in livestock and indigenous wildlife. Although *T. evansi* is pathogenic to most domestic and many wild mammals, its effect on the host varies depending on the virulence of the trypanosome strain, host species, and other factors such as concurrent infections and general stress in the host as well as local epizootiological conditions. The clinical signs of surra in most domestic and wild animals are fever and anaemia, followed by emaciation, oedema, cachexia and enlargement of the lymph nodes and spleen. *T. evansi* infections in cattle and water buffalo are typically chronic with associated weight loss and anaemia in some regions, e.g. in Indonesia. In cattle and water buffalo, a wide range of other clinical signs recorded include fever, salivation, diarrhoea, oedema, jaundice, conjunctivitis, lacrimation, mucopurulent nasal discharge, dyspnoea, alopecia, urticaria, swelling of superficial lymph nodes, abortion and infertility, decreased milk yield,

weakness, incoordination and paralysis. Formation of antigen–antibody complexes during the host response to *T. evansi* infection may cause inflammatory reactions in the central nervous system, myocardium and skeletal muscle (Luckins and Dwinger, 2004). Factors such as stress due to movement, adverse weather conditions, nutritional deficiencies, physiological changes and concurrent disease may result in clinical trypanosomiasis in latently infected animals. Infected animals die within weeks or months in acute cases but chronic infections may continue for several years. Boid et al. (1980) conducted studies on serum proteins and immunoglobulins during infection with *T. evansi* in Sudanese camels. They showed that total protein concentration was increased above normal values in experimentally and naturally infected camels. Levels of some serum enzymes were changed in camels infected with *T. evansi.* Increases in levels of sorbitol dehydrogenase, SGPT (serum glutamic oxaloacetic transaminase) and serum SGOT (glutamic pyruvic transaminase) above pre-infection levels were recorded whereas alkaline phosphatase decreased during the period of patent parasitaemia (Mahmoud and Gray, 1980). Thus, huge economical losses have been reported in Africa, Asia and South America, where thousands of animals die from *T. evansi* infections (Brun et al., 1998; Giardina et al., 2003; Luckins, 1988).

First occurrence of *T. evansi* in Asia is shown in Fig. 3. In India, *T. evansi* was first isolated from infected camels and equids in the Dera Ismail Khan district of the Punjab in 1880. The Hindi term 'surra' meaning

rotten or emaciated is the name used almost universally to describe the diseases caused by *T. evansi* in livestock. Later, the parasite was reported from many other areas of India and surra came to be considered one of the most important equine diseases in the country, causing thousands of animals to die. During 1940-1942, over 12000 cases of surra were reported in India (Luckins, 1988).

Surra was reported from Myanmar (formerly Burma) in 1885. During the course of military action there, some 6500 mules imported from Yunnan Province in China infected with the disease in Myanmar. Some died in Myanmar, but nearly 5000 survived and returned to China.

In China, *T. evansi* was first introduced in 1885 from Burma into Yunnan province in the southwest and later to other parts of China. However, the first cases of trypanosomiasis in domestic animals were recorded in 1938 in horses and mules of the Chang Jiang (Yangtze) Valley. Then, trypanosomiasis of animals became widespread throughout most of the country.

Trypanosoma evansi was first detected in 1888 in mules from North Vietnam and further isolations from equids were reported in the ensuing years. The first cases of bovine trypanosomiasis were detected in South Annam in 1906.

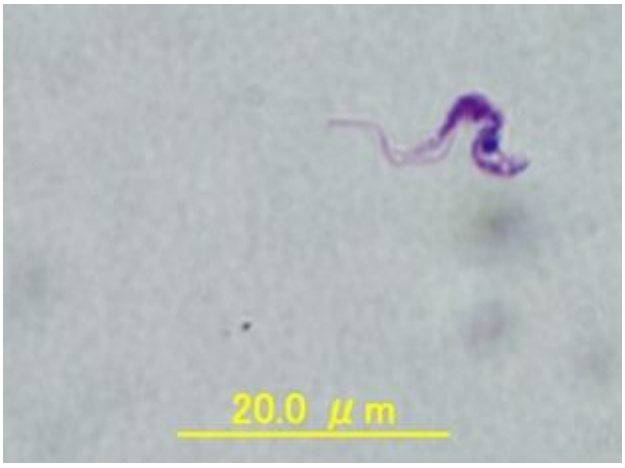


Fig. 1. A Giemsa-stained *Trypanosoma evansi* trypomastigote

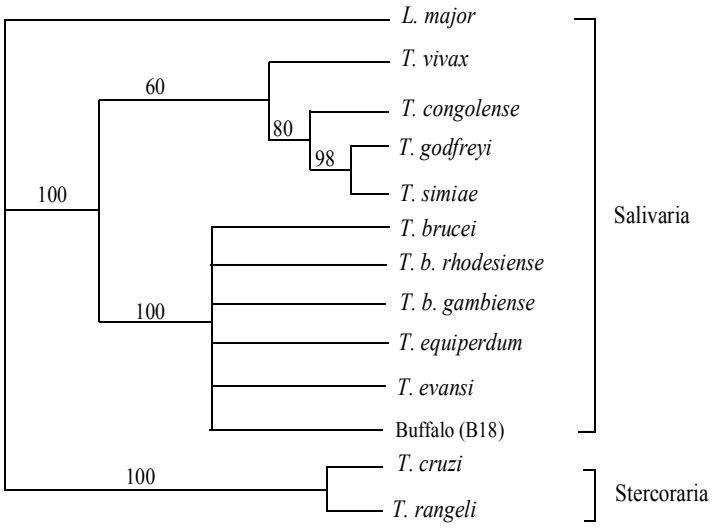


Fig. 2. Phylogenetic relationship of 18S rDNA obtained from the maximum likelihood methods among *Trypanosoma* species (including an isolate of B18 from a buffalo in Thailand) using *Leishmania major* as the outgroup (The numbers at the nodes represent the percentage of times the group occurred out of 100 trees.) (Khuchareontaworn et al., 2007).

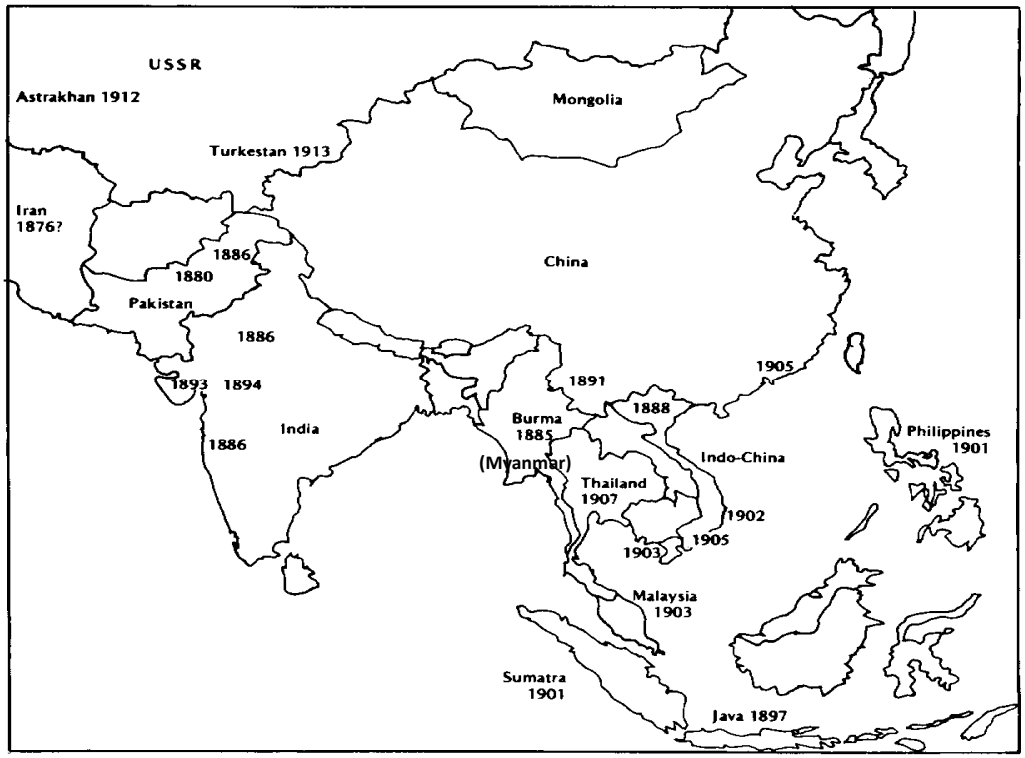


Fig. 3. Occurrence of *Trypanosoma evansi* in Asia. The dates indicate the earliest reports of disease associated with the demonstration of parasites in the animals.

In Malaysia and Thailand**,** *T. evansi* was identified in 1903 and 1907, respectively. In Indonesia, the first case of surra appeared in 1899 in equines and buffalo from Java. Surra appeared in Sumatra in 1901 and it was enzootic throughout low-lying areas of Java by 1917. Between 1920 and 1927, some 25000 cases of surra were recorded, 25% of cases in horses and all fatal.

By 1902, infection in buffalo and cattle were also recorded. In Soviet Asia, *T. evansi* was detected in Astrakhan in 1912 and in Turkestan in 1913. The prevalence of infection varied between 25% and 40% and epidemics of surra killed hundreds of camels (Luckins, 1988; Lun et al., 1993).

2. Chemotherapeutics of trypanosomiasis

Control of trypanosomiasis in livestock usually relies upon either curative or prophylactic treatment of animals with trypanocidal drugs. Currently, the most commonly used drugs for treatment of *T. evansi* infection are diminazene aceturate, suramin, isometamidium, homidium and cymelarsan (Table 1). Quinapyramine was discontinued due to its capacity to induce multi-drug resistance (Holmes et al., 2004).

Table 1. Currently available trypanocidal drugs for use in domestic livestock (Holmes et al., 2004)

Drug	Trade names	Dose (mg/kg)	Route	Activity	Animal
Diminazene aceturate	Berenil®	3.5-7	i.m	*T. congolense*	Cattle
	Many others			*T. vivax*	Small ruminants
				(T. brucei)	[Dogs]
				(T. evansi)	[Equidae]
Homidium chloride	Novidium®	1	i.m	*T. congolense*	Cattle
Homidium bromide	Ethidium®			*T. vivax*	Small ruminants
					Pigs
					[Equidae]
Isometamidium chloride	Samorin®	0.25-0.5	i.m	*T. congolense*	Cattle
	Trypamidium®	0.5-1	i.m	*T. vivax*	Small ruminants
	Veridium®			*T. brucei*	Equidae
				T. evansi	Camels
Quinapyramine dimethylsuphate	Trypacide sulphate®	3.0-5.0	s.c	*T. congolense*	Camels
Quinapyramine dimethylsuphate: chloride (3:2 w/w)	Trypacide Pro-salt®	3.0-5.0	s.c	*T. vivax*	Equidae
				T. brucei	Pigs
				T. evansi	Dogs
				T. simiae	
Suramin	Naganol®	7-10 g/animal	i.v	*T. evansi*	Camels
					Equidae
Melarsenoxide cysteamine	Cymelarsan®	0.25	s.c/i.m	*T. evansi*	Camels

i.m, intramuscular; s.c, subcutaneous; i.v, intravenous; ( ) limited activity; [ ], small therapeutic index.

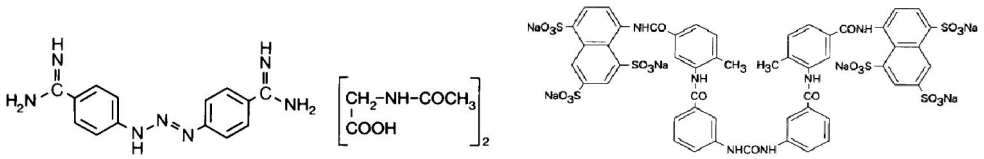
Diminazene is an aromatic diamidine derived from Surfen C. The molecule is marketed as the diaceturate salt and consists of two amidinophenyl moieties linked by a triazene bridge (Fig. 4a) p,p-diamidinodiazo-aminobenzene diaceturate tetrahydrate and N-1,3-diamidinophenyltriazene diaceturate tetrahydrate. Diminazene has subsequently become the most commonly used therapeutic agent for trypanosomiasis in domestic livestock. It is highly effective against *Babesia* spp., *T. congolense* and *T. vivax*, but less active against *T. b. brucei* and *T. evansi* infections. Trypanosomes resistant to other drugs (except quinapyramine) are commonly susceptible to diminazene (Peregrine and Mamman, 1993). Diminazene interferes with nucleic acid synthesis and binds to DNA *in vitro* (particularly to kinetoplast DNA) by a non-intercalative mechanism, thereby blocking DNA and RNA synthesis (Mehlhorn, 2008). It appears to enter *T. b. brucei* group parasites via the P2 nucleoside transporter that is also capable of transporting other diamidines and melamine-based arsenicals. Moderate adverse drug reactions are frequently reported include nausea, vomiting and albuminuria, but severe events including reversible paralysis and coma are rare. The main adverse drug reactions seen in animals are severe cerebral haemorrhages (Holmes et al., 2004).

