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ESTABLISHMENT OF SHORT-TERM *IN VITRO* CULTIVATION OF THAI ISOLATE *THEILERIA* SP. FOR SCREENING OF ANTI-THEILERIAL SUBSTANCES

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CHANUTREE KERDMANEE : ESTABLISHMENT OF SHORT-TERM *IN VITRO* CULTIVATION OF THAI ISOLATE *THEILERIA* SP. FOR SCREENING OF ANTI-THEILERIAL SUSBTANCES. THESIS ADVISORS: PEERAPAN TAN-ARIYA,Ph.D.,NOPPORN SARATAPHAN, D.V.M., KOSUM CHANSIRI, Ph.D., WITAYA THAMAVIT, D.V.M., 146 p. ISBN 974-665-122-6

The purpose of this study was successful short-term *in vitro* cultivation of erythrocytic stage of Thai isolate *Theileria* parasite.

Of the four types of culture media used in the experiment, M199 supplemented with 40% fetal bovine serum (FBS) was proved to be the best culture medium for this purpose. The mean percentage of living parasites obtained by using this medium was significantly higher than in other culture media, i.e., M199+fetal calf serum (FCS), RPMI 1640+FBS and RPMI 1640+FCS ($p<0.05$). In addition, all forms of parasites, i.e., ring, match, 2 piroplasm, 3 piroplasm and 4 piroplasm (quadruplet form) were present in significant number in this type of medium after 72 hours of cultivation. However, the optimal growth and development of parasite indicated by the mean percentage of parasitized cells with living parasite and multiplied piroplasm form were observed during H24 to H48. The optimal percentage of starting parasitemia and cell suspensions was found to be ranged from 4.5%-18% and 10%-12.5%, respectively. Under these optimal growth conditions, the short-term *in vitro* cultivation was applied for assessment of anti-theilerial activities of four anti-hemosporozoal drugs and seven medicinal plant extracts using both microscopic and radioisotopic methods. Only chloroquine (CQ), quinine (QN) and *Vernonia cinerea* stems extract were demonstrated to possess an inhibitory effect against Thai isolate *Theileria* parasite, though their 50% inhibitory concentration (IC_{50}) values were much higher than those previously reported with Japanese isolates. There was no significant difference between the IC_{50} values of CQ, QN and *V. cinerea* stems extract from both methods (CQ: 13.09 *vs* 12.00 μM; QN: 285.96 *vs* 260.00 μM; *V. cinerea* stems extract: 888.61 *vs* 745.00 μg/ml) ($p=0.256$; $p=0.065$; $p=0.175$).

Based on these results, this established a short-term *in vitro* culture method as being very meaningful for further studies, e.g., screening of anti-theilerial agents or ongoing research involving the erythrocytic stage of *Theileria* parasite.

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เรือเอกหญิง ชนุตรี เกิดมณี: ศึกษาวิธีการเพาะเลี้ยงเชื้อไธเลเรีย เพื่อการตรวจกรองหาสารที่มีฤทธิ์ต้านเชื้อ (ESTABLISHMENT OF SHORT-TERM *IN VITRO* CULTIVATION OF THAI ISOLATE *THEILERIA* SP. FOR SCREENING OF ANTI-THEILERIAL SUSBTANCES) คณะกรรมการควบคุมวิทยานิพนธ์:พีรพรรณ ตันอารีย์,ป.ร.ด.,นพพร ศราธพันธุ์, D.V.M., โกสุม จันทร์ศิริ, Ph.D., วิทยา ธรรมวิทย์, D.V.M., 146 หน้า. ISBN 974-665-122-6

จากการศึกษาครั้งนี้ประสบผลสำเร็จในการเพาะเลี้ยงเชื้อแบบระยะสั้นของไธเลเรียระยะที่อยู่ในเม็ดเลือดแดงสายพันธุ์ไทยในห้องปฏิบัติการเป็นครั้งแรก ผลจากการทดลองเพาะเลี้ยงเชื้อในอาหารเลี้ยงเชื้อทั้ง 4 ชนิด ได้แก่ RPMI 1640+fetal calf serum (FCS), RPMI 1640+fetal bovine serum (FBS), M199+FCS และ M199+FBS พบว่าเปอร์เซ็นต์ของเชื้อที่มีชีวิตใน M199+FBS มีค่าสูงกว่าผลที่ได้จากอาหารเลี้ยงเชื้อชนิดอื่น และหลังจากการเพาะเลี้ยงเชื้อเป็นเวลา 72 ชม. สามารถตรวจพบเชื้อได้ครบทุกระยะ ซึ่งได้แก่ ระยะวงแหวน, ไม้ขีดไฟ, 2 จุด, 3 จุดและ 4 จุด เมื่อทำการเพาะเลี้ยงเชื้อโดยเพิ่มเปอร์เซ็นต์เม็ดเลือดแดงที่ติดเชื้อและติดตามผลเป็นเวลา 120 ชม. พบว่าเปอร์เซ็นต์เม็ดเลือดแดงที่ติดเชื้อที่มีชีวิตมีค่ามากสุดเมื่อทำการเพาะเลี้ยงเชื้อไปแล้ว 24-48 ชม. และเมื่อทำการทดลองหาเปอร์เซ็นต์ parasitemia และเปอร์เซ็นต์ cell suspension ที่เหมาะสมเพื่อใช้ในการเริ่มต้นการเพาะเลี้ยงเชื้อพบว่าอยู่ในช่วง 4.5% - 18% และ 10% - 12.5% ตามลำดับ จากการเพาะเลี้ยงเชื้อที่สภาวะเหมาะสมดังกล่าว ทำให้ปริมาณของเชื้อที่มีชีวิตมากขึ้นและมีเชื้ออยู่ครบทุกระยะ ในการศึกษาตรวจกรองหาสารที่มีฤทธิ์ต้านเชื้อไธเลเรียของยาต้านสปอโรซัวในเลือด 4 ชนิดและสารสกัดจากสมุนไพร 7 ชนิดโดยใช้เชื้อไธเลเรียสายพันธุ์ไทยที่เพาะเลี้ยงได้และทำการตรวจสอบโดยวิธีนับเชื้อภายใต้กล้องจุลทรรศ์และวิธี radioisotope พบว่า คลอโรควิน, ควินินและสารสกัดจากต้นหญ้าดอกขาวเท่านั้นที่มีฤทธิ์ต้านเชื้อไธเลเรีย แม้ว่าค่าความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อไธเลเรียได้ 50% (IC_{50}) ของคลอโรควิน, ควินินและสารสกัดจากต้นหญ้าดอกขาวสูงกว่ารายงานที่ได้จากเชื้อสายพันธุ์ญี่ปุ่น อย่างไรก็ตามเมื่อเปรียบเทียบค่า IC_{50} ของสารทั้ง 3 ชนิดจาก 2 วิธีพบว่าให้ผลไม่แตกต่างกันอย่างมีนัยสำคัญ (คลอโรควิน: 13.09 *vs* 12.00 μM; ควินิน: 285.96 *vs* 260.00 μM; สารสกัดจากต้นหญ้าดอกขาว : 888.61 *vs* 745.00 μg/ml) (p=0.256; p=0.065; p=0.175)

จากการศึกษาครั้งนี้ชี้บ่งว่าการเพาะเลี้ยงเชื้อไธเลเรียแบบระยะสั้นในห้องปฏิบัติการสามารถนำไปใช้ในการศึกษาหายาต้านเชื้อไธเลเรียสายพันธุ์ไทยได้และยังสามารถนำไปใช้ในการศึกษาวิจัยเกี่ยวกับเชื้อไธเลเรียที่ต้องใช้เชื้อระยะที่อยู่ในเม็ดเลือดแดงอีกด้วย

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LIST OF ABBREVIATIONS

/	per
%	percent
µl	microliter(s)
µm	micrometer(s)
b.w.	body weight
°C	degree Celcius
cm^2	square centimeter(s)
CQ	chloroquine
Da	dalton
e.g.	exampli gratia
et al.	et all
etc.	et centera
FBS	fetal bovine serum
FCS	fetal calf serum
Fig.	Figure
g	Acceleration gravity
gm	gram
H, h	hour
[^{3}H]	tritium
IC_{50}	50% inhibitory concentration
i.e.	id est
i.m.	intramuscular

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LIST OF ABBREVIATIONS

(continued)

KBq	kilobecquerel
Kg	kilogram(s)
M	molar
M199	Mixture 199 medium
mg	milligram(s)
min	minute
ml	milliliter(s)
mM	millimolar
N	normal (concentration)
PCR	polymerase chain reaction
QN	quinine
RPMI	Rosewell Park Memorial Institute Medium Number 1640
SD	standard deviations
sp	species
U.S.A	United States of America
vs	versus
v/v	volume per volume
wt	weight
$\overline{X}$	mean

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CHAPTER I

INTRODUCTION

Theileria parasite, a causative agent of theileriosis, is a tick-transmitted intracellular protozoan that commonly found in domestic livestock in Africa, Asia and the Middle East including Thailand (1-3). Theileriosis caused by several species of *Theileria* is a serious constraint on livestock development and cattle industry. The taxonomy and nomenclature of *Theileria* parasites are confusion; the parasites share similar characteristics but have often been called by different name (1). The most pathogenic species of bovine theileriosis are *Theileria parva* and *T. annulata.* These two species cause lymphoproliferative disease with high mortality and morbidity in cattle. The pathogenesis is mainly due to schizont development in the host's lymphocytes. *T. parva* causes East Coast Fever, which is confinely found in eastern and central Africa, while *T. annulata* causes tropical theileriosis which is found in a broad region of the world, including southern Europe, northern Africa, the Middle East, southern Russia, India and southern China. The less pathogenic species are *T. sergenti, T. buffeli* and *T. orientalis.* The first two species were reported from Japan and Australia whereas *T. orientalis* was reported from worldwide. These three species cause benign theileriosis. The pathogenesis of the benign *Theileria* species was found to be related with the proliferation of the parasites in the host's red blood cells (1). A major clinical symptom of benign theileriosis is mild anemia but the majority of infected cattle have become long-lasting carriers of this parasite and occasionally they

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develop severe and fatal anemia under some stress conditions or following co-infection with other pathogenic microorganisms (4-7).

At present, identification of *Theileria* species and their vectors in Thailand are still uncertain. There is little information on the study of the species and strain characterization of Thai bovine *Theileria* parasites. Recently, Kakuda *et al* (8) analyzed phylogeny of benign *Theileria* species from cattle in Thailand, China and the U.S.A. based on the major piroplasm surface protein (MPSP) and small subunit ribosomal RNA (ssrRNA) genes. From the comprehensive comparison of MPSP and ssrRNA genes among these *Theileria* species, the isolates from China, Thailand and the U.S.A seemed to belong to a single species and related more closely to *T. sergenti/buffeli/orientalis* group parasites than to malignant species of *Theileria* (*T. parva* and *T. annulata*).

In the treatment of the disease, administration of drugs has been the major measure for controlling this disease. It has been reported that buparvaquone, naphthoquine, is very effective in curing cattle artificially and naturally infected with *T. parva* and *T. annulata* (9) and against *T. sergenti* infection (10). Though, anti-protozoal compounds, i.e., diminazene aceturate (Berenil®), primaquine, acriflavine hydrochoride and chloroquine have been demonstrated to be efficient against hemosporozoal infections including *Theileria* parasites, however, these drugs are unsatisfactory for systemic treatment because of their severe side effects (11,12). Recently, an alkaloid of *Pegnum harmala* L., Chinese herb, was proved to be effective against naturally hemosporozoal infections including *T. sergenti* (13,14). Though bovine theileriosis has been abundantly found throughout in Thailand for many decades but not much attention is paid to it due to the benign symptoms. Moreover,

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the study on the economic loss due to this infection has not been undertaken. However, it is generally accepted that theileriosis has an effect on the productivity of meat and milk of cattle industry. Nonetheless, no specific available regimen for treatment of this disease has been documented in Thailand. The infected cattle with severe symptoms were usually treated by anti-babesial drugs. The target for drug treatment and prophylactic measures against theileriosis is the piroplasm forms in the erythrocytes since these forms were found to be corresponded with clinical symptoms. Medicinal plant products have received high attention from many groups of investigators for treatment many diseases including theileriosis. To improve the cattle industry products, the treatment of theileriosis is necessity. Due to the limitation of anti-theilerial drugs in Thailand, searching for medicinal plant products for treatment of this disease is one of the interesting aspects of research. To fulfill this purpose, the establishment of *in vitro* culture for Thai bovine *Theileria* parasite for screening the anti-theilerial compounds is unavoidable. Although the short term *in vitro* culture method of piroplasm forms of malignant bovine *Theileria* species, i.e., *T. parva* and *T. annulata*, are available (15,16) but no study has been tried for Thai bovine *Theileria* sp. Thus, the aim of this study is to develop the *in vitro* culture method for Thai isolate *Theileria* parasites and to apply the established method for assessing susceptibility of the parasites to anti-hemosporozoal drugs and medicinal plant extracts.

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SPECIFIC OBJECTIVES

1. To establish the short term *in vitro* cultivation of Thai bovine *Theileria* sp.
2. To validate the established method for screening anti-theilerial compounds.

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CHAPTER II

LITERATURE REVIEW

1. *Theileria* parasite

Theileria parasite is a tick-transmitted protozoan parasite of wild and domestic animal, particularly cattle. There are several species of *Theileria* recorded in cattle (2). The most pathogenic species or malignant bovine *Theileria* parasites are *Theileria parva* and *T. annulata,* which cause lymphoproliferative disease with high mortality rates and morbidity in cattle. This disease results from the schizont playing a major role in pathogenicity. *T. parva* causes East Coast fever, which has a limited distribution in eastern, central and southern Africa, while *T. annulata* is more widely distributed, extending from southern Europe to southern Asia. The less pathogenic are *T. sergenti* in Japan, *T. buffeli* in Australia and *T. orientalis* in elsewhere (2). These parasites cause benign theileriosis. This disease is resulted from repeated cycles of the intraerythrocytic stage (piroplasm) of the parasites (4,5,7). A major symptom is mild anemia but the majority of infected cattle become long-lasting carriers of this parasite and occasionally they develop severe and fatal anemia under some stress. Benign bovine *Theileria* parasites are distributed in Asian countries and their pathogenicities vary from pathogenic (anemia) to almost non-pathogenic (6,16,17).

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1.1 The taxonomy of the benign *Theileria* species in cattle

The taxonomy of parasites of the genus *Theileria* of domestic animals appeared straightforward to most specialists about 38 years ago. However, as more criteria for distinguishing species and strains became available, the situation became more complex (2). Especially, the identities of the benign species of *Theileria* occurring outside Africa are very confusing. A representative of this group of parasites appeared in Japan, Australia, Korea, Britain, the United States of America, Malaysia and probably in many other countries. The name of *T. sergenti* has been retained by Japanese researchers (6), *T. buffeli* adopted by Australian researchers (16), and researchers in many other countries including Europe prefer the name *T. orientalis* (17); consequently, these parasites are frequently referred to as the *T. sergenti/buffeli/orientalis* group. *T. buffeli* was described by Schein in 1908 in Vietnam, where this parasite was found in the Asian water buffalo (*Bubalus bubalis*). *T. sergenti* was indicated as *Gonderia(=Theileria) sergenti* in the Vladivostock area of eastern Siberia by Yaimoff and Dekhtereff in 1930 (18). *T. orientalis* was also found in the same region by Yakimoff and Soudat Schenkoff in 1931 (19). The name of *T. buffeli* was also given to the Australian *Theileria* species by Callow in 1984 (16). In 1985, Uilenberg and his colleagues compared stocks of *Theileria* from Japan, Australia, Britain, Iran, the U.S.A. and Korea by morphological and serological aspects. They concluded that those stocks all belonged to one species, and propose the name as *T. orientalis*. Until 1987, Stewart and his workers showed that *T. buffeli* was not transmitted by the tick--*Haemaphysalis longcornis,* which has been proven as a vector of *T. sergenti.* This observation suggested the difference between Australian and Japanese *Theileria* species (22). Recently, *T. buffeli/orientalis/sergenti* were

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classified into two groups, i.e., *T. buffeli/orientalis* and *T. sergenti* based on differences in tick vectors (23), protein analysis of piroplasms by two-dimentional gel electrophroresis (24), serological dissimilarities (25) and the nucleotide sequences of the genes encoding immunodominant piroplasm surface proteins (26).

1.2 The distribution and classification of the benign *Theileria* species

Benign *Theileria* parasites in cattle are distributed in Asian countries (China, Taiwan, Korea, Japan, Indonesia, Malaysia, Thailand), Australia, European countries and in the U.S.A. The specific name of *T. sergenti* has been adopted for the parasites in Japan and Korea (27,28) and *T. buffeli* for Australian isolates (16,20,21). Although the exact nomenclature of these parasites are still indefinite (29), the parasites which are isolated from many countries, including Europe, sometimes are called *T. orientalis* (17,30). Recently, molecular biological studies on the gene encoding the major piroplasm surface protein (MPSP) have indicated that parasites in this group are divided into at least four types i.e., I, C, B_1 and B_2. The classification is based on the allelic forms of MPSP genes (31-33). The field parasites of Japan, Korea, Australia, European countries and Thailand were found to have mixed populations of *Theileria* parasite since various combinations of MPSP genotypes were observed (8,34-37). The parasites of *Theileria* infected Australian cattle consisted of a mixture of B_1 and C types. *T. buffeli* Warwick stock and *T. orientalis* Essex stock contain single parasite population of B_1 type. In 1996, Kukota and colleagues revealed that nucleotide sequences of the gene encoding for Australian C-type MPSP are similar to that of Japanese C-type parasite of *T. sergenti.*

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1.3 Transmission

1.3.1 Transstadial transmission by tick vectors

The commonly known biological vectors of the Japanese *Theileria sergenti* are Kaiseriana ticks, *Haemaphysalis (Kaiseriana) longicornis, H. (K.) cornigeraias* (38), *H. (K.) mageshimaensis* (39) and *H. (K.) bancrofti* (23). However, *T. sergenti* has also been transmitted by other haemaphysaline tick species, e.g. *H. flava, H. kitaokai, H. concinna* (38), *H. punctata* (17) and *H. megaspinosa* (23). In contrast, the transmission of *T. sergenti /buffeli/orientalis* by ticks of genera *Amblyomma, Boophilus, Dermacentor, Ixodes* and *Rhipicephalus* is also feasible (17,38). The transmission of *T. sergenti* via *R. appendiculatus*, which is a vector of *T. parva*, was also unlikely (23).

In 1987, Stewart and his co-workers revealed that *H. humerosa* is the major vector of the Australian *Theileria* parasite. *H. longicornis* -- a vector of *T. sergenti* and found in Japan, was reported for its possibility to transmit the Australian *Theileria* (21), whereas the data of Fujisaki and his colleagues (23) disagreed.