Based on the trypanocidal activity of the dyes trypan red, trypan blue, and afridol violet, research in Germany resulted in the introduction of suramin into therapy in 1920 (Fig. 4b). Suramin is soluble in water, but

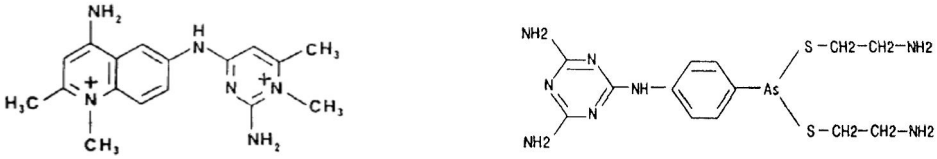
solutions deteriorate quickly in air. It is a relatively slowly acting trypanocide (>6 hours *in vitro*) with high clinical therapeutic activity against both *T. brucei gambiense* and *T. brucei rhodesiense*. Suramin reacts reversibly with a variety of biomolecules *in vitro,* inhibiting many trypanosomal and mammalian enzymes and receptors unrelated to its antiparasitic effects, including purinergic and AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptors (Suzuki et al., 2004). Suramin-treated trypanosomes exhibit damage to intracellular membrane structures other than lysosomes. Very little amount of suramin penetrates into the CSF (cerebrospinal fluid), consistent with its lack of efficacy in CNS (central nervous system) trypanosomiasis. Therefore, suramin is used primarily to treat early stages (before CNS involvement) of African trypanosomiasis (Pépin and Khonde, 1996). Malaise, nausea, and fatigue are also common immediate reactions. The most common problem encountered after several doses of suramin is renal toxicity, manifested by albuminuria, and delayed neurological complications, including headache, metallic taste, paresthesias, and peripheral neuropathy (Voogd et al., 1993).

Quinapyramine is a bis-quaternary compound (Fig. 4c) introduced in the 1950s for field use as a therapeutic (Antrycide sulphate®) and prophylactic drug (Antrycide Prosalt®) for animal trypanosomiasis. It has been used successfully against *T. congolense, T. vivax* and *T. b. brucei* infections in cattle, sheep and goats. Infections of *T. evansi, T. equinum* and *T. equiperdum* have also been treated effectively using quinapyramine. Due

(a) (b)



(c) (d)



(e) (f)

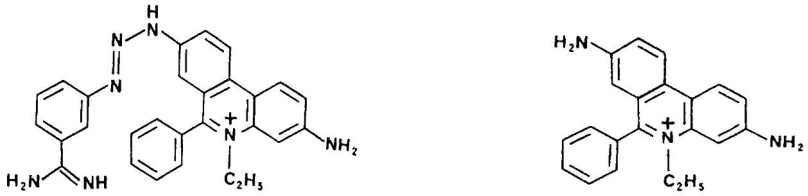


Fig. 4. Molecular structures of diminazene aceturate (a), suramin (b), quinapyramine (c), melarsenoxide cysteamine (d), isometamidium (e) and homidium (f).

to development of drug resistance, the drug was withdrawn from the market in many parts of Africa in the 1970s (Griffin and Allonby, 1979; Leach and Roberts, 1981). However, the drug has been re-introduced on the market in the recent past, under two different names. Trypacide sulphate® is recommended for subcutaneous treatment of clinical cases while Trypacide Pro-salt® (quinapyramine sulphate: quinapyramine chloride, in the ratio of 3:2) is recommended for prophylaxis (Kinabo, 1993; Zhang et al., 1991).

Melarsenoxide cysteamine (Cymelarsan®) (Fig. 4d) was discovered in 1985 as member of the melaminyl thioarsenite group of compounds invented in the 1940s. The drug product is presented as a white powder highly soluble in water. Melarsenoxide cysteamine is primarily effective against infections caused by *T. evansi* in camels, cattle, horses and buffaloes (Lun et al., 1991). Compared to suramin, isometamidium and diminazene, Melarsenoxide cysteamine has shown to be more efficacious against *T. evansi* and *T. equiperdum* (Zhang et al., 1991). The mechanism of these compounds is believed to act primarily in two ways. The first mechanism is by interference with energy generation processes through inhibition of pyruvate kinase. The second mechanism is by interaction with trypanothione (Flynn and Bowman, 1974). There are also indications that the drug may be of value in eliminating *T. b. gambiense* and *T. b. rhodesiense* in their reservoir hosts such as cattle and swine (Kinabo, 1993).

Isometamidium (Samorin®, Trypamidium®) and homidium

(chloride salt; Novidium®; bromide salt or ethidium bromide: Ethidium®) are phenanthridinium compounds. Isometamidium (Fig. 4e) differs from homidium (Fig. 4f) by an additional moiety of m-amidinophenyl-azo-amine which in fact is part of the diminazene molecule. Both isometamidium and homidium are active against *T. congolense* and *T. vivax*. Additionally, isometamidium is also of value against infections caused by *T. b. brucei* and *T. evansi* infections in donkeys, horses and camels. Homidium was extensively used in the 1960s and 1970s but its usefulness has been greatly reduced due to widespread resistance (Scott and Pegram, 1974). The primary mode of action of phenanthridinium drugs is blockade of nucleic acid synthesis through intercalation between DNA base pairs, inhibition of RNA polymerase (Richardson, 1973), DNA polymerase and incorporation of nucleic acid precursors into DNA and RNA (Lantz and Van Dyke, 1972). The mechanism of resistance to isometamidium is associated with reduced accumulation of the drug in the parasite (Sutherland and Holmes, 1993). One of the major adverse properties of phenanthridinium drugs, particularly isometamidium, is tissue damage at injection site (Kinabo and Bogan, 1988).

As a result of side effects associated with existing trypanocidal drugs and development of drug resistant trypanosomes in many regions (Anene et al., 2001; Kibona et al., 2006; Matovu et al., 2001), 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).

One possible source for such affordable treatment lies in the use of natural products.

Natural products have served as an important source of drugs since ancient times and about half of the useful drugs today are derived from natural sources. In many parts of the world, extensive use is made of plants in traditional medicine. Antiparasitic plant-derived molecules have been used as lead compounds to develop semi-synthetic or synthetic drugs with better efficacy and safety (Tagboto and Townson, 2001). The majority of the world's population depends on traditional medical remedies, on account of the limited availability and affordability of conventional medicines. It is estimated that some 20000 species of higher plant are used medicinally throughout the world. 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). Many well-known drugs listed in the modern pharmacopoeia have their origins in nature, including, for example, quinine from the bark of the *Cinchona* tree and artemisinin from the herb *Artemisia annua* for the treatment of malaria. The numerous plant-derived natural products with antiprotozoal activities, including various alkaloids, terpenoids, flavonoids, and quinonoids have been reported. 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).

This study focused on antitrypanosomal activities of medicinal plants and natural compounds against *T. evansi*. As earlier described,

research in development of new compounds for the treatment of surra is very important. The aim of this study is isolation of antitrypanosomal compounds from a traditional medicinal plant, *Brucea javanica,* examination of antitrypanosomal activities and cytotoxic activities of isolated compounds and screening of antitrypanosomal and cytotoxic activities of Myanmar medicinal plants.

CHAPTER I

Isolation of quassinoids from a medicinal plant, *Brucea javanica*

1. INTRODUCTION

Natural compounds in plants offer valuable sources of novel drug discovery. *Brucea javanica* (L.) Merr. (Simaroubaceae) is a small spreading tree that has compound leaves with 3-15 leaflets (Fig. I-1a). Bases of leaflets are oblique and have serrate margin that are formed by the end of a vein bearing a marginal gland. Flowers in a raceme and stamens are red while its fruits of 1-4 druplets are purple to black in colour when ripen (Fig. I-1b and c). *B. javanica* is widely distributed throughout Asia and is known by various local names such as "ya dan zi", "ya tan tsu", "kho-sam", "macassar kernels", "makassaarse pitjes", etc. Its fruits have been used as a traditional medicine for various diseases, including cancer, amoebic dysentery and malaria in Indonesia (Subeki et al., 2007), Myanmar, Thailand, or China (Lin et al., 1990).

Extensive studies of the genus *Brucea* have led to the identification of many compounds, such as quassinoids, nigakilactones, alkaloids, triterpenoids, and flavonoids. Quassinoids are the bitter principles found in various species of Simaroubaceae plants in the tropics (Sakaki et al., 1984; Yoshimura et al., 1984). According to their basic structures, quassinoids are categorized into five distinct groups, C-18, C-19, C-20, C-22 and C-25 types. The C-20 quassinoids can be further classified into two types, tetracyclic and the pentacyclic. Currently, there are over 150 quassinoids that have been isolated and fully characterized (Guo et al., 2005). In the past few decades, much attention has been devoted to quassinoids because of their wide range

(a)

(b)

(c)

Fig. I-1. Tree (a) and fresh (b) and dry fruits (c) of *Brucea javanica*.

of biological activities *in vitro* and/or *in vivo,* including antitumor (Fukamiya et al., 1992; Ohnishi et al., 1995), antimalarial (O'Neill et al., 1986), antiviral, anti-inflammatory (Kitagawa et al., 1996), antifeedant, insecticidal, antiamoebial (Gillin et al., 1982), antibabesial (Elkhateeb et al., 2008; Subeki et al., 2007) and herbicidal activities.

The quantity and the existence of quassinoids in the plants appear 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. In addition, rapid identification of the bioactive compounds of natural product mixtures remains a critical factor for drug discovery. In order to determine the chemical nature of compounds, isolation of a substance in pure form using various separation techniques is the first step. The coupling of chromatographic methods such as silica gel and high pressure liquid chromatography (HPLC) is an important tool for obtaining the pure compounds.

In this study, contents of quassinoids in *B. javanica* were analyzed by fractionation with column chromatography and ultra performance liquid chromatography (UPLC) with an electrospray ionization triple quadrupole mass spectrometry (MS/MS). UPLC-MS/MS generates higher chromatographic performance by using smaller stationary phase particle size columns. This column decreases band-broadening, thereby giving high

efficiency of separation, which concurrently increases resolution and sensitivity (Jacob et al., 2007).

The aim of this study, therefore, is to isolate quassinoid compounds from the fruits of *B. javanica* and compare the contents of quassinoids in *B. javanica* from different geographic origins.

2. MATERIALS AND METHODS

2.1 Isolation of quassinoids by column chromatography

2.1.1 Plant materials

Dried 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).

2.1.2 Extraction and isolation

From the ethyl acetate (EtOAc)-soluble fraction of the Indonesian plant materials, 8 quassinoids were isolated (Subeki et al., 2007). Quassinoids isolation in brief; silica gel column chromatography (MeOH–$CHCl_3$, 0:1, 3:97, 1:4, 7:3, 1:0) of the EtOAc-soluble portion of

the boiled H_2O extract of *B. javanica* fruit yielded five fractions. The MeOH–$CHCl_3$ (1:4) eluate gave, after silica gel column chromatography using hexane–EtOAc (1:1), 10 fractions. The fifth fraction gave bruceine A on crystallization from MeOH, whereas the seventh, eighth, and ninth fractions yielded bruceantinol, bruceine B, and bruceine C, respectively, on crystallization from hexane–EtOAc (9:1). The MeOH–$CHCl_3$ (7:3) eluate was subjected to silica gel column chromatography using MeOH–EtOAc (1:19) to give two new quassinoids, together with bruceine D and yadanziolide A (Fig. I-2). Identification of the known compounds was accomplished by comparing their spectroscopic data with those in the literature (Lee et al., 1979; Yoshimura et al., 1985).

In addition to these compounds, 7 quassinoids were isolated from the Chinese plant material (Elkhateeb et al., 2008) (Fig. I-3). Extraction of plant in brief; dried ground fruit (500 g) was defatted with *n*-hexane (3L×3) and the marc was successively extracted with chloroform (3L×3). The extract was filtered and chromatographed on a silica gel column, eluted with a gradient of MeOH-$CHCl_3$ (from 1:19 to 2:3) to give three fractions, Fr. I {MeOH-$CHCl_3$ (1:19), 1000 ml}, Fr. II {MeOH-$CHCl_3$ (1:4), 1000 ml} and Fr. III {MeOH-$CHCl_3$ (2:3), 1000 ml}. After purified by HPLC (Capcell Pak C_{18}, 5 μm, 15 mm × 250 mm, Shiseido) with CH_3OH-H_2O (3:2) and detection using a UV spectrophotometer at 254 nm, brusatol and bruceantin were obtained from Fr. I. Fr. II was further purified by HPLC (Capcell Pak C_{18}, 5 μm, 15 mm × 250 mm, Shiseido) with

CH_3OH-H_2O (3:2) and detection with a UV spectrophotometer at 254 nm to give dehydrobruceine A, dehydrobrusatol and dehydrobruceine B. From Fr III after purification by HPLC (Capcell Pak C_{18}, 5 μm, 15 mm × 250 mm, Shiseido) with the solvent CH_3OH-H_2O (3:2) and detection by UV spectrophotometer at 254 nm to give bruceoside A and yadanzioside G.