1.3.2 Transmission by mechanical vector

It has been reported that *Tabanus trigeminus* acts as a vector for mechanical transmission under special conditions (40). Mechanical transmission of *T. sergenti* is also achieved by the long-nosed cattle louse, *Linognathus vituli*, as it is one of the most important harmful blood sucking insects found on calves at any season and area of Japan (41). Its ability to transmit *T. sergenti* can play an important role in winter and in-house infection. It is postulated that the infection may be established by regurgitation of piroplasm in the gut contents of the lice (42).

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1.3.3 Transmission by prenatal infections in calves

In 1994, Onoe and co-workers (43) observed prenatal infections of *T. sergenti* in calves at 1 or 2 days after birth. The results of this study indicated a possibility of intra-uterine transmission of *T. sergenti* from infected cows. However, no additional experiments have been established to confirm this hypothesis.

1.4 Life cycle

The life cycle of *T. sergenti* has been investigated in both the mammalian host and the tick vector, the parasite undergoes a series of transformations into different forms (Fig. 1). In general, when ticks feed on animals that are infected with the parasites, they ingest red blood cells containing the piroplasms. Once the parasites are transferred to the tick gut, they differentiate themselves to male and female gamonts and fuse to form zygotes (41,44). Studies of the morphological development of *T. sergenti* in the midgut of nymphs of *H. longicornis* and *H. mageshimaensis* revealed that the zygotes contain round and oval forms with the reddish-violet colour in Giemsa-stained gut smears. The nucleus is often situated in the periphery but does not always stain. These forms are detected in the gut through the course of moulting of ticks and increased in size from 2.50 to 8.58 μm in diameter. The spindle- or club-shaped structure, situated in invaginated zygotes at the periphery of cytoplasm, is the developing kinete and possesses an average size of 5.2 x 1.5 μm. This size is approximately one time smaller than the developed kinete in the haemolymph (10.0 μm) of *H. longicornis* 25 to 42 days after the nymphal stage has fed on infected cattle (44). After entering and developing in the gut, the kinetes move to the salivary gland and enter a particular cell type. The parasites develop to sporoblasts, which may give rise to 30,000 to 50,000 sporozoites. A number of sporozoite forms of the parasites in

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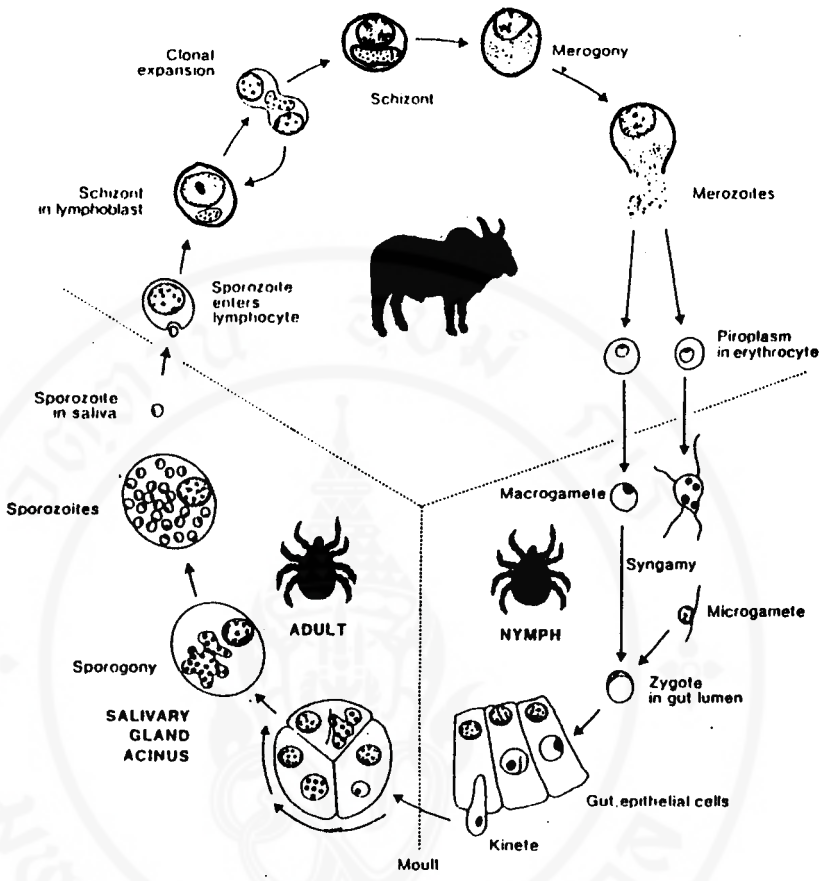

Figure 1 Life cycle of *Theileria* parasite

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tick's saliva are transmitted to the bovine host and enter the lymph node, where sporozoites could develop into schizonts.

In 1991, Kawasu and co-workers (45) described the schizont stage of the Japanese *T. sergenti* in the lymph nodes of splenectomised calves. The data were similar to those of Korean *Theileria* (17) and Australian *Theileria* (46). In 1993, Sato and colleagues (47) showed that schizogony not occurs only in the lymph nodes but also in liver and spleen. The huge cell, schizonts (macroschizont) range from 50 to 200 μm in diameter and contain both regular and irregular shapes of 1 to 7 μm in diameter. The ultrastructure of the schizonts reveal that one or more nucleus can be formed in the unit of enlarged cell. The schizont can be detected in the draining lymph node between 4 and 8 days after sporozoite inoculation. Host cells of schizont are considerably enlarged by parasitism. At this stage, the morphology of *T. sergenti* schizonts is similar to that of malignant *Theileria* species. Microschizonts in the phase of merozoite formation are observed as the basophilic regular shaped granules under light microscopy on days 8 after sporozoite inoculation. At this phase, merozoites are budding from the schizonts. The round shape merozoites of 0.75 μm in diameter further develop into piroplasms and are able to infect ticks. Morphological findings on the intraerythrocytic stage (piroplasm) of *T. sergenti*, the main causative agent of the disease, show that the parasites with various shapes such as rod- and comma-shaped, ovoid and tetrad are observed in the peripheral erythrocytes. The rod- or comma-shapes (match form) are suggested to be detected mainly in the acute stage of disease, and the ovoid shape (ring form) appears in the chronic stage (48,49) (Fig. 2).

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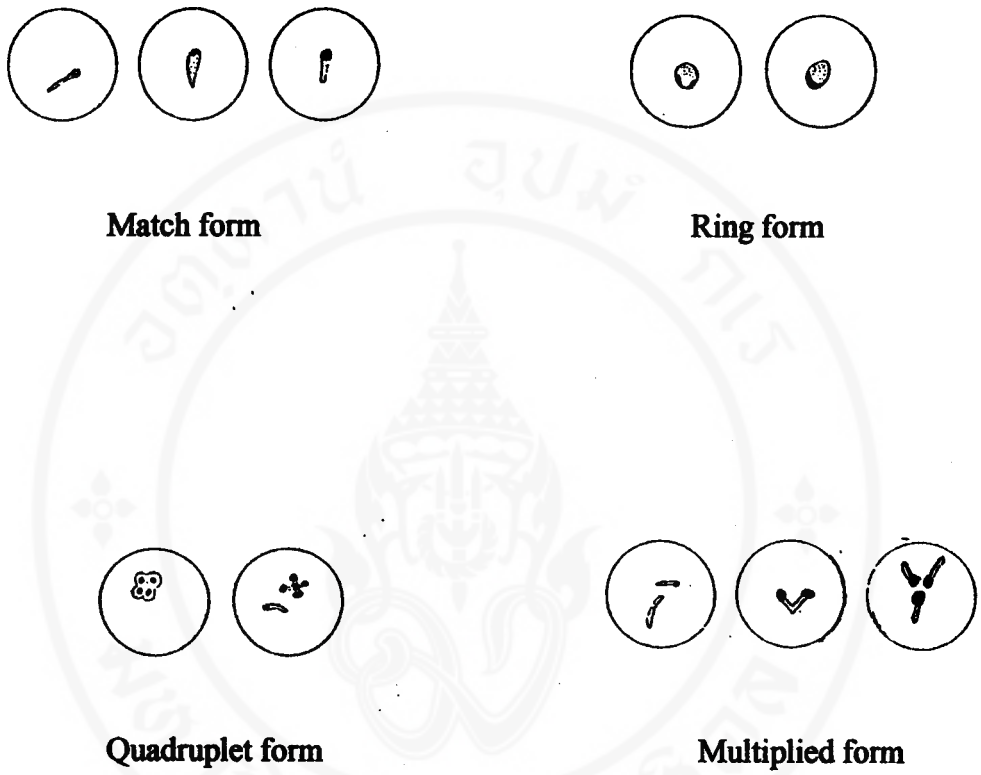

Figure 2 Piroplasm forms of *Theileria* parasite in erythrocytes

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1.5 Treatment and prophylactic measures

It has been emphasized that the pathogenesis of the benign theileriosis is mild anemia. Nonetheless, the spleen, liver and lymph nodes are commonly enlarged. The schizonts and the piroplasms have quite different responses to chemotherapeutic agents and thus, treatment must differ with the species involved and the stage of infection. Theoretically, if schizogony of macroschizont was arrested by therapy, microschizonts and piroplasms would not be produced. However, most clinical cases have passed this point before a diagnosis is made. Schizogony can be arrested by administrating the antibiotics, oxytetracycline or chlortetracycline, just prior to or at the time when the infection takes place and maintaining treatment at therapeutic levels throughout the incubation period. If treatment is delayed until symptoms appear, no response would be expected. The broad-spectrum antibiotics have no place as therapeutics. Recently, the anti-theilerial drug baparvaquone has been found to be effective to curing cattle artificially infected with *T. sergenti* (10). In 1992, Kamiyama and co-worker (50) showed that chloroquine was the most effective drug and quinine showed moderate inhibitory effect among anti-hemosporozoal drugs tested against *T. sergenti* in short-term culture. In addition, it has been reported anti-babesial drugs, i.e., Berenil® and Imizol® were used to prevent and cure piroplasmosis including *T.sergenti* in China (51). The death rate was reduced from 26% to 5.9%. Not many kinds of drug are available for therapeutic purposes against *T. sergenti*.