The dried powder of Indonesian *B. javanica* fruits was extracted with MeOH-H_2O and partitioned using EtOAc into water and EtOAc-soluble fractions. The water-soluble fraction was further chromatographed on DIAION HP-20 and Sephadex LH-20 columns. The sample was finally purified over a silica gel column to give bruceine D (Fig. I-4).

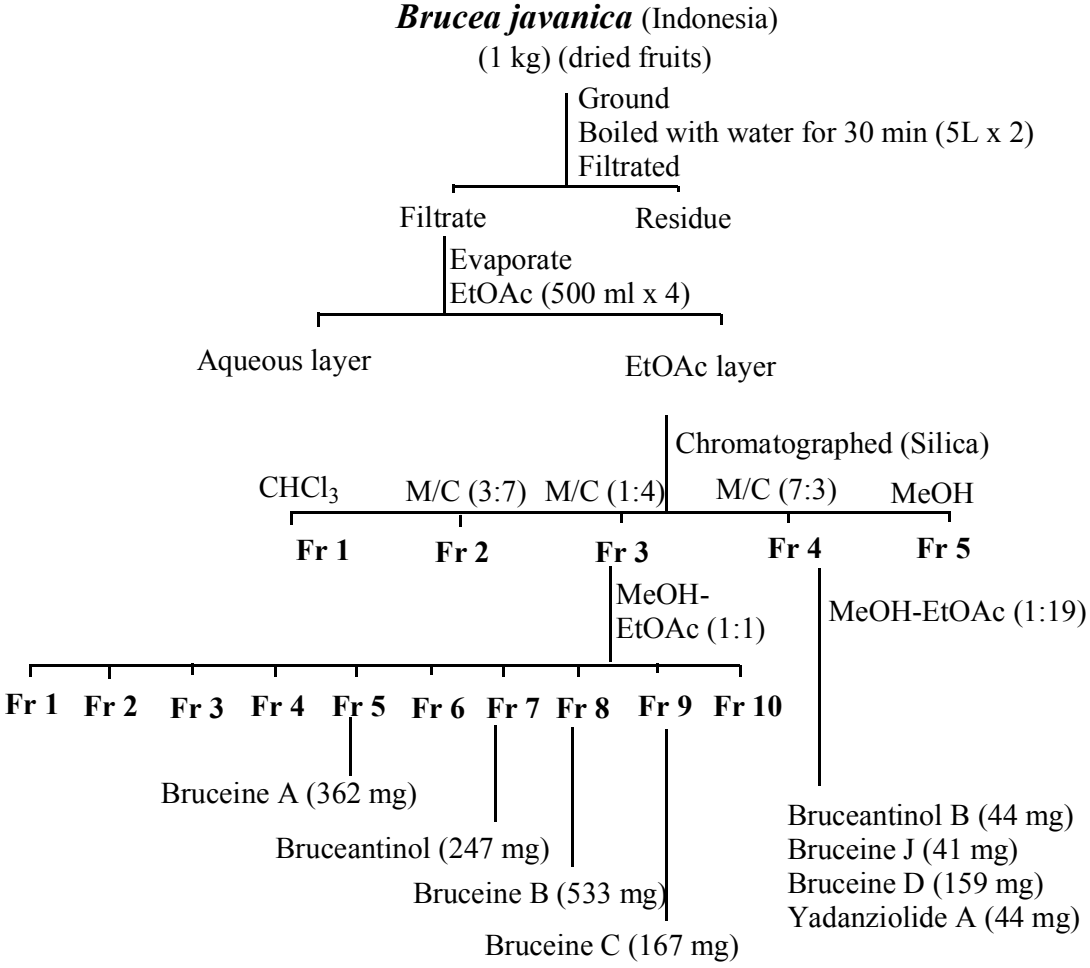
2.1.3 Identification

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 nuclear magnetic resonance (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).

From the EtOAc-soluble fraction of the Indonesian plant material, bruceine A, bruceine B, bruceine C, bruceine D, bruceantinol, bruceantinol B, bruceine J, and yadanziolide A 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). The physical appearances and molecular formulas are;

1) bruceine A-- white powder; $C_{26}H_{34}O_{11}$ (MW = *m/z* 522)
2) bruceine B-- white powder; $C_{23}H_{28}O_{11}$ (MW = *m/z* 480)
3) bruceine C-- white powder; $C_{28}H_{36}O_{12}$ (MW = *m/z* 564)
4) bruceine D-- white powder; $C_{20}H_{27}O_{9}$ (MW = *m/z* 411)
5) bruceine J-- amorphous solid; $C_{25}H_{31}O_{11}$ (MW = *m/z* 508.5)
6) bruceantin-- colorless amorphous powder; $C_{28}H_{36}O_{11}$ (MW = *m/z* 548)
7) bruceantinol-- colorless amorphous powder; $C_{30}H_{38}O_{13}$ (MW = *m/z* 606)
8) bruceantinol B-- amorphous solid; $C_{29}H_{35}O_{13}$ (MW = *m/z* 578.5)
9) brusatol-- colorless amorphous powder; $C_{26}H_{32}O_{11}$ (MW = *m/z* 520)
10) dehydrobruceine A-- colorless amorphous powder; $C_{26}H_{32}O_{11}$ (MW = *m/z* 520)
11) dehydrobruceine B-- amorphous powder; $C_{23}H_{26}O_{11}$ (MW = *m/z* 478)
12) dehydrobrusatol-- colorless amorphous powder; $C_{26}H_{30}O_{11}$ (MW = *m/z* 518)
13) bruceoside A-- white powder; $C_{32}H_{42}O_{16}$ (MW = *m/z* 682)
14) yadanzioside G-- colorless amorphous powder; $C_{36}H_{48}O_{18}$ (MW = *m/z* 768)
15) yadanziolide A-- white powder; $C_{20}H_{26}O_{10}$ (MW = *m/z* 427).



M/C = methanol + chloroform solvent

Fig. I-2. Isolation of quassinoids from *Brucea javanica* (Subeki et al., 2007).

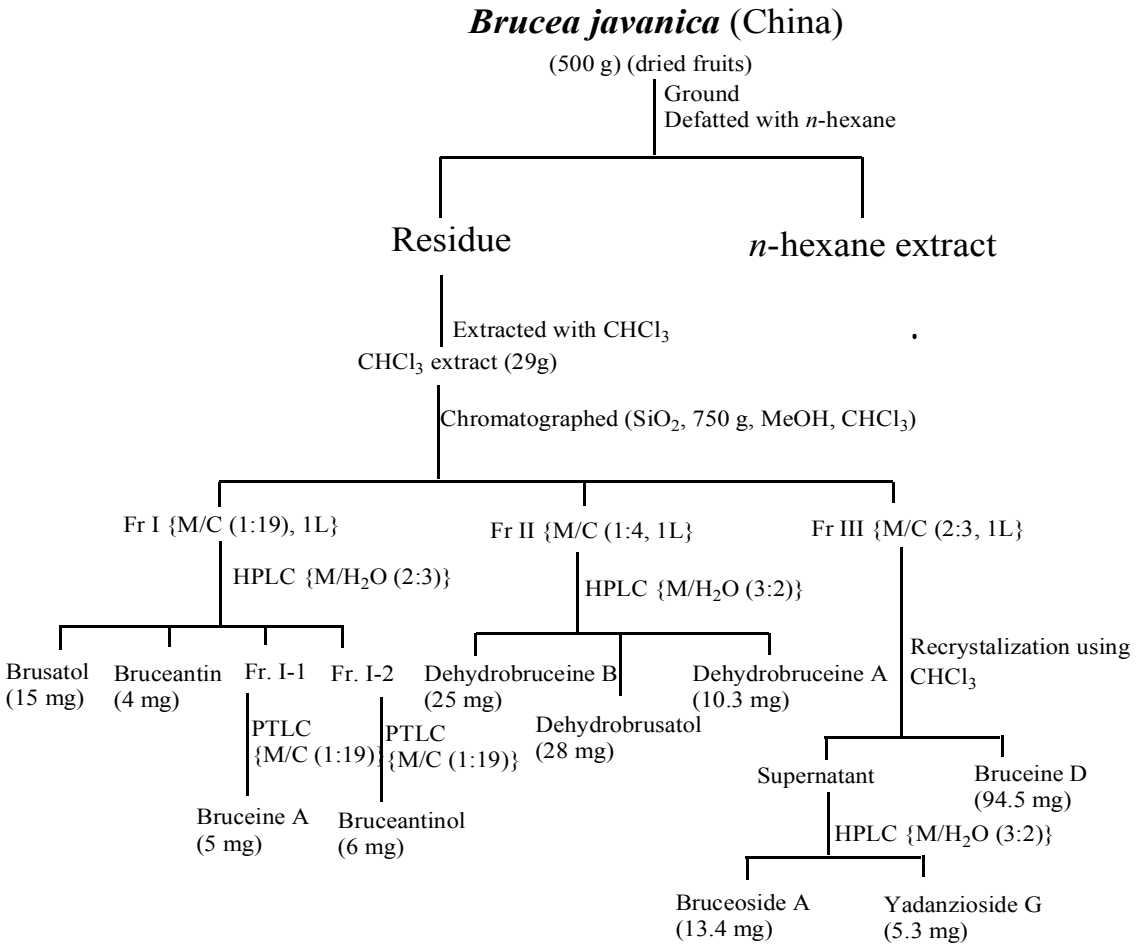


Fig. I-3. Isolation of quassinoids from *Brucea javanica* (Elkhateeb et al., 2008).

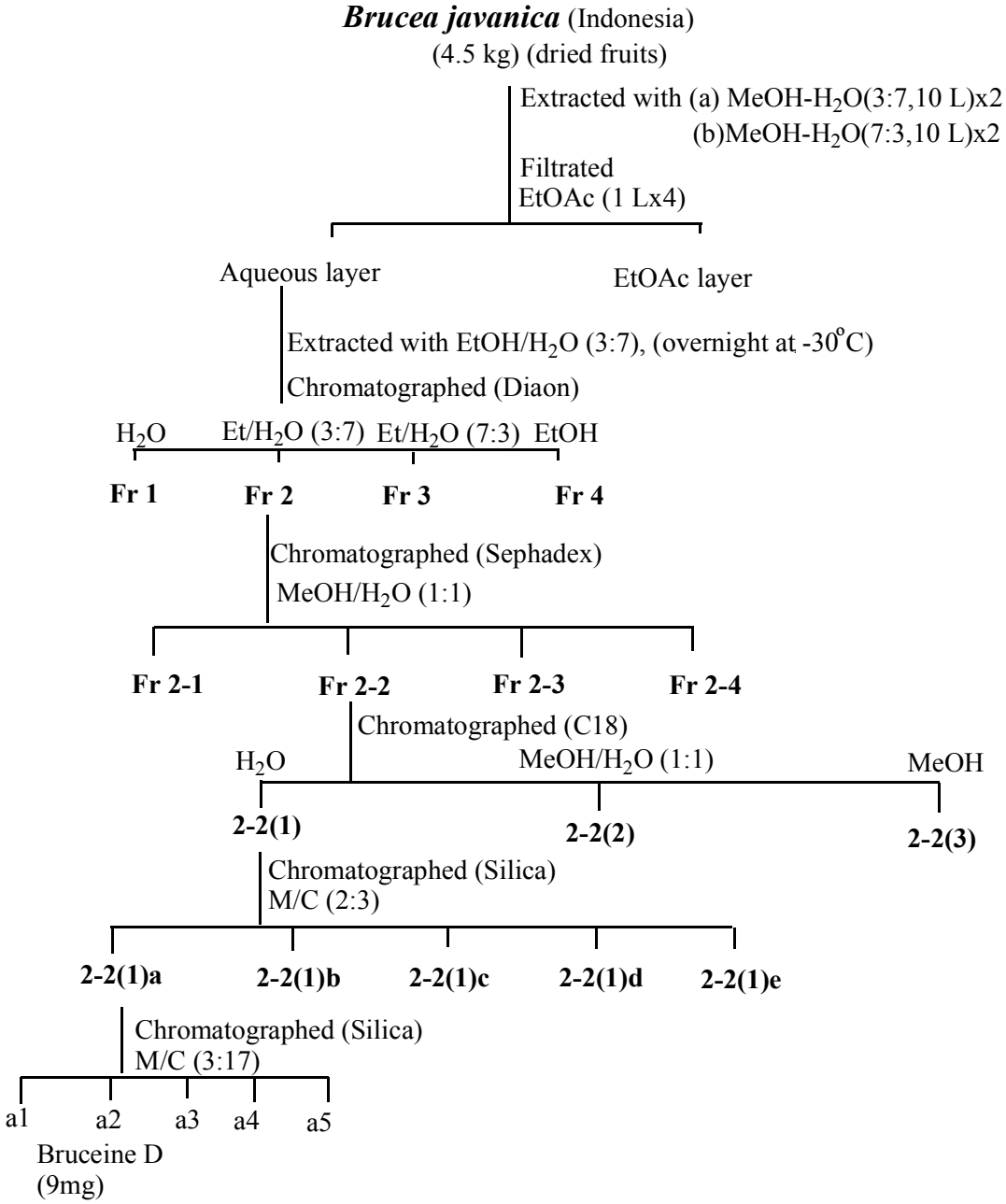


Fig. I-4. Isolation of bruceine D from water soluble fraction of *Brucea javanica*.

2.2 Determination of quassinoid contents in B. javanica from different geographic origins

2.2.1 Plant materials

Dried fruit samples of *Brucea javanica* were collected from Indonesia in 2005 and China in 2007 as described above. Additionally, two Myanmar samples were collected at Theingyi market, Yangon, in 2007 (Myanmar I) and 2008 (Myanmar II). One gram of each sample was extracted with 70% ethanol (10 ml/g). After filtration, extracts were dried and 20 ng of samples were measured.

2.2.2 Reference standards

Purified bruceine A, bruceine B, bruceine C, bruceine D and brusatol were diluted (0.1, 1, 10, and 100 ng) and analyzed by UPLC as described below.

2.2.3 Instrumentation

UPLC was performed on a Waters ACQUITY UPLC system (Waters, Milford, USA). Detection was performed on a Waters Micromass Quattro Premier^{TM} Tandem Quadrupole Mass Spectrometer (Waters Inc., Micromass, Quattro Premier XE). The instrument was equipped with autosampler and column thermostats. The instrument was controlled using Waters MassLynx 4.0 software and data were evaluated using Waters TargetLynx software.

2.2.4 UPLC-MS/MS conditions

MS optimizations were performed in MS scan mode and in product scan mode. All quantifications were performed in multiple reactions monitoring (MRM) mode. The tune page parameters and conditions for each of the MRM transitions were optimized by infusing the neat standard solution into the mass spectrometer at 10 µg/ml. To ensure that the tune page parameters are compatible with the UPLC flow during the tuning, an UPLC flow of 0.3 ml/min at solvent 20% aqueous MeOH with 0.05% AcOH (solvent A): MeOH with 0.05% AcOH (solvent B) (1:1) was introduced into the mass spectrometer at the same time by utilizing a T unit (Upchurch Scientific, Oak Harbor, WA, USA). To collect MRM data during the UPLC experiments, the ionization source conditions; the capillary voltage of 3.0 kV, source temperature of 120ºC, desolation temperature of 350°C were used. The desolation and cone gas flows were 800 l/h and 50 l/h, respectively. During each UPLC injection, the mass spectrometer was set to collect data in MRM mode using electrospray ionization in negative ion mode.

2.2.5 UPLC conditions for MS/MS MRM

UPLC analysis was performed using a Waters ACQUITY ethylene-bridged (BEH) C18 column (2.1 x 100 mm, 1.7 µm) at 38°C. The UPLC system was coupled to a Waters Micromass Quattro Premier Tandem Quadrupole Mass Spectrometer. The analytes were eluted from the

column with a mixed solvent of 20% aq. MeOH with 0.05% AcOH (solvent A) and MeOH with 0.05% AcOH (solvent B) using a linear gradient mode. In order to examine the compounds, the combination of A and B was 70:30 from 0 s to 0.2 min, and from 0.2 min to 2 min the combination of A and B was linearly converted from 70:30 to 10:90. The combination of 10:90 was maintained from 2 min to 3 min. The column was finally eluted with A:B (0:100) from 3.1 min to 4.0 min at a flow rate of 0.3 ml/min. Sample extract volumes of 5 µl were injected into the system. With these UPLC-MS/MS conditions, quassinoids were analyzed by MRM. Calibration curves were constructed by plotting the peak area (AUC) of each quassinoid (0.1-100 ng) for estimation of the amounts of quassinoids in crude extracts from plant samples.

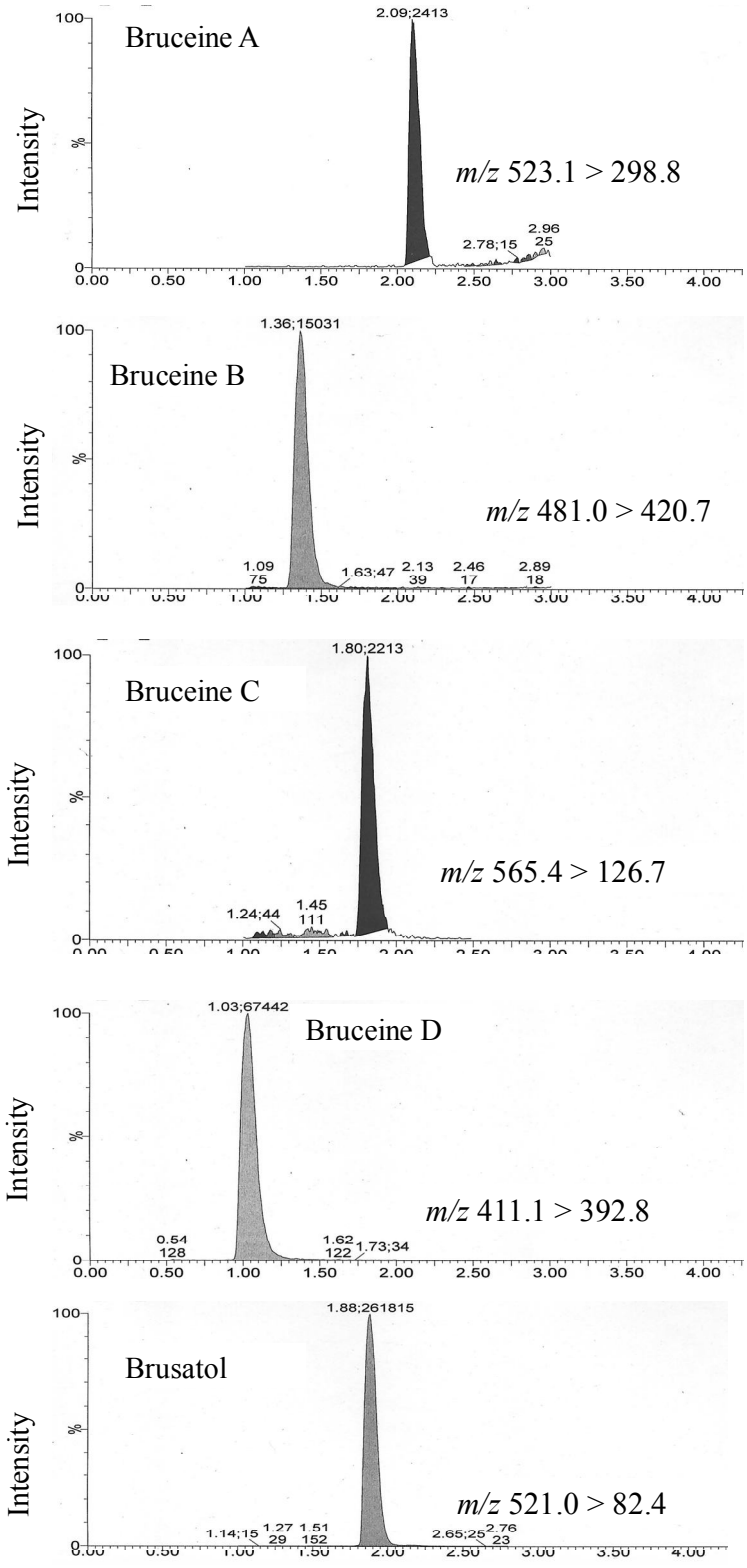


Fig. I-5. UPLC-MS/MS charts of standard quassinoids (10 ng).

3. RESULTS AND DISCUSSION

Identification of each quassinoid was successfully performed by NMR and mass spectrometry in the previous studies (Elkhateeb et al., 2008; Subeki et al., 2007). Using the same methodology, this study revealed that only bruceine D was isolated from the water-soluble fraction of *B. javanica* fruits, indicating that most quassinoids in this plant species are water-insoluble.

This study also showed the development and validation of a rapid UPLC-MS/MS method for the quantitative analysis of quassinoids. Purified bruceine A, B, C, D, and brusatol showed single peak at retention time with 2.1, 1.3, 1.8, 1.0, and 1.8 min, respectively (Fig. I-5). Fragments of quassinoids were detected by selected reaction monitoring using mass-to-charge (*m/z*) transition of *m/z* 523.1 > 298.8 for bruceine A, *m/z* 481.0 > 420.7 for bruceine B, *m/z* 565.4 > 126.7 for bruceine C, *m/z* 411.1 > 392.8 for bruceine D, and *m/z* 520.0 > 82.4 for brusatol.

The standard curves were created by measurement of the peak area (AUC) in MRM chromatograms for each quassinoid (0.1-100 ng) (Fig. I-6). Linear regression was calculated from dose response graph using Microsoft Excel. The regression equations: $y = 1.4364x + 1.9417$, $y = 1.0982x + 3.0652$, $y = 1.0273x + 2.3682$, $y = 0.9081x + 3.8888$ and $y = 1.1390x + 4.1360$ were used for calculation of amount of bruceine A, B, C, D and brusatol, respectively. The peak areas of bruceine A, B, C, D, and brusatol were determined for crude methanol extracts from *B. javanica* samples

from different countries and their amounts in samples were estimated (Table I-1). The amount of bruceine C was much higher than other quassinoids in all samples. As expected, the amounts of these quassinoids were different with different origins. Bruceine A, which was the most active against *T. evansi*, was contained at the most in the Indonesian sample. Differences in the amounts of quassinoids between the harvest times in Myanmar samples may be due to the seasonal variation at similar climate conditions or the geographical variation even in the same countries.

The present studies of fractionation with column chromatography and UPLC MS/MS analysis suggest that the quantity and composition of quassinoids in the same plant species depend on geographic factors as described by Pavanand et al. (1986). It is well known that the concentration of biologically active constituents varies with the stage of plant growth and development (Mendonça-Filho, 2006). Thus, the time of harvest of medicinal plants should be considered to obtain fairly amounts of objective compounds. Rapid and accurate quantitative method developed in this study will be useful for the screening of the detection and quantification of quassinoids or other bioactive compounds from crude plant extracts. Further studies including the development of simple and low-priced method for isolation of active compounds in a large quantity are required.

Table. I-1. Retention time (min), peak areas (AUC) and estimated amount (ng) of quassinoids detected from *Brucea javanica* samples from Myanmar, Indonesia and China

		AUC (estimated amount in ng)			
Quassinoid	Retention time (min)	Myanmar I	Myanmar II	Indonesia	China
Bruceine A	2.1	2943 (0.2)	5183 (0.3)	11080 (0.8)	9360 (0.6)
Bruceine B	1.3	3040 (2.9)	6928 (7.1)	11924 (12.9)	6683 (6.8)
Bruceine C	1.8	12355 (59.0)	21391 (103.7)	10197 (48.4)	7449 (35.1)
Bruceine D	1.0	78312 (8.2)	61314 (6.5)	52186 (5.7)	72301 (7.6)
Brusatol	1.8	3855 (0.2)	3337 (0.2)	2008 (0.1)	94589 (9.0)

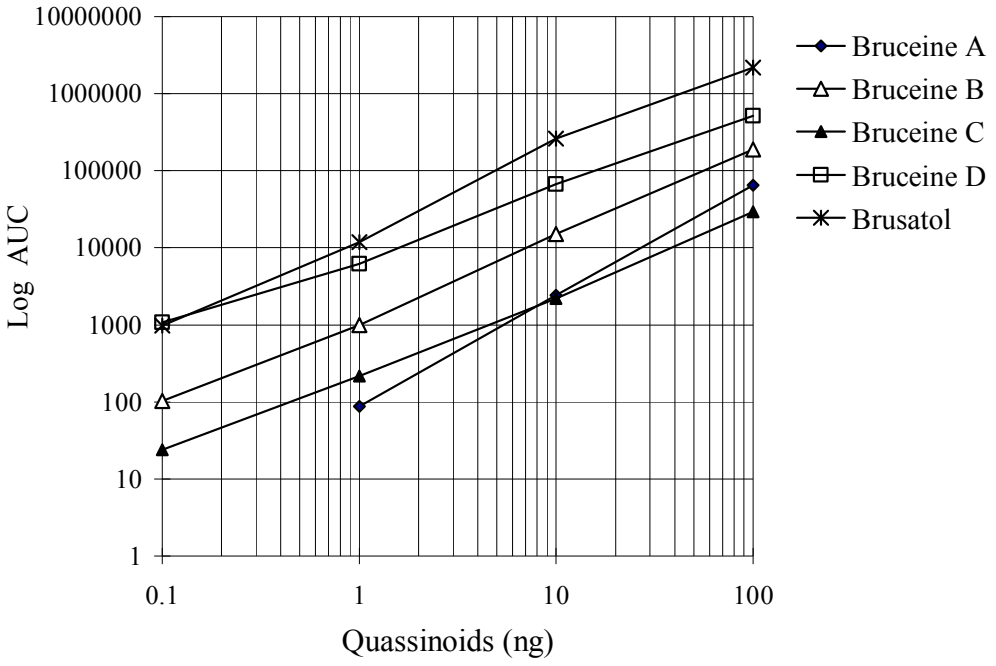


Fig. I-6. The peak area (AUC) for reference standard quassinoids (0.1-100 ng).