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1.5.1 Chloroquine

Chloroquine (CQ)($C_{18}H_{26}ClN_3$) is antimalarial drugs act as the quinoline blood schizontiocides to exert their effect (Fig. 3). This drug probably mediates its activity by inhibiting hematin polymerization (52-54). It is known that the quinoline blood schizontiocides such as CQ bind hematin. Binding to monomeric and dimeric hematin, either free in solution or complexed to a growing hemozoin chain,may prevent further hematin incorporation into hemazoin (53-55). Alternatively, interaction with the hemozoin, β-hematin polymer may induce depolymerization (56). It is believed that these processes result in higher concentrations of free hematin in the food vacuole. As free hematin, especially if complexed to CQ, is capable of inducing membrane lysis (57), it is likely that this accounts for the parasiticidal activities of the quinoline blood schizontiocides. Other targets remain under investigation, for instance, recently two uncharacterized *P. falciparum* proteins of 33 and 42 kDa were found to bind a photoreative analogue of CQ in a specific manner (58). Whether these proteins are related to killing of *P. falciparum* by chloroquine is unknown. The 33 kDA protein has been identified as *P. falciparum* lactate dehydrogenase (PfLDH). However, it has been shown that this enzyme was not associated with CQ activity (59). CQ has shown to have an effect against benign *Theileria* parasite (50), however, the mechanism of drug action has not been investigated.

1.5.2 Quinine

Quinine (QN)($C_{20}H_{24}N_2O_2$) is a powerful blood schizontocidal antimalarial against asexual erythrocytic stages of all four species of plasmodia that infect man, including CQ resistant *P. falciparum* (Fig. 3)(61). The drug has no action on the exoerythrocytic form (62). The gametocytes of *P. vivax*, *P. ovale* and *P. malariae* and immature

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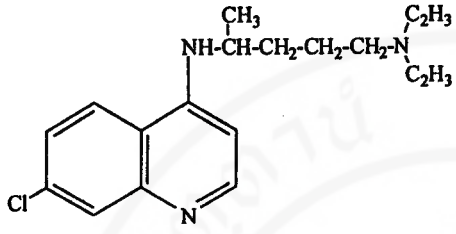
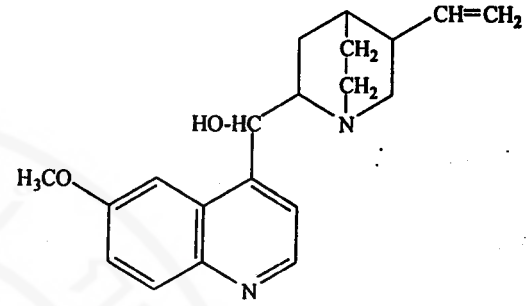
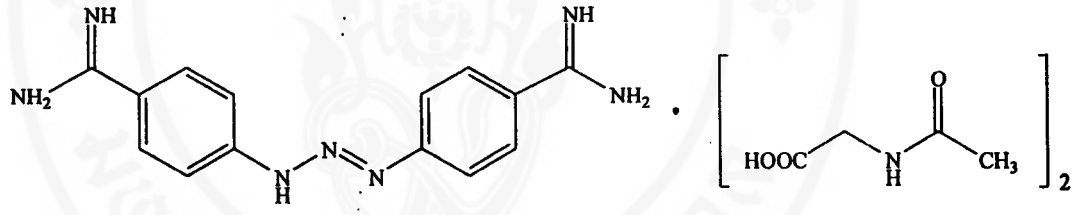


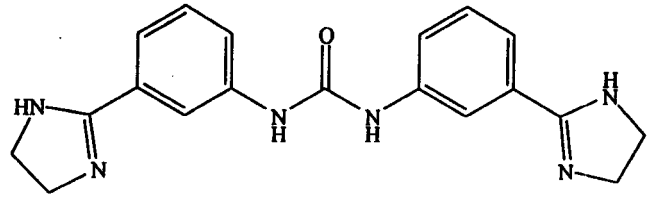
Chloroquine



Quinine



Berenil®



Imizol®

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Figure 3 Structural formulas of anti-hemosporozoal drugs.

gametocytes of *P. falciparum* are also sensitive to QN (62). Same as CQ, though it was proved to be effective against benign *Theileria* parasite but its mechanism has not been studied (50). The mechanisms of action of QN remain to be elucidated. The suggested mechanisms could be either through the intercalation with parasite DNA or the complex with ferriprotoporphyrin IX in the parasites, which results in the lysis of the parasites and the host cells (62).

1.5.3 Berenil®

Berenil® is a trade name of diminazene aceturate ($C_{22}H_{29}N_9O_6$). This compound is an aromatic diamidine derived form Surfen C (Fig. 3) (63). The molecule is marketed as the diaceturate salts and consists if two amidinophenyl moieties linked by a trizene bridge: *p,p*-dimidinodiazoaminobenzene diaceturate tetrahydrate; *N*-1,3-diamidinophenyltriazene diaceturate tetrahydrate. In aqueous solution the compound is stability, diminazene is marketed in combination with the stabilizer phenyldimethyl pyrazolone (antipyrine). Solutions of the preparation can be used without loss of activity for up to 10-15 days when stored at room temperature (64).

In initial experiments, diminazene was shown to be highly active against both *Trypanosoma* and *Babesia* sp. (65,66). The compound was introduced into the market as a trypanocide and babesiacide for domestic livestock in 1955 (67).

Diminazene aceturate is currently marketed under the trade names Azidide®, Berenil®, Ganasag®, Ganasegur® and Veriben®, as both a trypanocide and babesiacide for domestic livestock. For all animals the general i.m. dose is 3.5 mg/kg b.w. Diminazine aceturate is recommended only for use as a therapeutic agent since it is rapidly excreted and therefore thought to have little prophylactic activity (66). Furthermore, using an *in vitro* cultivation assay, Intramuscular doses of 5 mg

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diminazene aceturate/kg b.w. have also been shown to protect cattle against challenge, with *B. bovis* for up to 14 days following treatment (68). Thus, diminazene exhibits prophylactic activity in cattle for periods varying from a few days to a few weeks. Variation in duration of prophylaxis appears to be dependent on the sensitivity of the parasites to diminazene and the dose of drug used.

Babesiosis in domestic animals is commomly treated with diminazene aceturate. The standard i.m. dose recommended by the manufacturer is 3.5 mg/kg b.w. (Hoechst Veterinar, Germany). Literature relating to the use of diminazene for treatment of bovine, equine, canine, feline ovine, porcine and human babesiosis has been adequately reviewed by Kuttler (69). In addition to trypanocidal and babesiacidal activity, diminazene aceturate has also been effectively used as a therapeutic agent for visceral and cutaneous leishmaniais in man (70,71) and for bovine francaiellosis (72). As anti-theilerial agent, Berenil® could reduce the death rate of sicked cattle from 26% to 5%.

1.5.4 Imizol®

Imizol® is a trade name of Imidocarb or 3,3'-bis-(2-imidazolin-2-yl)-carbanilide dihydrochoride) ($C_{19}H_{20}N_6O_7$) (Fig. 3). Beveridge (73) reported that the compound had a greater babesiacidal effect and a better chemoprophylactic index than did quinuronium, diamidine and amicarbalide derivatives against *Babesia rodhaini* in mice and rats. In Ireland, Wood 1971 (74) found that imidocarb was very effective against *B. divergens* infection for therapy and for prophylactic treatment. Hart *et al* (75) reported that the compound protected cattle from naturally occurring *B. argentina* infection for 44 days.

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Imidocarb is highly effective against acute infection of *B. bigemina* and *B. argentina,* according to several workers (74,76-78). Apperently, imidocarb acts directly on the *Babesia* sp. parasites, causing an alteration in number, size, vacuolation, and morphology of the nucleus and cytoplasm. These changes in *Babesia sp.* parasites apparently occur due to the malfunction of the metabolic and enzymatic processes that eventually kills the organism. Simultaneously, the drug causes changes in the tissue reactively, stimulates the active mesenchyme and reticuloendothlial system, increases the phagocytosis or the number of macrophages, helps to produce defensive substances, and increases the immunologic properties of the host. Imidocarb effectively controls parasitemia; the day following injection, the parasites disappear.

The potential use of imidocarb as a prophylactic drug is indicated in a number of circumstances. To protect susceptible cattle which are moved from a tick and *Babesia*-free country to a tick infested area, imidocarb can be used to replace hazardous vaccination procedures with virulant *Babesia* spp. Furthermore, application of imidocarb and exposure of cattle to infected ticks with *Babesia* sp. might help in the development of natural co-infectious immunity without hazardous losses. Administration with Imizol® and Berenil®, a significant reduction of percentage of death rate of infected cattle was achieved (51).

1.6 Medicinal Plants

The use of medicinal plants for treatment of *T. sergenti* infection has long been reported in China (12,79). Alternative treatment with medicinal plants, such as total alkaloid of *Peganum harmala* L. has been used in the treatment of experimental and natural hemosporozoal infections including *T. sergenti.* Recently, more than 5 Thai medicinal plants have been shown to exert anti-malarial activities (80). It was

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suggested that these Thai medicinal plants may be effective against piroplasm stage of *Theileria* parasites.

1.6.1 *Alyxia reinwardtii*

Alyxia reinwardtii is a plant with wide distribution in Thailand. Locally, various parts of the plant have been used as perfume, flavoring and medicine (Fig. 4) (81). The fine ground stem is used in the manufacture of incense and other aromatic products. Water extracts of the stem are used in traditional or religious rites and to impart aroma to pipe tobacco. According to traditional medicinal practice, the leaves and fruits of this plant can be used to reduce fever; the flowers are effective in treating mental confusion and hallucination associated with high fever; stopping hiccup and correcting unspecified gall bladder ailments. The roots are effective in reducing fever, the stems are used to treat fainting, heart failure and abdominal discomforts due to gaseous distention or other unspecified causes. These claims in traditional medicinal practice involve the actions of the plant on mainly the central nervous system and the gastrointestinal system. The coumarin is one of the most widely occurring organic compounds in this plant (81). Based on this general approach, the action of this plant may be related to some of those of coumarins reported in the literature e.g. spasmolytic action (82,83), hemostatic action (84), anti-helmintic action (85), antiveratrinic action (86), and vasodilator action (85,87). In addition, other autonomic effectives may also be present even though no related claim has been made in traditional medicine e.g. effects on other smooth muscles like the bronchial and intestinal and the secretory cells.

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Figure 4 *Alyxia reinwardtii* BL. Var *lucida* Markgr.

Figure 5 *Barringtonia acutangula* Gaerth.

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1.6.2 *Barringtonia acutangula*

Barringtonia acutangula is commonly known as Aram, Bantana, Hijal, Hijgal, Hijjala, ijal, indian oak, Kadamu, Neora, Oak indian and Paniha. The roots are used for a laxative (Fig. 5)(88). The whole plant is anticephalelgic and antiscorbutic. The leaves are administered for antidysenteric, treatment of wound. The seeds are employed for antibechic, antiemetic and antipyretic. The barks are used to stupefy fish (89). Among its chemical constituents, the plant contains acutangulic (90,91), β-amyrin (91), barrigenic acid (92), barringtonic acid (91), barringtogenic acid (90), barringtoside A, barringtoside B, barringtoside C (93), ellagic acid, ellagic acid,3-3'-di-O-methl, ellagic acid,3-O-methy (94), olean-12-ene-23-28-dioic acid, 2α-3β-19α-trihydroxy, 28-O-β-D-glucopyranoside (95), oleanolic acid, β-sitosterol, γ-sitosterol (90), stigmasterol-3-O-β-D-glucoside (90), tangulic acid (91).

1.6.3 *Oxylum indicum*

Oxylum indicum (F. Bignoiaceae) is known in the common names as Phae kaa, Kaa-do-dong (Karen-Kanchanaburi), Do-ka, Dok-ka, Du-kae (Karen-Mae Hong Son), Be-ko (Malay-Narathiwat), Ma litmai, Ma linmai, Litmai (Northern), Lin faa (Loei), Maak-lin-kaang, Maak-lin-saang (Shan-Northern)(Fig. 6)(96). The plant is a deciduous tree having gray, smooth or somewhat fissured bark, covered with lenticels. Leaves are opposite, 2-4 times pinnate with terminal leaflet consisting of petiole and rachis robust; leaflets ovate to oblong, acuminate at the apex, base narrowed, rounded or slightly cordate, usually asymmetric, glabrous. Flowers are in terminal racemes; consisting of large tubular without lobes. Fruits are very long, broad hanging capsules. Seeds are flat with broadly winged around. The plant is scattered along the edges of evergreen forests, frequently in villages and disturbed habitats.

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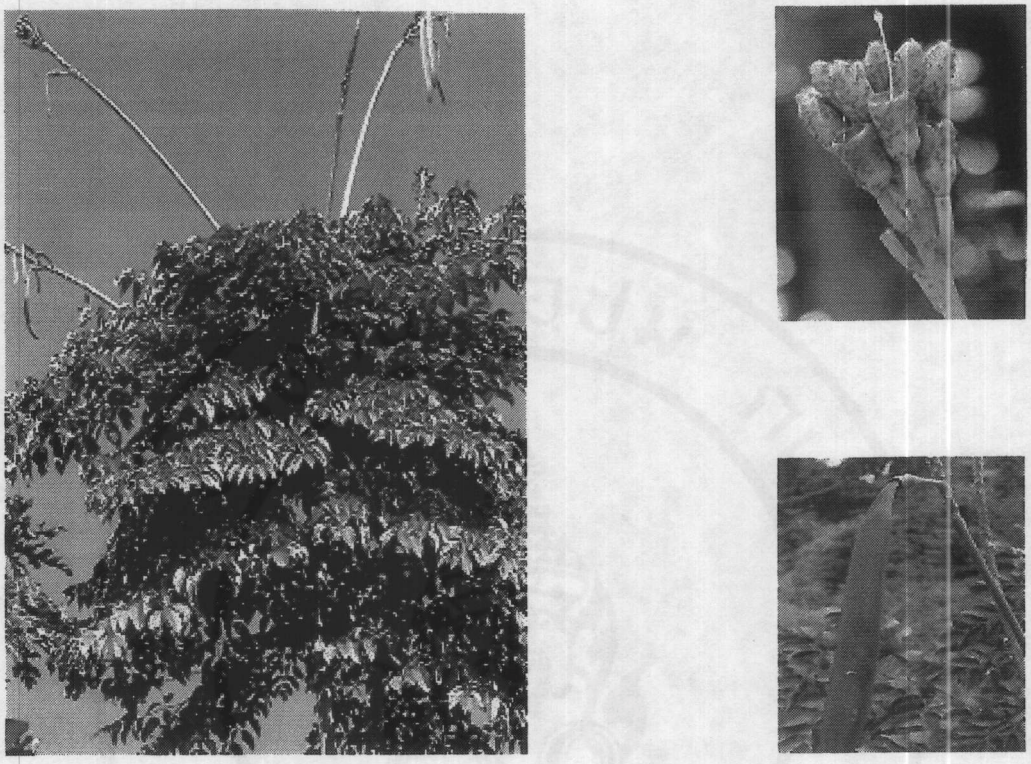

Figure 6 *Oroxylum indicum*, Linn. Kurz

Figure 7 *Alstonia scholaris*, Linn., R.Br.

They are widely distributed throughout India, Ceylon, Burma, south China (Yunnan), Indo-China, Malay Peninsula, Sumatra, Java, Borneo, Timor and the Philippines. The roots are used for antipyretic, antidiarrhoeal, antidysentery, appetite-stimulating and anti-inflammatory action. The whole plant are applied in the treatment of oozing eczema due to lymphatic disorders, vomiting, infantile jaundice and infectious disease in early childhood (under 5 years old), abscesses; for wound healing and as a blood purifier, antipyretic, blood tonic, expectorant, antidysentery, carminative and anti-inflammatory. Roots consist of many chemical constituents, i.e., baicalein, D-galactose, oroxylin-A, sitosterols, wogonin (97). Prunetin and β-sitosterol were found in the heartwood (98) and baicalein, chrysin and 6-methylbaicalein in the barks (98). Aloe emodin was isolated from the leaves (100).

1.6.4 *Alstonia scholaris*

Alstonia scholaris R. Br. (F. Apocynaceae) is a tall evergreen tree occurring widely in India, in sub-Himalayan areas and through Southeast Asia countries to Indochina, known as Dita Bark or Devil's Bark, *A. scholaris* has been widely used in folk medicine as an astringent, alternative, tonic, anthelmintic and antiperiodic in fever and has been claimed to be useful in restoring stomatch muscle tone after debility due to fever and in the treatment of some forms of rheumatism (Fig. 7) (101,102).

Although popular in Europe early in this century, dita bark declined in popularity since no real pharmacological evidence was available. The barks were also a source of poor quality caouthouc and the woods were used in the manufacture of furniture, boxes, coffins, etc (101).

The use of *A. scholaris* in folk medicine and interest in the indigenous plants of Thailand stimulated a re-appraisal of the alkaloids present in the stems and roots. Philippinos commonly employ crude extracts of the bark of *Alstonia* species as a "cure" for malaria. The chief constituents from *A. scholaris* are ditamine and echitamine (103,104). Another non-nitrogenous bitter, alstonine, has been reported from *A. constricta* (105). Alstonine has been stated to be the active antimalarial principle in *A. scholaris* and is being developed as a drug at the Walter Reed Institute (106).

1.6.5 *Vernonia cinerea*

Vernonia cinerea Less. (F. Compositae) known in the local names; Mor Noi (Central), Yar Sarm Wan (Chiang-Mai), Karn Thub (Chanta-Buri), Yar Dok kaow, Yar-La-ong (Bangkok) is the annual herb, different parts such as stem, leaves, root, flower and seed are used in folklore medicine (Fig. 8) (107). The whole plant (108) is used for antipyretic, antidiarrhoea, antihepatitis, anti-infective, bronchitis, antispasmodic activity of the urinary bladder and as the astringent, tonic, diuretic. Also, it is reputed for treatment of asthma, bronchitis and tuberculosis in India, Indonesia, Philippines and Gobon. The leaves are used for antiamoebic, antipyretic (109). The leaf sap is also used treatment of pneumonia. The roots are used for anthelmintic, antidiarrhoea, antipyretic and diuretic (108). The flowers are administered for conjunctivitis (110). The seeds are employed in abdominal pain, anthelmintic (111). The chemical studies of many *Vernonia* spp. have been reported and found to contain triterpenes, steroids, steroid glycosides, steroidal sapogenins, coumarins, coumarin glycoside and flavonoids (112).