4. SUMMARY

Brucea javanica is widely distributed and its fruits have been used as traditional medicine against various diseases, including cancer, amoebic dysentery, and malaria. The dried fruits of *B. javanica* were extracted with 70% aqueous methanol and partitioned using ethyl acetate to yield aqueous and organic layers. In the previous studies, C-20 types of quassinoids such as bruceine A, bruceine B, bruceine C, bruceine D, bruceantinol, bruceantinol B, bruceine J, and yadanziolide A were isolated and purified from the organic layer of the Indonesian plant materials using various kinds of chromatography techniques and the structures were determined using NMR. In addition, brusatol, bruceantin, dehydrobruceine A, dehydrobruceine B, dehydrobrusatol, bruceoside A, and yadanzioside G were isolated from the organic layer of the Chinese plant materials. This study showed that the water-soluble fraction of the Indonesian *B. javanica* contained bruceine D.

A rapid UPLC-MS/MS method for the quantitative analysis of quassinoids was developed. The peak area (AUC) in MRM chromatograms for each quassinoid can be used for estimation for the amounts of some quassinoids in crude methanol extracts from *B. javanica* in different origins. As expected, the amounts of quassinoids in the plant materials were different with different origins. The amount of bruceine C was much higher than those of other quassinoids in all samples. Bruceine A, which was the most effective against *T. evansi*, was contained at the most in the

Indonesian sample.

The present studies of fractionation with column chromatography and UPLC-MS/MS analysis suggest that the quantity and composition of quassinoids in the same plant species depend on geographic factors. Thus, the time of harvest of medicinal plants should be considered to obtain fairly amounts of objective compounds. Rapid and accurate quantification method developed in this study will be useful for the screening of the detection and quantification of quassinoids or other bioactive compounds from crude plant extracts. Further studies including the development of simple and low-priced method for isolation of active ingredients in a large quantity are required.

CHAPTER II

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

1. INTRODUCTION

Control of trypanosomiasis in livestock usually relies upon either curative or prophylactic treatment of the animals with trypanocidal drugs. Currently, the most commonly used trypanocidal drugs have been associated with side effects, and the development of drug resistant trypanosomes has occurred in many regions (Anene et al., 2001; Kibona et al., 2006; Matovu 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). Quassinoids are the bitter principles found in various species of the Simaroubaceae in the tropics (Sakaki et al., 1984; Yoshimura et al., 1984). Quassinoid compounds from *B. javanica* exhibited inhibitory activities on protozoan parasites such as *Plasmodium falciparum* (O'Neill et al., 1986 and 1987), *Entamoeba histolytica*, *Giardia intestinalis*, and *Toxoplasma gondii* (Wright et al., 1993). Furthermore, quassinoids also exhibited *in vitro* inhibitory activity against *Babesia gibsoni* (Elkhateeb et al., 2008; Subeki et al., 2007).

This study comprises the first report on antitrypanosomal activity of quassinoid compounds *in vitro* against *Trypanosoma evansi* and the

structure-activity relationship is discussed. Cytotoxic activity of quassinoids against MRC-5 cells (human lung diploid fibroblast cell line) was also examined and selectivity index (SI) was calculated.

2. MATERIALS AND METHODS

2.1 Antitrypanosomal test

2.1.1 Plant materials, extraction, isolation and identification

Plant materials, extraction, isolation and identification of quassinoids are described in Chapter I.

2.1.2 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 Chemicals, Japan), 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.

2.1.3 In vitro test for antitrypanosomal activity

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

2.2 Cytotoxicity assay

MRC-5 cells (human lung diploid fibroblast cell line, purchased from RIKEN Cell Bank, Japan) were seeded in each well of 96-well culture plates at a concentration of 2.5×10^4 cells/ml in 100 µl of MEM medium (SAFC Biosciences, USA) supplemented with 3% HEPES

(Sigma) and 10% heat inactivated FBS (fetal bovine serum, Gibco, USA). After 24 h incubation at 37°C in 5% CO_2 in air, the medium was aspirated and 100 µl of fresh culture medium containing two-fold serial dilutions of quassinoids were added to final concentrations of 0.2-100 µM. After 6 days incubation, 10 µl of Alamar Blue® (TREK Diagnostic Systems, USA) was added to the cultures 6 h before the end of incubation. Absorbance at 570 and 600 nm was measured using a plate reader (SpectraMax M5-H, Molecular Devices, USA). The Alamar Blue assay measures cell viability and proliferation based on detection of metabolic activity (Räz et al., 1997). The Alamar Blue colorimeteric/fluorometric growth indicator incorporates an oxidation-reduction indicator that changes colour in response to chemical reduction by metabolically active cells. Growth related reduction causes the indicator to change from oxidized (blue, non-fluorescent) to reduced (red, fluorescent). IC_{50} values were calculated as described above. The selectivity index (SI) was determined by dividing the IC_{50} value for MRC-5 cells by the IC_{50} value for trypanosomes.

3. RESULTS

3.1 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. II-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 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. II-1). Bruceantinol B was 7700 times less active than bruceantinol.

3.2 Cytotoxic activities of quassinoids against MRC-5 and SI values

IC_{50} values of bruceine A, B, C, and D against MRC-5 cells and SI values are shown in Table II-1. Among quassinoids examined, bruceine D showed the highest SI value of 4186. Bruceine A and C showed a similar SI value (2966 and 2604, respectively), and bruceine B had the lowest SI value of 1896.

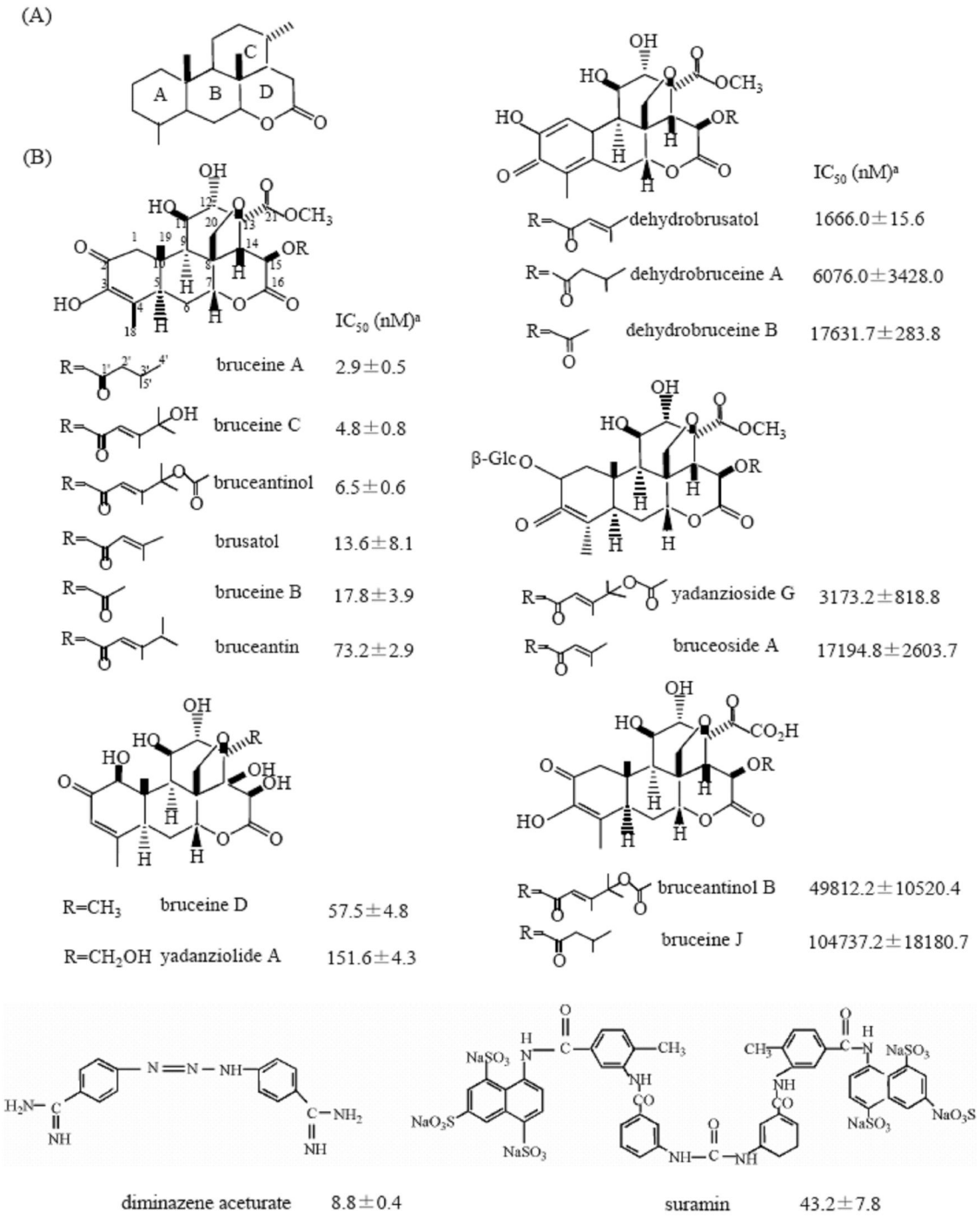


Fig. II-1. Chemical structures and *in vitro* antitrypanosomal activities of quassinoids isolated from *B. javanica* fruits. (A) Skeleton of tetracyclic, C-20 quassinoid, indicating rings A to D. (B) Chemical structures and IC_{50} values of quassinoids, diminazene aceturate, and suramin against *T. evansi.*

[a]The IC_{50} value is the mean ± standard deviation of two to three independent experiments.

Table II-1. Cytotoxic activity of quassinoids against MRC-5 and selectivity index

Compounds	IC_{50} (μM)[a]	SI
Quassinoids		
Bruceine A	8.6 ± 1.4	2966
Bruceine B	32.8 ± 17.3	1896
Bruceine C	12.5 ± 3.2	2604
Bruceine D	240.7 ± 33.5	4186
Standard drug		
Diminazene aceturate	3799.4 ± 397.0	400000

[a]The IC_{50} value is the mean ± standard deviation of three independent experiments.

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. II-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 to 2000 times higher than the related compounds of bruceine A, bruceine B, and brusatol.

A comparison of the *in vitro* anti-protozoal activities of quassinoid compounds against different protozoan species is shown in Table II-2. Quassinoids with a diosphenol moiety in ring A appeared to show greater

Table II-2. *In vitro* antiprotozoal activity of quassinoids isolated from *B. javanica*

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

Data from Wright et al., [a]1988, [b]1993 [e]Data from Subeki et al., 2007

Data from O'Neill et al., [c]1987, [d]1986 [f]Data from Elkhateeb et al., 2008

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 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 trypanosomes 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 derivatives, quinines, 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 and 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. b. rhodesiense* blood stream forms with a very high SI (SI > 10^6), but it was found to be very nephrotoxic in goats.

In the present study, quassinoid compounds, a group of degraded triterpenes, from a medicinal plant, *B. javanica*, were examined for their *in vitro* antitrypanosomal activities. Among quassinoids examined, bruceine A had the highest activity against *T. evansi* bloodstream trypanosomes with IC_{50} of 2.9 nM and the high SI of 2966 on MRC-5 cells. However, the SI was 64.7 on KB cells (human nasopharynx carcinoma cells) calculated from a result by Anderson et al. (1991). Similarly, the SI values of bruceine B, C, and D were 1896, 2604, and 4186 on MRC-5 cells, respectively, in this study and were 64.4, 7.8, and 49.0 on KB cells, respectively (Anderson et al., 1991). These results suggested that cytotoxicity of quassinoid compounds varies with cell types used.

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., 2009). 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 (Gutteridge, 1985; Homeida et al., 1981; Peregrine and Mamman, 1993). Thus, further work should be carried out to evaluate potent trypanocidal drugs for the treatment of different animal species.

In order to reduce adverse side-effects and improve their activities and specificities, structural modifications should be considered to produce more specific compounds. Alternately, different drug delivery systems such as using liposomal formulations (Date et al., 2007; Lian and Ho, 2001) can be explored. Despite the mechanism is not clear, several studies have suggested that positively charged liposomes (stearylamine-bearing liposomes) could attach on the negatively charged external surface of *Trypanosoma* organisms. Stearylamine molecules are translocated from the liposomal membrane to the plasma membrane of cells by the membrane fusion, eventually causing cell lysis (Tachibana et al., 1988; Yongsheng et al., 1996). Meanwhile, drugs encapsulated in the liposomes may also be transferred to the parasites.

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 and activities against other trypanosome species, are necessary. Evaluation of their *in vivo* effects in animal models is also required. 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.

5. SUMMARY

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*. In addition, bruceine A, B, C, and D had relative low cytotoxicity with selectivity index values in the range of 1900-4200 against human lung diploid fibroblast MRC-5 cells.

CHAPTER III

Evaluation of Myanmar medicinal plants for their *in vitro* antitrypanosomal and cytotoxic activities

1. INTRODUCTION

Myanmar (formerly known as Burma) is located in Southeast Asia with supreme natural environment and abundant plant resources. Peoples in Myanmar have inherited their own traditional medicine to maintain their health and treat various alignments such as malaria, diarrhea and fever for over millennia of history. However, Myanmar is non-endemic to human trypanosomiasis and leishmaniasis, and medicinal plants had not been paid attention to these neglected tropical diseases except two reports on antileishmanial activities (Mori et al., 2008; Takahashi et al., 2004). In Myanmar, agriculture and animal production (milk and meat) are the major industrial sectors. Thus, control of zoonoses and livestock diseases are also very important for the development of national economy.