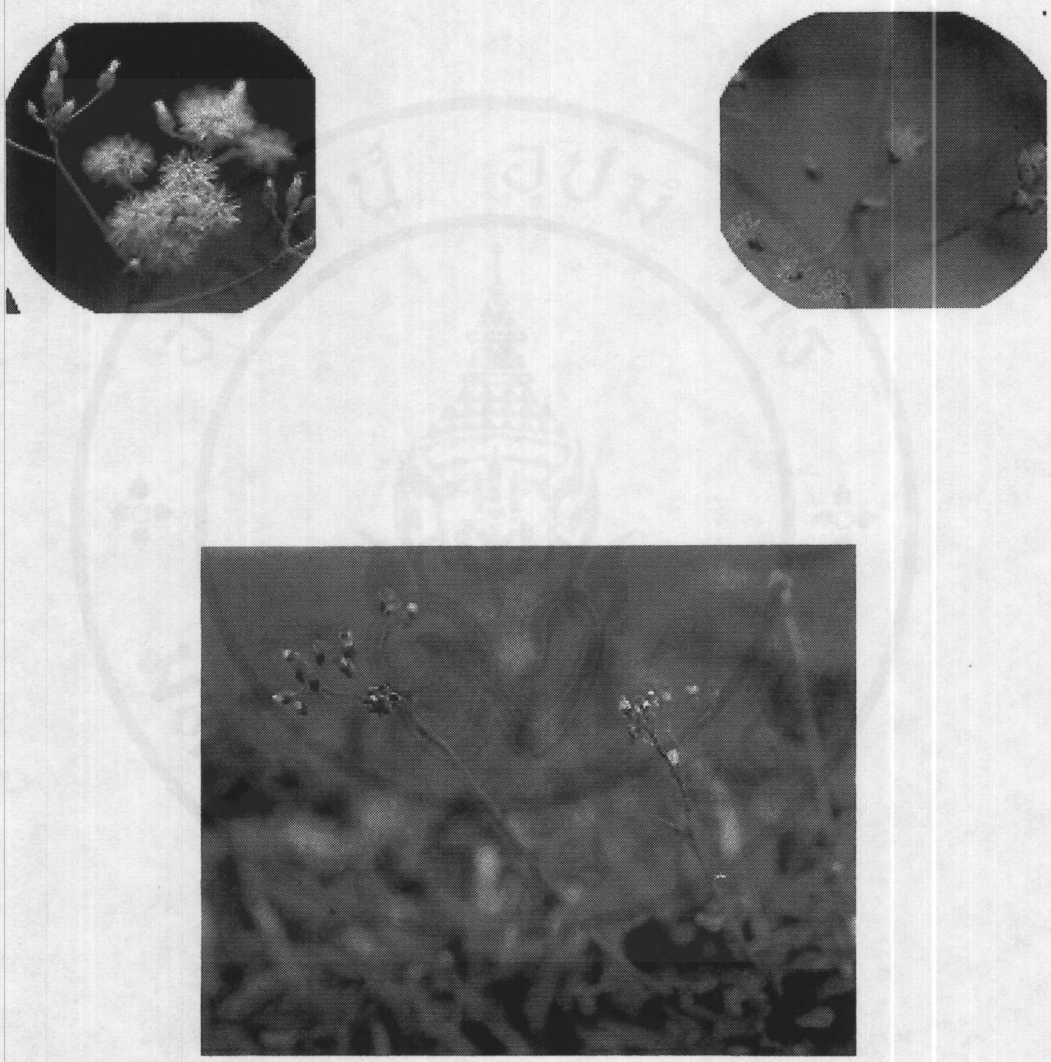

Figure 8 *Vernonia cinerea* L., Less.

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1.7 Control of benign theileriosis

To control *T. sergenti* infection in Japan, living parasite in erythrocytes (piroplasm) has previously been used as a vaccine (38). However, its use was then prohibited because of the transmission of infectious agents such as bovine leukemia virus (113). An injection of piroplasms occasionally does not induce any protective immunity in calves because of antigenically different strains of *T. sergenti* in Japan. However, Tanaka *et al* (112) analyzed the p32 antigen and demonstrated that passive immunization of anti-p32 monoclonal antibody inhibited progressing of the disease in terms of anemia. Thus, they considered p32 as a candidate vaccine. Lastly, Onuma *et al* (36) developed two vaccine candidates, the recombinant p32 antigen and synthetic peptides including KEK motifs derived from p32 of I and C type parasites. Twelve months old calves were used in this experiment. Two weeks after the last immunization, all calves were splenectomized and then challenged with sporozoites derived from I-and C-type parasites. Vaccine effect was considered as the allele-specific inhibition of parasites by PCR, lower parasitemia level and the reduction in the severity of clinical symptoms when compared to that of control calves. It was found that a cocktail vaccine, containing KEK peptide derived from I-and C- type parasites with manna-coated liposome as adjuvant is necessary for the control of *Theileria* parasite infection. A lived recombinant p32 vaccine using vaccinia virus and bovine herpesvirus as vector has been tried by this group. Effective vaccines against intracellular pathogens may induce cell-mediated immunity rather than humoral immune responses. Thus, there may be a useful recombinant lived vaccine for the control of *T. sergenti* infection in the near future.

1.8 Situation of bovine theileriosis in Thailand

In Thailand, the first report of *Theileria* parasites in Thai native cattle from the southern part was studied by monitoring parasitemia of the splenectomised calves (115). Recently, *Theileria* parasite DNAs in a blood sample collected form dairy cattle and beef cattle in various places were analysed by immunodominant major piroplasm surface protein genes and their allele variants by polymerase chain reaction (37). The results showed that there are three groups of *Theileria* isolates, the first group showed positive PCR amplification by specific p32 primer. The second group was positive to both specific p32 and p33/34 primers. The third group represented *Theileria* sp. of no PCR amplification when the two set of primers were used. However, Kakuda and collegues (8) have designed a new specific primer to amplify the gene coding major piroplasm surface protein (MPSP) of the third group. According to the sequences of MPSP of *Theileria* parasites isolated from cattle in China, Thailand and the U.S.A., more than 89% and 83% similarity of the nucleotide and amino acid level were observed, respectively. By using the allele-specific PCR, Sarataphan *et al* (37) found that the majority of *Theileria* parasites in Thailand was C-type MPSP, whereas the mixed population of these types are minute in size as shown in Table 1. However, there is little information on the study of bovine theileriosis. Thus, the life cycle of parasite in cattle infected with Thai isolate *Theileria* species remains unknown and their vector in Thailand is still uncertain. Moreover, it is proposed that benign *Theileria* groups are potentially abundant throughout the country (3). The parasite infection, by itself, cause a disease characterized by mild anemia and hyperthermia, but severe and fatal cases are not rare under conditions in the field. In addition, these parasites can induce abortion and mortality in the animals due to their weakness

Table 1 Distribution of MPSP alleles of *Theileria* parasite stocks and isolates (36)

Stocks	MPSP allesles			
	I	C	B1	B2
T. sergenti Ikeda stock	+	-	-	-
T. sergenti Chitose stock	+	+	-	-
T. sergenti Fukushima stock	-	+	-	-
T. buffeli Warwick stock	-	-	+	-
T. orientail Essex stock	-	-	+	-

Country	No. of isolate	I	C	B1	B2	Thai
Japan	12	+	+	-	-	-
	5	+	-	-	-	-
	3	-	+	+	-	-
	2	-	+	+	-	-
Korea	14	+	+	-	-	-
	12	+	+	+	-	-
	5	+	+	-	+	-
	4	+	-	-	-	-
	1	+	-	-	+	-
China	4	-	+	-	-	-
	1	+	+	-	-	-
Taiwan	11	-	+	-	-	-
	4	-	+	+	-	-
Australia	2	-	+	+	-	-
Italy	6	-	+	+	-	-
Thailand*	14	-	-	-	-	+
	6	-	+	-	-	-
	5	-	+	-	-	+
	2	-	+	+	-	-
	1	-	+	+	-	+

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* results obtained from Sarataphan *et al* (37)

causing the considerable loss in productivity of domestic animals (116). Bovine theileriosis may be controlled by one or more of the following methods: i) management with particular emphasis on movement control; ii) vector control in order to preventing transmission of disease; iii) treatment of clinical disease using specific chemotherapeutics; iv) immunization with live vaccines; and v) the use of cattle resistant to tick or the disease. To date, there is no effective drug for treatment of bovine theileriosis in Thailand. The anti-babesial drugs, i.e., Berenil® and Imizol® are the current products, which used for control the disease. Therefore, the need for new drugs to replace the current products lead to search for provide better kinds of drugs that are highly effective against Thai isolate *Theileria* infections.

2. Cultivation

The *in vitro* cultivation of bovine *Theileria* parasites has been shown to be of value as a model for *Theileria* established in cattle. *In vitro* cultivation of this parasite can be divided into two methods which depending on the life cycle that of the long-term cultivation of schizont stage within the lymphoid cells and that of the short-term cultivation of piroplasm stage within erythrocytes.

2.1 The long-term cultivation of schizont stage within the lymphoid cell

Cultivation of the schizont stage *in vitro* led to the development of a vaccine against *T. annulata* infection (117). In addition, this system was used for investigating the mode of multiplication of intralymphatic forms of *T. parva*, *T. annulata* and *T. lawrencei* (118). It has been revealed that prolonged subcultivation of schizonts *in vitro* resulted in attenuation of *T. annulata* (118). Cell-mediated immunity to

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Theileria-transformed cell lines for the control of East Coast fever disease was studied by using *in vitro* cultivation assays (120,121). To date, cultivation of schizont-infected lymphoid cells is established in various regions either for research on the malignant *Theileria* parasites (T. *annulata* and *T. parva*) and vaccine development, or for vaccine production, for which mass cultivation of cells is a necessity. The long-term cultivation of schizont stage within the lymphoid cell consists of preparing *Theileria* infected bovine lymphoid cell lines, which are used as a supporting feeder layer. Many of cell lines could be provided by several cell types, e.g., normal bovine leukocytes from buffy coat, bovine lymphocytes from lymph node and spleen, Hela cells, bovine kidney and lamb testis cells. Then, cell lines are infected with isolated schizont of *Theileria* parasites. The infected cell lines are cultured in growth media supplemented with serum and the essential supplements (glutamine, penicillin and streptomycin). Before use, the pH and osmolarity of culture media are corrected to 7.0-7.3 and 280-320, respectively. Schizont-infected cells are grown in tissue culture flasks or glass bottles and incubated at 37°C. Subculturing are performed as otherwise stated by dispensing cells with ethylene diamine tetra acetic acid (EDTA) and transferring them into a new vessel. Recently, successful long-term *in vitro* cultivation of *T. annulata* schizonts in media supplemented with homologous and heterologous sera were reported (122). The results suggested that normal bovine serum and goat sera could be successfully used in placed of fetal bovine serum in the growth medium (RPMI-1640 medium) for long-term *in vitro* propagation of *T. annulata* schizont.

2.2 The short-term cultivation of piroplasm stage within the erythrocytes

Under the current situation, there are only few studies on *in vitro* cultivation of intraerythrocytic stage. Thus, this method has not been successful, as it has been

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established for intralymphatic stage. In 1985, studying the parasite development in short-term cultivation revealed the mode of intraerythrocytic multiplication of *T. annulata*. (14). Infected bovine blood was obtained from two calves infected with sporozoite stabilates of *T. annulata*. Then, blood was centrifuged, and the plasma and buffy layer were removed. The remaining concentrated erythrocytes were used to prepare an erythrocyte suspension in complete medium. The medium and serum in the complete medium preparation were routinely supplemented with the essential substances (gentamicin sulphate and L-glutamine). The complete medium were consisting of 60% (v/v) medium 199 with Hanks' salts, 25 mM HEPES buffer and 40% (v/v) heat-inactivated fetal bovine serum (FBS). The erythrocyte suspensions were deposited into 2 cm^2 flat-bottomed wells or into 25 cm^2 screw-top tissues culture flasks. Plates were placed in humidified plastic boxes and flask stood vertically before being gassed with 5% CO_2 in air and incubated at 37°C. Parasite numbers were recorded initially and every 24-48 hour, and smears were prepared with a cytocentrifuge. All smears were air-dried, fixed with methanol, stained for 40 min in 5% (v/v) solution of Giemsa stained and examined under a compound light microscope. When blood was taken for culture establishment on selected days post-splenectomy, the majority of the parasitized erythrocytes contained single piroplasms with less than 10% containing 2, 3 or rarely 4 piroplasms. In cultures established with blood from infected cattle 20-60% of single piroplasms was divided into quadruplet forms by day 6 *in vitro*. Transmission electron microscopic studies of *T. annulata* in culture showed that piroplasms possess intracytoplasmic food vacuoles and cytostomes during a pre-division trophozoite stage. The onset of intraerythrocytic multiplication was marked by the appearance of rhoptries and electron-dense plaques

beneath the parasite's plasmalemmal membrance. The plaques developed into short segments of subplasmalemmal double membranes, which were closely associated with the rhoptries. It was concluded that multiplication of *T. annulata* in erythrocytes occurred by schizogony, as nuclear division preceded cytoplasmic division and the final separation of merozoites. The four merozoites produced by intraerythrocytic schizogony had the same ultrastructural features as the *T. annulata* merozoites produced by intralymphotic schizogony. Clusters of four merozoites, identical to those observed in stationary cultures, were also seen in the erythrocytes of persistently infected cattle and appeared to represent the most significant dividing forms of *T. annulata in vitro.* In the same year, the mode of intraerythrocytic multiplication of *T. parva* was also studied by light and electron microscopy (15). Piroplasms of *T. parva* isolate from Mugaga, Kenya were maintained in the short-term stationary erythrocyte cultures. The method of this cultivation is similar to the cultivation of *T. annulata.* From their study, division of single piroplasms into four intraerythrocytic merozoites was observed within the first 24 h *in vitro.* After 8 days of cultivation, 10-20% of parasitized erythrocytes contained quadruplet merozoite forms resulting from multiplication *in vitro.* Electron micrographs showed that intraerythrocytic multiplication occurred by a schizogonous process that proceeded from the initial formation of rhoptries and electron-dense cytoplasmic plagues beneath the parasite's plasmalemmal membrane, to nuclear division and the final separation of a maximum of four differentiated merozoites. The merozoites formed by intraerythrocytic schizogony *in vitro* had ultrastructural features identical to merozoites produced by intralymphocytic schizonts of *T. parva.* The short-terms cultivation of piroplasm stage had applied to studying invasion of bovine erythrocytes by *T. sergenti* piroplasm *in*

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vitro. Merozoite invasion assays were carried out *in vitro* assay for the above (4,5). Isolated parasites were cultured in minimum essential medium (MEM) added with 10% heat-inactivated fetal calf serum (FCS). Washed intact erythrocytes were suspended in MEM with 10% FCS to give 5% cells to the medium. Ten parts of free parasite suspensions were mixed with 48 and 72 hours. A few drops of the mixture were sampled from the preparations at each incubation time, and by transmission electron microscopy (TEM). The isolated piroplasm of *T. sergenti* completely invaded erythrocytes *in vitro* within 10 min. Most of parasites that invaded blood cells were the ring form. The observation by TEM revealed that piroplasms completely invaded erythrocytes, however, the rate of erythrocytes invaded by the parasites was extremely low. Piroplasms consistently penetrated into the erythrocyte by the nuclear hemisphere during the invasive process. At present, the continuous culture of the erythrocytic stage of *Theileria* parasites have not been achieved yet. Since the continuous *in vitro* cultivation of *Babesia bigemina* has been reported (123); therefore, this method would lend to the cultivation of *Theileria* parasite under similar conditions. For *Babesia sp.* cultivation, infected blood was collected from an infected calf, defribinated by shaking with glass bead and propagated *in vitro*. Culture conditions included washing of infected and normal bovine erythrocytes in a Vym's solution, and the use of a 5% to 10% (v/v) erythrocyte suspension in Medium 199 supplemented with 20% to 50% fresh normal bovine serum. Sterile, disposable 24-well tissue-culture cluster plates were used throughout the experiments. Top and bottom (A and D) rows in the plates were filled with 1.5 ml of sterile tap water to prevent excessive evaporation. The final infected normal RBC suspensions were distributed in duplicate wells. Plates were incubated at 37 ± 0.1 °C in a 5% CO_2, 2% O_2, 93% N_2 atmosphere. Spent medium was

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collected and replaced with freshly prepared serum-supplemented media at 24 hours intervals. Culture samples (3 to 5 μl) were obtained daily at the time observation. This *in vitro* procedure of *B. bigemina* with slight modification will be applied to establish the short-term cultivation of piroplasm stage of Thai isolate *Theileria* species and to screen for new anti-theilerial drug for local use.

2.3 Assessment of *in vitro* drug sensitivity assay of Thai *Theileria* parasite

2.3.1 Microscopic method

Drug sensitivity assay of malaria parasites was performed by using an *in vitro* micro-technic method (124). This method requires inexpensive equipment and short experimental time that makes it more feasible to apply in the filed. The micro-technique is performed on sterile flat-bottom tissue culture plates (96 well-microtitration plate) which are dosed with the testing drugs. The drug concentrations in the wells are the test doses of different drug concentrations. The parasitized cells used in the test are obtained from the continuous cultures. The period of incubation (124-126) is 24 h. The test is evaluated by quantitative parasite method after incubation period. The parasitized cells against 5,000 erythrocytes are counted. By reference to the number of survival parasites observed in control well (untreated culture), 50% inhibitory concentration (IC_{50}) is then calculated. This method will be applied for the drug sensitivity test of *Theileria* parasite in the present study.

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2.3.2 Radioisotopic method

Drug sensitivity assay of malaria parasite was also evaluated by using radioisotopic method (50). Semi-automated microdilution technic based on the use of radioisotope labeling and microtiter system was developed by Desjardins and co-worker (127). This method has been proved to provide a rapid and quantitative measurement of activities for certain testing compounds or medicinal plant extracts against cultured intraerythrocytic stage of *Theileria* parasite (50). Microtitration plates are used to prepare serial dilution of the compounds to be tested. Parasites are pre-cultured for 6 h and then the [^{3}H] hypoxanthine is added to the wells and further incubated for 24 h. At the end of second incubation period, the parasites are harvested and then the incorporation of [^{3}H] hypoxanthine into the nucleic acid of the parasites is measured by a liquid scintillation counter. The IC_{50} values were estimated from separate experiments using a wider range of drug concentrations.

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CHARTER III

MATERIALS AND METHODS

1. Experimental animals

Four naturally *Theileria* sp. infected calves, aged between 4 to 7 months, were used as sources of infected blood samples in this study. All calves were from Rattanapum District, Songklha Province, in the South of Thailand. They were singly infected with unclassified species of Thai isolate *Theileria.* The percentage of parasitemia was found to be varied from 0.01% to 0.05%. In order to achieve higher percentage of parasitemia for the *in vitro* culture, all calves were splenectomized at National Institute of Animal Health, Department of Livestock Development, Kaset Klang, Chatuchak, Bangkok by Dr. Nopporn Sarataphan. After 5 days of splenectomy, daily parasitemia was monitored by making thin blood smears from jugular's vein. The smears were fixed in absolute methanol and stained with Field stain (see Appendix 1). The percentage of parasitemia was determined microscopically under 100x objective lens. The percentage of parasitemia was followed up daily. Once the percentage of parasitemia reached 0.4-18% or higher, sixty milliliters of the infected blood samples would be collected for the study.