The findings of antibabesial and antitrypanosomal activities of quassinoids isolated from the fruits of a medicinal plant, *Brucea javanica*, (Bawm et al., 2008; Nakao et al., 2009; Subeki et al., 2007), suggesting a promise use of medicinal plant extracts for protozoan diseases in livestock as well. Although few surveys for trypanosomiasis in livestock were performed in Myanmar, surra has been reported in horses, cattle, buffalo, pigs, dogs, cats, deer, hog deer and even elephants with varying clinical manifestations in Thailand, the neighboring country to Myanmar (Tuntasuvan and Luckins, 1998).

This study was aimed to evaluate *in vitro* antitrypanosomal and cytotoxic activities of crude extracts from Myanmar medicinal plants.

2. MATERIALS AND METHODS

2.1 Plant materials

A total of 55 fresh plant specimens from 45 plant species were collected at National Herbal Park in Naypyitaw and National Botanical Garden in Pyin-oo-lwin, Myanmar in January 2009. Species identification was done by Mr. Hla Myint and Dr. Kyaw Kyaw Swe, respectively. These plants were fixed in 70% ethanol immediately after collection and deposited in Laboratory of Bioorganic Chemistry, Graduate School of Agriculture, Hokkaido University. Seven dry plant specimens from 7 plant species were prepared at Pathein University after identification of the plant species. Nine dry plant specimens from 9 plant species were obtained at Thein-gyi market in Yangon. Only *Andrographis paniculata* was overlapped in both fresh (stem and twigs with leaves) and dry (leaf and stem) samples.

2.2 Preparation of crude plant extracts

Each fresh plant specimen (15-30 g) was extracted with 40 ml of 70% ethanol for two weeks. Dry plant specimens (10-20 g), except the powder of *Brucea javanica* fruits, were also cut into small pieces and extracted with 40 ml of 70% methanol for 7 days at room temperature. The choice for use of ethanol for fresh sample extraction is due to the availability and low toxicity at the time of collecting plant samples. We used methanol for dry sample extraction due to higher scores in methanol

than ethanol for the screening of antimicrobial components from plants (Eloff, 1998). The extracts were passed through a filter paper (Advantec Toyo Kaisha, Ltd., Japan) and concentrated in a rotary evaporator at 37°C, yielding dried crude extracts in the range of 0.7-28.0% weight of the starting materials. The extracts were dissolved in DMSO and diluted with HMI-9 medium with 0.5% DMSO before use.

2.3 In vitro test for antitrypanosomal activity

Bloodstream trypomastigote forms of *Trypanosoma evansi* (H3 strain, isolated from deer in Thailand) were maintained in HMI-9 medium as described in Chapter II. Antitrypanosomal activity tests were performed in a 96-well microtiter plate using the plant crude extracts described above and a standard trypanocidal drug, diminazene aceturate. Two-fold serial dilutions of these extracts were prepared in HMI-9 medium with 0.5% DMSO. Each well contained plant crude extracts (1.9-1000 μg/ml) and 5×10^4 parasites/ml in 100 μl of HMI-9 medium. Plates were incubated at 37° C in 5% CO_2 in air for 72 h. Six hours before the end of incubation, 10μl of Alamar Blue® (TREK Diagnostic Systems) were added to cultures and absorbance at 570 and 600 nm were measured with a plate reader (SpectraMax M5-H, Molecular Devices). The 50% inhibitory concentration (IC_{50}) value was calculated as described in Chapter II. All tests were performed twice or three times, with each plant extract concentration in triplicate.

2.4 Cytotoxicity assay

MRC-5 cells were seeded in each well of 96-well culture plates at a concentration of 2.5×10^4 cells/ml in 100 µl of MEM medium as described in Chapter II. After 24 h incubation at 37° C in 5% CO_2 in air, the medium was aspirated and 100 µl fresh culture medium containing two-fold serial dilutions of plant crude extracts were added to the final concentrations of 3.9-1000 µg/ml. After 6 days incubation, 10 µl Alamar Blue® were added to each well for 6 h, followed by colorimetric readings and IC_{50} values were calculated as described in Chapter II. The selectivity index (SI) was determined by dividing the IC_{50} value for MRC-5 cells by the IC_{50} value for trypanosomes.

3. RESULTS

The local name and traditional uses of 57 medicinal plants in Myanmar are presented in Table III-1. In general, when IC_{50} values of plant extracts against *T. evansi* were <100 µg/ml, the cytotoxicity tests against MRC-5 cells were performed. Some other extracts showing IC_{50} values of 100-200 µg/ml were also examined for their cytotoxic activities. Antitrypanosomal activities of all 71 plant extracts from various plant species collected in Myanmar and cytotoxic activities of 13 plant extracts with selectivity indices (SI) are shown in Table III-2.

According to the method by Osorio et al. (2007), antitrypanosomal activities of medicinal plant extracts were classified into four categories,

highly active ($IC_{50} \leq 10$ µg/ml), active ($10 < IC_{50} \leq 50$ µg/ml), moderately active ($50 < IC_{50} \leq 100$ µg/ml) and non-active ($IC_{50} > 100$ µg/ml). When plant extracts showed SI ≤ 10, these samples are considered to have good selectivity and will be considered for further bio-guided fractionation. Of 71 plant extracts, only one dry plant extract from rootbark of *Vitis repens* showed highly active (IC_{50} = 8.6 µg/ml and SI = 24.4) against *T. evansi*. Two dry plant samples from fruits of *Brucea javanica* (IC_{50} = 27.2 µg/ml and SI = 11.4) and leaves/stem of *Vitex arborea* (IC_{50} = 48.6 µg/ml and SI = 15.1) were active. Three fresh plant samples from leaves of *Eucalyptus globulus* (IC_{50} = 51.1 µg/ml and SI = 12.2), fruits of *Jatropha podagrica* (IC_{50} = 52.3 µg/ml and SI = 12.5) and leaves of *Rhoeo discolor* (IC_{50} = 75.8 µg/ml and SI = 5.6) were moderately active. Three dry plant samples from leaves/stems of *Andrographis paniculata* (IC_{50} = 54.7 µg/ml and SI = 1.0), rhizomes of *Combretum acuminatum* (IC_{50} = 90.7 µg/ml and SI = 9.4) and leaves/stems of *Phyllanthus simplex* (IC_{50} = 96.1 µg/ml and SI = 1.0) were moderately active against *T. evansi*.

Table III-1. List of medicinal plants investigated and traditional uses in Myanmar

Scientific name (family name)	Myanmar Name	Part used	Tradional Uses
Adhatoda vasica Nees. (Acanthaceae)	Mu-ya-gyi	Leaf	Pulmonary diseases, dry cough
Ageratum conyzoides Linn. (Compositae)	Khwe-thay-pann	Leaf	Antibacterial
Alpinia officinarum Hance. (Zingiberaceae)	Pade-kaw-lay	Rhizome	Anti-diuretics, aches, rheumatism
Alstonia scholaris R. Br. (Apocyanaceae)	Taung-mu-yo	Leaf	Antiseptic, astringent, amoebic dysentery
Andrographis paniculata Nees. (Acanthaceae)	Say-kha-gyi	All parts	Malaria, antipyretic, tonic, diabetes
Artemesia annua Linn. (Compositae)	Daw-na	Leaf	Malaria
Artemesia vulgaris Linn. (Compositae)	Daw-na	Leaf	Malaria
Asparagus racemosus Willd. (Liliaceae)	Ka-nyut	Root	Diarrhoea, fever, blood tonic
Azadirachta indica (Meliaceae)	Ta-mar (neem)	Leaf	Diabetes, malaria, skin diseases
Barleria prionitis Linn. (Acanthaceae)	Leik-suu-shwe	Leaf	Diuretic, oedema, pile
Blumea balsamifera DC. (Compositae)	Phon-ma-thein	Leaf	Gastric disease, indigestion, arthritis
Brucea javanica (L.) Merr. (Simaroubaceae)	Ya-dan-si	Fruits	Dysentery, tumor, malaria
Cinnamomum tamala F. Nees. (Lauraceae)	Thit-kyan-bo	Bark	Cholecystitis, laryngitis, dysentery, antiseptic
Combretum acuminatum (Combretaceae)	Na-nwin-kha	Rhizome	Malaria, dysentery
Crataeva religiosa Forst. (Capparidaceae)	Khan-thet	Leaf	To promote digestion, tumour, as tonic agent, paresis and paralysis, as antiaging agent

Table III-1 continue-

Crinum latifolium Linn. (Amaryllidaceae)	See-pwa-gamon	Leaf	Dysentry, diarrhoea
Crinum pratense Herb. (Amaryllidaceae)	Pa-daing	Leaf	Gonorrhoea, arthritis, dilatation of pupil
Curcuma comosa (Zingiberaceae)	Na-nwin	Rhizome	Malaria, dysentery
Eichomia crassipes (Pontederiaceae)	Bay-dar	All parts	Malaria, fever
Elettaria cardamomum Maton. (Zingiberaceae)	Chin-paung-phalar	Fruits	Dysentery, malaria, diarrhoea
Eucalyptus globulus Labill. (Myrtaceae)	Yu-ka-lip	Bark, root	Malaria
Euonymus kachinensis (Celastraceae)	Ma-shaw	Leaf	Relief poisons, sore, antimicrobial
Eupatorium odoratum Linn. (Compositae)	Bi-sat	Leaf	Antiseptic, anti tumor
Euphorbia hirta Linn. (Euphorbiaceae)	Kywe-kyaung-min-se	All parts	Antimicrobial
Euphorbia longana (Euphorbiaceae)	Taw-kyet-maut	Leaf, flower	Diarrhoea
Gentiana kurroo Royle. (Gentianaceae)	Say-pu-le	Root	To promote digestion, menstrual disorders
Holarrhena antidysenterica Wall. (Apocynaceae)	Lettok-gyi	Leaf, bark	Amoebic dysentery, diarrhoea, astringent
Hydrocotyle asiatica Linn. (Umbelliferae)	Myin-khwar	All parts	Longevity, leprosy
Ichnocarpus frutescens R.Br. (Apocyanaceae)	Twin-net-ka-doe	Leaf	Tonic, effective on the heart, gallbladder
Jatropha podagrica HK. (Euphorbiaceae)	Ta-bin-shwe-hti	Leaf, stem	Pulmonary and gastrointestinal diseases, malena
Kalanchoe laciniata DC. (Crassulaceae)	Mee-malaung-ban	Leaf	Dysentery, diarrhoea
Mansonia gagei Drummond. (Sterculiaceae)	Ka-ra-met	Leaf, bark	Fever, dysentery
Morinda angustifolia Roxb. (Rubiaceae)	Ye-yo	Leaf, fruit	Fever, tonic, dysentery, diarrhoea
Ocimum sanctum Linn. (Labiatae)	Pin-sane	Leaf, stem	Deodorant, headache, cough
Origanum majorana Linn. (Labiatae)	Taw-yon	Leaf	Antimicrobial, anti inflammation, diuretic
Orthosiphon aristatus (Blume) Miq. (Labiatae)	Thagya-ma-gaik	Leaf	Diabetes, arthritis, diuretics
Orthosiphon stamineus (Labiatae)	-	Leaf	Diabetes, arthritis, diuretics

Table III-1 continue-

Phyllanthus niruri Linn. (Euphorbiaceae)	Kyet-tha-hin	All parts	Fever, dysentery
Phyllanthus simplex Retz. (Euphorbiaceae)	Taung-zee-phyu	Fruits	Fever, dysentery
Physalis peruviana Linn. (Solanaceae)	Baut	Fruits	Fever
Piper attenuatum Buch. Ham. (Piperaceae)	Sa-yo	Leaf, fruit	Malaria
Piper nigrum Linn. (Piperaceae)	Nga-yoke-kaung	Leaf, fruit	Malaria
Plumbago rosea Linn. (Plumbaginaceae)	Kant-choke-ni	Leaf	Dysmenorrhea, amenorrhea, skin disorder
Plumbago zeylanica Linn. (Plumbaginaceae)	Kant-choke-phyu	Leaf	Leucoderma, scabies, anthelmintic
Rhoeo discolor (L.Her) Hance. (Commelinaceae)	Mee-kwin-gamon	Leaf	Dysentry, diarrhoea
Sansevieria cylindrica (Liliaceae)	Sin-swe-gamon	Leaf	Dysentry, diarrhoea
Sansevieria zeylanica Wild. (Liliaceae)	Naga-set	Leaf	Anti venom
Saxifraga virginiensis (Saxifragaceae)	Kyaut-kwe	Leaf, stem	Diuretic, oedema
Scoparia dulcis Linn. (Scrophulariaceae)	Danta-thu-kha	All parts	Toothache, haemoptysis, anti-emetric
Tacca pinnatifida Forst. (Taccaceae)	Pin-pwar	Root	Fever
Talinum patens L. Wild. (Portulacaceae)	Kaw-li-thein	Leaf	Fever, dysentery
Vernonia cenerea Less. (Compositae)	Pyar-mee-swe	All parts	Fever
Vitex arborea Desf. (Verbenaceae)	Kyet-lel-san	All parts	Fever
Vitis repens Wight & Arn. (Vitaceae)	Tapin-taing- mya nan	Root, bark	Sore, carbuncles, ulcers, hepatitis and jaundice tumors and hypertension
Withania somnifera Dunal. (Solanaceae)	Da-har-tha-kaing	Bark	Inflammation, diuretic, aphradisiac, tonic
Zingiber officinale Rosc. (Zingiberaceae)	Gyin	Rhizome, leaf	Asthma, hiccough, cholera, earache
Zizyphus rugosa Lank. (Rhamnaceae)	Zee-thee	Fruits	Diarrhoea, tachycardia, skin infection

Table III- 2. *In vitro* antitrypanosomal and cytotoxic activities of crude plant extracts

Scientific name	Parts	IC_{50} (µg/ml)[a]		SI
		Antitrypanosomal	Cytotoxicity	
		T. evansi	MRC-5	
Fresh Sample				
Adhatoda vasica	L	458.1 ± 189.1	nd	
Ageratum conyzoides	L	440.6 ± 75.3	nd	
Alpinia officinarum	R	242.2 ± 20.5	nd	
Alstonia scholaris	L	391.7 ± 5.8	nd	
Andrographis paniculata	STL	467.4 ± 8.8	nd	
Artemesia annua	L	316.2 ± 99.4	nd	
Artemesia annua	Fl	326.9 ± 47.3	nd	
Artemesia vulgaris	LS	196.9 ± 112.5	nd	
Asparagus racemosus	R	325.9 ± 125.6	nd	
Azadirachta indica	L	167.8 ± 12.5	nd	
Barleria prionitis	L	>1000	nd	
Barleria prionitis	L	252.4 ± 23.9	nd	
Blumea balsamifera	L	429.5 ± 19.4	nd	
Crinum latifolium	L	443.1 ± 103.9	nd	
Crinum pratense	L	251.8 ± 72.1	nd	
Crinum sp.	L	400.1 ± 51.6	nd	
Curcuma comosa	Rh	236.9 ± 6.6	nd	
Elettaria cardamomum	L	157.9 ± 15.8	nd	
Eucalyptus globulus	L	51.1 ± 1.5	622.95 ± 299.7	12.2
Euonymus kachinensis	L	232.2 ± 62.2	nd	
Eupatorium odoratum	L	414.7 ± 89.8	nd	
Euphorbia hirta	L	118.7 ± 7.1	nd	
Euphorbia longana	LFl	280.3 ± 69.2	nd	
Holarrhena antidysenterica	L	180.6 ± 54.8	nd	
Hydrocotyle asiatica	L	590.9 ± 25.9	nd	

Table III-2 continue-

Ichnocarpus frutescens	L	205.5 ± 86.1	nd	
Jatropha podagrica	L	736.5 ± 186.1	nd	
Jatropha podagrica	F	52.3 ± 13.5	652.7 ± 202.9	12.5
Kalanchoe laciniata	LT	396.3 ± 46.5	nd	
Mansonia gagei	L	789.6 ± 80.8	nd	
Morinda angustifolia	L	330.3 ± 11.0	nd	
Ocimum sanctum	LFl	578.0 ± 251.3	nd	
Origanum majorana	L	287.6 ± 73.0	nd	
Orthosiphon aristatus	L	495.0 ± 51.1	nd	
Orthosiphon stamineus	L	144.7 ± 36.4	628.9 ± 66.8	4.3
Physalis peruviana	F	625.1 ± 86.4	nd	
Piper attenuatum	L	282.8 ± 141.9	nd	
Piper attenuatum	F	469.6 ± 4.1	nd	
Piper nigrum	L	170.8 ± 49.4	nd	
Plumbago rosea	L	554.5 ± 144.7	nd	
Plumbago rosea	Fl	156.7 ± 68.8	557.05 ± 269.4	3.6
Plumbago zeylanica	L	268.8 ± 0.9	nd	
Rhoeo discolor	L	75.8 ± 16.0	424.9 ± 160.0	5.6
Rhoeo discolor	Fl	135.5 ± 34.0	>1000	
Rhoeo sp.	L	808.1 ± 16.1	nd	
Rhoeo sp.	Fl	490.3 ± 77.2	nd	
Sansevieria cylindrica	LT	208.6 ± 29.5	nd	
Sansevieria zeylanica	LT	255.6 ± 56.9	nd	
Sansevieria zeylanica	L	400.8 ± 116.4	nd	
Saxifraga virginiensis	LS	255.6 ± 27.5	nd	
Talinum patens	L	244.6 ± 128.2	nd	
Zingiber officinale	L	305.2 ± 141.8	nd	
Zingiber officinale	R	358.6 ± 208.6	nd	
Zizyphus rugosa	F	373.8 ± 145.7	nd	
Zizyphus rugosa	S	257.6 ± 56.8	nd	

Table III-2 continue-

Dry Sample				
From Pathein				
Crataeva religiosa	LS	107.1 ± 11.6	691 ± 489.3	6.4
Eichomia crassipes	LS	157.8 ± 47.6	nd	
Oldenlandia diffusa	LS	504.8 ± 21.7	nd	
Phyllanthus niruri	LS	336.9 ± 53.5	nd	
Phyllanthus simplex	LS	96.1 ± 34.6	98.8 ± 26.0	1.0
Vernonia cenerea	LS	789.8 ± 2.6	nd	
Vitex arborea	LS	48.6 ± 10.9	735.15 ± 374.5	15.1
From Yangon				
Andrographis sp.	LS	616.2 ± 0.1	nd	
Andrographis paniculata	LS	54.7 ± 33.1	55.1 ± 20.0	1.0
Brucea javanica	F	27.2 ± 7.9	309.15 ± 1.6	11.4
Cinnamomum tamala	B	445.9 ± 75.5	nd	
Combretum acuminatum	Rh	90.7 ± 11.6	853.15 ± 39.3	9.4
Gentiana kurroo	R	155.3 ± 80.1	nd	
Tacca pinnatifida	R	208.4 ± 153.5	nd	
Vitis repens	RB	8.6 ± 1.5	209.9 ± 125.5	24.4
Withania somnifera	SB	353.2 ± 174.8	nd	
Reference drug				
Diminazene aceturate		0.01140 ± 0.001	>1000	

Plant part: L, leaf; R, root; STL, stem and twigs with leaves; F, fruit; Fl, flower; LFl, leaf and flower; LS, leaf and stem; LT, leaf and twig; Rh, rhizomes; RB, rootbark; SB, stembark

[a]The IC_{50} value is the mean ± standard deviation of three independent experiments.

(a) (b)

(c) (d)

Fig. III-1. *Vitis repens* Roots (a), *Vitex arborea* plant (b), *Eucalyptus globulus* leaves (c) and *Jatropha podagrica* fruits (d).

4. DISCUSSION

In the present study, we have tested 71 crude extracts from 60 ethnobotanically selected medicinal plant species from Myanmar. They are traditionally used for treatment of various alignments including malaria, diarrhea and fever in Myanmar (Department of Traditional Medicine, 2006; Forest Department, 2000; Myanmar medicinal plant database; Soe and Ngwe, 2004). As far as we know, these plant species except *Brucea javanica, Andrographis paniculata*, and *Plumbago zeylanica* were examined for their antiprotozoal activities for the first time.

Vitis repens Wight & Arn. (Vitaceae) (Fig. III-1a) is a tendrillar climber and widely distributed in Shan state, eastern hills of Myanmar, where it is used for the treatment of sore, carbuncles, ulcers, hepatitis/jaundice, peptic ulcer, tumors/hypertension, and so on. The present study showed that an extract from dry rootbark of this plant species exhibited high activity against *T. evansi* (IC_{50} = 8.6) with high SI of 24.4. The genus *Vitis* commonly contains various oligomers of resveratrol, such as vitisinols A-D, (+) -σ- viniferin, (-)-viniferal, ampelopsin C, miyabenol A, (+)-vitisin A, and (+)-vitisin C (Li et al., 1996; 1998). Some resveratrol derivatives showed potent antiplatelet and antioxidative activities (Huang et al., 2005). Neither antiprotozoal nor antimicrobial activity of *V. repens* was found in the literature.

As described in Chapter I, *Brucea javanica* (L.) Merr., a plant species of the family Simaroubaceae, is distributed widely throughout

Asian countries, where the fruits have been used for various ailments including cancer, amoebic dysentery, and malaria. This plant contains a variety of quassinoid compounds, the bitter principles, which exhibit *in vitro* inhibitory activities on protozoan parasites such as *Plasmodium falciparum*, *Entamoeba histolytica*, *Giardia intestinalis, Toxoplasma gondii*, *Babesia gibsoni* and *T. evansi*. In this study, crude extract from fruits of *B. javanica* showed the second strong activity against *T. evansi* (IC_{50} = 27.2 μg/ml and SI = 11.4), confirming our previous studies. However, another study showed that crude extracts from *B. javanica* in Thailand had almost no antitrypanosomal activity against *T. b. brucei* (500 μg/ml) with high cytotoxic effect against KB cells (SI = 0.018) (Camacho et al, 2003). This might be due to differences in the study protocols.

Vitex arborea Desf. (Verbenaceae) (Fig. III-1b) is a small to medium-sized evergreen tree distributed in Asia. In Myanmar, this plant is used for the treatment of fever and diarrhea. *V. doniana* has pharmacological activity against diarrhoeal in mice (Agunu et al., 2005).

Fresh plant extracts from leaves of *Eucalyptus globulus* (Fig. III-1c) and fruits of *Jatropha podagrica* (Fig. III-1d) showed moderate activity with high selectivity. Many species of the genus *Eucalyptus* in the Myrtaceae family have been used for treatment of malaria and respiratory diseases. Plants in the genus *Eucalyptus* contain monoterpenoid, citronellal, and eucalyptol (1, 8-cineole) (Juergens et al., 2004). Essential oil of *E. globulu*s showed antimicrobial activities against *Haemophilus influenzae,*

H. parainfluenzae, Stenotrophomonas maltophilia, and *Streptococcus pneumoniae* (Cermelli et al., 2008). However, no antitrypanosomal activities of *Eucalyptus* species have been reported before this study.

Jatropha podagrica (Euphorbiaceae) is a shrub commonly found in Africa, Asia, and Latin America. Different parts of this plant is used for various diseases such as stomatitis, glossitis, menstrual disorder, disorder after delivery, injury, haemorrhage, antipyretic, diuretic, and purgative in Myanmar (Department of traditional medicine, 2006) and Nigeria (Aiyelaagbe et al., 2007). *Jatropha* species including *J. podagrica* are known to have various biological activities, such as antibacterial (Aiyelaagbe et al., 2007; Marquez et al., 2005) and molluscicidal activity (dos Santos and Sant'Ana, 1999). The major constituents of *Jatropha* species are diterpenoids such as japodagrin and japodagrone, which have shown antibacterial activity against some gram-positive bacteria (Aiyelaagbe et al., 2007). Despite jatrogrossidione, diterpene from *J. grossidentata* showed a strong *in vitro* leishmanicidal (IC_{100} = 0.75 µg/ml) and trypanocidal activity (IC_{100} = 1.5-5.0 µg/ml) (Schmeda-Hirschmann et al., 1996), no reports were available regarding antitprotozoal activity of *J. podagrica*.

Four other plant species exhibited moderate activities with low SI. These include extracts of fresh leaves of *Rhoeo discolor* (IC_{50} = 75.8 µg/ml and SI = 5.6), dry leaves/stems of *Andrographis paniculata* (IC_{50} = 54.7 µg/ml and SI = 1.0), dry rhizomes of *Combretum acuminatum* (IC_{50} = 90.7 µg/ml and SI = 9.4), and dry leaves/stems of *Phyllanthus simplex* (IC_{50} =

96.1 μg/ml and SI = 1.0). *Rhoeo discolor* (L. Her) Hance. (Commelinaceae) is used for dysentery in Myanmar. Major constituents of this plant are flavonoids, anthocyanins, saponins, carotenoids, terpenoids, and steroidal compounds (Idaka et al, 1987). Rosales-Reyes et al. (2008) reported the antitumoral activity of this plant. *Andrographis paniculata* is annual shrub which grows abundantly in Asia and is widely used for treatment of malaria and cancer. The aerial part of this plant has a very bitter taste. The ethnopharmacological properties of *A. paniculata* are well documented. Several biological activities such as anti-malarial properties (Mishra et al., 2009; Siti Najilaa et al, 2002), anticancer (Kumar et al., 2004), microfilaricidal (Merawin et al., 2010), and antimicrobial (Singha et al., 2003) activities have been reported. Xanthones, constituent of this plant showed anti-malarial, antitrypanosomal, and antileishmanial activities (Dua et al., 2004; 2009). Rhizomes of *Combretum* sp. is used for the treatment of diarrhea and malaria in Myanmar, and used for the treatment of diarrhea, sterility, and pyomyositis in Uganda (Odda et al., 2008). Furthermore, plant extract of *Combretum* sp. showed antiviral (Maregesi et al., 2008) and larvicidal activities (Odda et al., 2008). *Phyllanthus simplex* Retz. (Euphorbiaceae) is a fibrous perennial herb widely distributed in tropical and sub-tropical region. It has been used for the treatment of dysentery and fever in Myanmar, and for the treatment of gonorrhea, jaundice, hyperglycemia, diabetes, and liver disease in India (Shabeer et al., 2009) and Cuba (del Barrio and Parra, 2000). Plants from *Phyllanthus* spp.

contain lignans, alkaloids, flavonoids, phenols, and terpenes. Bioactivities such as anti-babesial and anti-plasmodial (Subeki et al., 2005), and antiviral (del Barrio and Parra, 2000; Fernández Romero et al., 2003; Yang et al., 2007) activities have been reported.

In conclusion, the present study revealed that some medicinal plants (eg. *Vitis repens*) in Myanmar may offer a potential use for treatment of *T. evansi* infection. The most promising plant extracts should be prioritized for phytochemical and *in vivo* studies as well as the determination and purification of active compounds.

5. SUMMARY

Peoples in Myanmar have inherited their own traditional medicine and practiced it for over millennia of history. In the present study, *in vitro* antitrypanosomal and cytotoxic activities of Myanmar medicinal plants were examined. A total of 55 fresh and 16 dry medicinal plant specimens from 60 plant species were extracted with 70% ethanol and 70% methanol, respectively. The *in vitro* antitrypanosomal activity against trypomastigotes of *Trypanosoma evansi* and cytotoxic activity against MRC-5 (human lung diploid fibroblast) cells were determined for these extracts using Alamar Blue® colorimetric assay. The 50% inhibitory concentration (IC_{50}) on parasite and cell growth for each extract was determined, and the selectivity index (SI) was calculated. Three of 55 fresh samples and 6 of 16 dry samples showed IC_{50} values with <100 µg/ml against *T. evansi*. *Eucalyptus globulus* and *Jatropha podagrica* showed higher antitrypanosomal activities (IC_{50} = 51.1 and 52.3 µg/ml, respectively) with higher selectivity indexes (SI = 12.2 and 12.5, respectively) than other fresh samples. Among dry samples, *Vitis repens* showed the highest antitrypanosomal activity (IC_{50} = 8.6 µg/ml, SI = 24.4). In conclusion, some medicinal plants used in Myanmar for the treatment of various ailments such as malaria, dysentery, tumor, and pulmonary diseases may offer a potential use for the treatment of *T. evansi* infection.

CONCLUSION

Trypanosoma evansi is an animal-pathogenic flagellated protozoan parasite. The parasite 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 the parasites are mechanically transmitted by biting arthropods, especially horse flies (*Tabanus* spp.) and stable flies (*Stomoxys* spp.), from one infected host to another. The disease causes great economical losses in areas of Africa, Asia, and South America, where thousands of animals die from *T. evansi* infections. Currently, the most commonly used drugs for the treatment of *T. evansi* infection are diminazene aceturate, suramin, and quinapyramine. However, existing trypanocidal drugs have been associated with side effects, and the development of drug resistant trypanosomes has occurred in many regions. 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.

Natural compounds in plants offer a valuable source for novel lead drug discovery. A medicinal plant, *Brucea javanica* contains quassinoids, which are the bitter principles found in various species of the Simaroubaceae in the tropics. In Chapter I, the content of quassinoids in *B. javanica* was analyzed by fractionation with column chromatography, ultra performance liquid chromatography (UPLC), electrospray ionization triple

quadrupole mass spectrometry (MS/MS) and nuclear magnetic resonance. In the previous studies, C-20 types of quassinoids such as bruceine A, bruceine B, bruceine C, bruceine D, bruceantinol, bruceantinol B, bruceine J, and yadanziolide A were isolated and purified from the organic layer of the Indonesian plant materials. In addition, brusatol, bruceantin, dehydrobruceine A, dehydrobruceine B, dehydrobrusatol, bruceoside A, and yadanzioside G were isolated from the organic layer of the Chinese plant materials. In this study, the dried fruits of Indonesian *B. javanica* were extracted with 70% aqueous methanol and partitioned using ethyl acetate to yield aqueous and organic layers. From the water-soluble fraction, bruceine D was obtained. A rapid UPLC-MS/MS method for the quantitative analysis of quassinoids was developed. The peak area in multiple reaction monitoring chromatograms for each quassinoid can be used for estimation for the amounts of some quassinoids in crude methanol extracts from *B. javanica* in different origins. As expected, the amounts of quassinoids in the plant materials were different with different countries and places, suggesting that the quantity and composition of quassinoids in the same plant species depend on geographic factors. Thus, the time of harvest of medicinal plants should be considered to obtain fairly amounts of objective compounds. Rapid and accurate quantification method developed in this study will be useful for the screening of the detection and quantification of quassinoids or other bioactive compounds from crude plant extracts. Further studies including the development of simple and

low-priced method for isolation of active ingredients in a large quantity are required.

Quassinoid compounds are known to exhibit inhibitory activities on protozoan parasites such as *Plasmodium falciparum*, *Entamoeba histolytica*, *Giardia intestinalis*, *Toxoplasma gondii* and *Babesia gibsoni*. In Chapter II, the antitrypanosomal activity of quassinoid compounds against *T. evansi in vitro* was evaluated and the structure-activity relationship was discussed. Cytotoxic activity of quassinoids against human lung diploid fibroblast (MRC-5) cells was also examined. Among fifteen C-20 quassinoids, bruceine A, bruceantinol, bruceine C, brusatol, and bruceine B showed strong *in vitro* 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 with IC_{50} of 8.8 nM and suramin with IC_{50} of 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*. Bruceine A, B, C, and D had relative low cytotoxicity with selectivity index (SI) values in the range of 1900-4200 against MRC-5 cells.

The findings of antibabesial and antitrypanosomal activities

quassinoids isolated from the fruits of a medicinal plant, *Brucea javanica*, suggest a promise use of medicinal plant extracts for protozoan diseases in livestock as well. Myanmar has abundant plant resources and Myanmar peoples have inherited their own traditional medicine for over millennia of history. In Chapter III, it was attempted to discover antitrypanosomal medicinal plants from Myanmar. A total of 55 fresh and 16 dry medicinal plant specimens from 60 plant species were extracted with 70% ethanol and 70% methanol, respectively. The *in vitro* antitrypanosomal activity against trypomastigotes of *T. evansi* and cytotoxic activity against MRC-5 cells were determined. Three of 55 fresh samples and 6 of 16 dry samples showed IC_{50} values with <100 µg/ml against *T. evansi*. *Eucalyptus globulus* and *Jatropha podagrica* showed higher antitrypanosomal activities with IC_{50} values of 51.1 and 52.3 µg/ml, respectively, and higher selectivity indexes of 12.2 and 12.5, respectively, than other fresh samples. Among dry samples, *Vitis repens* showed the highest antitrypanosomal activity with IC_{50} of 8.6 µg/ml and SI of 24.4. In conclusion, some medicinal plants used in Myanmar for the treatment of various ailments such as malaria, dysentery, tumor, and pulmonary diseases may offer a potential use for the treatment of *T. evansi* infection.

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（様式４）

博　士　論　文　内　容　の　要　旨

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博　士　論　文　題　目

Studies on Antitrypanosomal Activity of Medicinal Plants

（薬用植物の抗トリパノソーマ活性に関する研究）

*Trypanosoma evansi*はトリパノソーマ科の鞭毛虫であり、ウマ、ラクダ、ウシ、スイギュウ、ヤギ、ヒツジ、ブタなどの血液中で増殖し、スーラとして知られるトリパノソーマ症を引き起こす。本疾病は吸血昆虫、とくにアブ（*Tabanus* spp.）やサシバエ(*Stomoxys* spp.)によって機械的に伝播されるため世界的に蔓延しており、アフリカ、アジアおよび南アメリカにおいては大きな経済的損失をもたらしている。本症の治療に用いるおもな薬剤はdiminazene aceturate、suraminおよびquinapyramineであるが、これら既存の抗トリパノソーマ薬は副作用が大きな問題となっており、また、薬剤耐性株の出現が多くの地域で報告されている。このことから、ヒトのトリパノソーマ症である睡眠病やウシのナガナ病と同様に、スーラの治療に向けた新たな化合物の研究は、緊急かつ重要な課題である。

植物由来の天然化合物はこれまで新薬開発の重要な資源となってきた。薬用植物の１つである*Brucea javanica*は多種類のクアシノイドを含むが、これは多くの熱帯性ニガキ科植物において見いだされる苦味成分である。第一章では、カラムクロマトグラフィー、超高速液体クロマトグラフィー（UPLC）、質量分析（MS/MS）および磁気共鳴法を用いて、*B. javanica*に含まれるクアシノイドの種類と各含有量を解析した。筆者らのグループはこれまでの研究において、bruceine A、 bruceine B、 bruceine C、 bruceine D、 bruceantinol、 bruceantinol B、 bruceine Jおよびyadanziolide AなどのC-20型クアシノイドをインドネシア産材料の抽出有機層から分離・精製したこと、また、brusatol、bruceantin、dehydrobruceine A、dehydrobruceine B、dehydrobrusatol、bruceoside Aおよびyadanzioside Gを中国産材料の有機層から分離したことを報告してきた。本研究においては、インドネシア産の本植物乾燥果実を70%メタノールで処理したのち、酢酸エチルを用いて水層と有機層に分離したが、水層にbruceine Dが含まれていることを初めて見出した。次に、これらの分離・精製したクアシノイド類を標準化合物として、UPLC-MS/MSを用いたクアシノイド迅速定量法を開発し、*B. javanica*のメタノール粗抽出液中に含まれるクアシノイド類の含有量の評価を行った。その結果、各種クアシノイドの含有量は*B. javanica*の産地によって異なること明らかとなり、同種植物のクアシノイド類の組成と成分含有量は地理的要因、薬用植物の収穫時期などに依存している可能性が示唆された。本迅速定量法は、未精製の植物抽出液からのクアシノイド類やその他の生理活性物質の検出と定量的スクリーニングに有用であると考えられる。今後は、有効成分を大量

に分離するための簡便かつ安価な手法の開発研究が必要である。

*Brucea javanica*より分離されたクアシノイド類は、その種類に応じて*Plasmodium falciparum*、 *Entamoeba histolytica*、*Giardia intestinalis*、*Toxoplasma gondii*、*Babesia gibsoni*などの原虫類の増殖を阻害することが知られている。第二章においては、クアシノイド化合物の*T. evansi*の血流型虫体に対する*in vitro*における増殖阻害活性と化合物構造との相関について検討した。また、クアシノイド類のヒト肺線維芽細胞（MRC-5）に対する細胞毒性を測定した。その結果、15種類のC-20型クアシノイドのうち、bruceine A、bruceantinol、bruceine C、brusatolおよびbruceine Bは2.9 nMから17.8 nMの範囲の50%増殖阻止濃度（IC_{50}）を示し、強い抗トリパノソーマ活性を示すことが明らかになった。これらの活性は、diminazene aceturate (IC_{50} = 8.8 nM)やsuramin (IC_{50} = 43.2 nM)に匹敵するものであった。一方、dehydrobruceine A、dehydrobruceine Bおよびdehydrobrusatolは、bruceine A、bruceine Bおよびbrusatolと比較して、各々約2100倍、900倍、1200倍活性が低く、構造-活性相関解析の結果から、C-15側鎖の性状とAリングのジオスフェノールの存在がクアシノイドの抗トリパノソーマ活性に重要であることが示唆された。また、bruceine A、B、CおよびDの抗トリパノソーマ活性のMRC-5細胞に対する選択指数（SI）は1900から4200の範囲であり、これらのクアシノイド類の細胞毒性は比較的低いことが示された。

薬用植物*Brucea javanica*の果実より分離されたクアシノイド類が抗バベシア活性ならびに抗トリパノソーマ活性を有するという発見は、家畜における原虫疾病に対する薬用植物抽出物の有用性を示唆している。豊富な植物資源を有するミャンマーでは、長い歴史の中で薬用植物による独自の伝承医薬療法が発達している。第三章では、ミャンマーの薬用植物の中から抗トリパノソーマ活性を有する植物を探索した。60種類の薬用植物より得られた55種類の新鮮材料と16種類の乾燥材料について、それぞれ70%エタノールと70%メタノールで粗成分を抽出し、*T. evansi*に対する*in vitro*における増殖阻害活性とMRC-5細胞に対する細胞毒性を測定した。その結果、本スクリーニング法において、3つの新鮮材料と6つの乾燥材料の粗抽出液が*T. evansi*に対して100 µg/ml未満のIC_{50}値を示したことから、これら9種の薬用植物を活性成分の分離・精製を実施する候補薬用植物として選出した。とくに、*Vitis repens*、 *Eucalyptus globulus*そして*Jatropha podagrica*は高い抗トリパノソーマ活性と選択指数を示し、その有望性が示唆された。結論として、ミャンマーにおいて、マラリア、赤痢、腫瘍及び肺疾患などの様々な疾患の治療に使用されているいくつかの薬用植物は、*T. evansi*感染の治療薬としての潜在的有用性をもつことが示された。