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2. Parasite isolates

Infected blood samples of unclassified species of Thai isolate *Theileria* parasite used for *in vitro* culture experiments were collected from splenectomized calves as already described in Materials and Methods 1. Fifty milliliters of blood sample was aseptically collected by jugular venipuncture into a sterile flask and defibrinated by shaking with glass beads. The blood sample was transported to Department of Microbiology, Faculty of Science, Mahidol University, Bangkok within 1-2 hours of the same day.

3. Culture media

Two types of culture media were used for cultivation of Thai isolate *Theileria* parasite. They were RPMI 1640 (GIBCO) containing L-glutamine but without sodium bicarbonate and Medium 199 (M199) with Hank's salts liquid (GIBCO). The first one was purchased as a powder formular while the second one was in a liquid formular. RPMI medium was prepared by dissolving 10.4 gm of RPMI 1640 powder, 1.5 gm of $NaHCO_3$, 25 mM *N*-12-hydroxyethylpiperrazine-*N*'-12-ethanesulfonic acid (HEPES) buffer (Sigma), 0.3 gm of L-glutamine, 2 μm hypoxanthine (Sigma) 1 mg of reduced glutathione (Sigma) and 50 mg of gentamicin sulfate (Sigma) with 960 ml of double distilled water and adjusted to pH 7.2-7.4 by using 1.0 N NaOH and 1.0 N HCl. Finally, the volume was brougth up to 1,000 ml and sterilized by filtration through a millipore filtration of 0.45 μm porosity. One hundred milliliters of the sterile media was transferred into sterile glass bottles, kept at 4-10°C and used within one month (see Appendix 1).

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4. Commercially prepared serum

Two types of sera were used in this study. They were fetal bovine serum (FBS) (Biochrome KG seromed®) and fetal calf serum (FCS) (Bio ◆ Whittaker). Both types of sera were inactivated at 56°C for 30 min in a waterbath and allowed to be cool at room temperature. Then, an approximate of 10-12 ml of each serum was distributed aseptically into sterile tubes, stored at -20°C and used within 6 months.

5. Working culture media

Before using for culture, both RPMI 1640 and M199 media were supplemented with 40% of either FBS or FCS (see Appendix 1). These working culture media were referred to as complete media with serum. All types of working culture media namely RPMI 1640+FCS, RPMI 1640+FBS, M199+FCS and M199+FBS were stored at 4 °C and had to be used within a week.

6. Non-infected bovine erythrocytes

Non-infected bovine erythrocytes used for adjusting the percentage of parasitemia and cell suspension were collected from healthy non-splenectomized calves that had no experience of *Theileria* infection. An approximate of 60 ml of blood was collected from the jugular vein into a sterile flask and defibrinated by shaking with glass beads. White blood cells were firstly removed from the blood samples by passing through a Whatman CF-11 cellulose column and washed once with 1x Vym's

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solution (see Appendix 1) by centrifugation at 1,000 g for 10 min at 15 °C. Supernatant and buffy coat were removed carefully, and packed erythrocytes were then resuspended with the equal volume of complete medium with serum to make 50%(v/v) of non-infected erythrocytes suspension. These non-infected erythrocytes were kept at 4°C and used within 1-2 months.

7. Establishment of short-term *in vitro* culture of *Theileria* parasite

7.1 Selection of working culture medium

This experiment was carried out in order to determine which one of the four types of working culture media is appropriate for culturing *Theileria* parasite. When the percentage of parasitemia of splenectomized calves reached 0.4%, (Materials and Methods 1). Sixty milliliters of infected blood sample was collected from the jugular vein into a sterile flask and defibrinated by shaking with glass beads. The method used for cultivation was based on that used for *Babesia* sp. (123) with slight modification. White blood cells were removed from the blood samples by passing through a Whatman CF11 cellulose column and washed once with 1x Vym's solution by centrifugation at 1,000 g for 10 min at 15°C. The packed erythrocytes were resuspended to make 50%(v/v) cell suspension in 4 types of working culture media namely, RPMI 1640+FCS, RPMI 1640+FBS, M199+FCS and M199+FBS. Then, the percentage of cell suspension was reduced from 50% to 10% with each type of working culture media. Each blood inoculum sample was finally dispensed in 1 ml aliquot into each of duplicate wells of 24 well-culture plates (Nunc^{TM}). Only the 8 inner wells were used for culture, whereas all the outer wells were added with sterile

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water to provide humidity. The culture plates were placed at 37°C in a 5% CO_2, humidified incubator, with a change of the corresponding medium (1 ml) at 24 h and at 48 h during 72 h of incubation. After the medium of each well was aspirated, the cells were mixed well, and 5 µl sample was taken from each well to make a thin blood smears which was fixed with absolute methanol and stained with Field stain. Duplicate thin blood smears were made from each well. Each smear was examined microscopically under 100x objective lens. The number of infected cells with various forms of parasites were counted against 5,000 erythrocytes and expressed as percentage. Duplicate counting was made for each slide. The number of erythrocytes containing dead parasites was also recorded. Changes of developmental stages and survival of parasites were compared among 4 different types of working culture media by using mean values. The working culture medium that provided the best result would be selected for using in the following experiments.

7.2 Observation of growth, development and survival of *Theileria* parasite during 120 h cultivation

The selected working culture medium from 7.1 was used for culture *Theileria* parasites for 120 h. The protocol of preparation the parasite inoculum was similar to that of 7.1. But the cultures were kept longer i.e., for 120 h. Evaluation of growth, development and survival of parasite were done in the same way as the previous experiment (7.1). In addition, the differential count of various forms (single piroplasm and multiplied piroplasm) of parasites against 5,000 erythrocytes was also determined. Mean values of all obtained data were used for statistical analysis.

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7.3 Observation of growth, development and survival of *Theileria* parasite in 24 h cultures initiated with varying percentage of parasitemia and cell suspension

Sixty ml of *Theileria* infected blood with up to 18% parasitemia was collected from splenectomized calf (see Materials and Methods 1). Fifty percent cell suspension in selected working culture medium was eventually prepared as already described (see Materials and Methods 7.1). The 50% cell suspension sample was then 2-fold diluted with 50%(v/v) of non-infected erythrocyte suspension (see Materials and Methods 6) to obtain varying percentage of parasitemia, i.e., 18%, 9%, 4.5% and 2.2% respectively. Each parasitemia sample was finally adjusted to 2-fold varying percentage of cell suspension, i.e., 50%, 25%, 12.5%, 6.3% and 3.2% respectively by using the selected working culture medium. One ml of each blood inoculum was dispensed into each of duplicate wells of 24 well-culture plates. The parasites were cultured for 24 h at 37°C in a 5% CO_2, humidified incubator. Duplicate thin blood smears were made from each well after discarding of supernatant. The number of infected cells harboring different stages of parasites were differentially count against 5,000 erythrocytes from thin blood smears. Growth, development and survival of parasites under various experimental conditions were compared by using mean values. Mean values of all obtained data were used for statistical analysis. The appropriate percentage of parasitemia and cell suspension were finally selected for the drug sensitivity assay.

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8. *In vitro* drug sensitivity assay

8.1 Testing drugs

Two antimalarial drugs, chloroquine (Sigma) and quinine (Sigma) and the other two anti-hemosporozoal drugs namely Berenil® (Hoechst, Germany) Imizol® (Cooper Animal Health., Ltd) were used in drug sensitivity assay of *Theileria* parasite. All drugs were diluted with selected working culture medium to initial concentration of 10 mM and filtered through 0.45 μM membrane before use (see Appendix 1).

8.2 Medicinal plant extracts

Medicinal plant extracts were prepared and provided by Associate Professor Kosum Chansiri of the Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University. Water extracts of different parts of the plants i.e. roots, stems and barks of Thai medicinal plants were used in this study. Selected five species of plants tested in this study included:

1. *Oroxylum indicum*, Linn. Kurz
2. *Alstonia scholaris*, Linn., R.Br.
3. *Barringtonia acutangula* Gaerth.
4. *Alyxia reinwardtii* BL. Var *lucida* Markgr.
5. *Vernonia cinerea* L., Less.

Stock solutions of medicinal plant extracts were prepared at a concentration 0.01 g/ml in the selected working culture medium and sterilized by filtration through a millipore filter of 0.45 μM porosity (see Appendix 1).

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8.3 Working solution

The stock solutions of testing drugs and medicinal plant extracts were two-fold serially diluted to desired concentrations by using selected working culture medium.

8.4 Microscopic method

Drugs sensitivity assay was performed in 96-well flat-microtitration plates (Nunc™) by using an *in vitro* micro-technic method (131), with slight modifications. Briefly stated, 50 µl of two-fold serial dilutions of working solutions of either testing and plant extracts were added into duplicate wells of 96- well flat-microtitration plate. Two wells were used as control wells by adding 50 µl of working culture medium. *Theileria* parasites were previously cultured in 24 well-culture plates for at least 18 h before being used in the assay. The 18 h-precultured infected cells were pooled washed and prepared to have an appropriate percentage of parasitemia and suspension in the selected working culture medium. Fifty µl of the prepared blood inoculum was added to both control and experimental wells. The microtitration plates were gently shaked by hand and placed at 37°C in a 5%CO_2, humidified incubator. After 24 h of incubation, thin blood smears were made from each well, fixed and stained with Filed stain. The number of infected cells containing living and dead parasites was counted against 100 parasitized cells. Each slide was examined twice in order to obtain quadruplet data from each concentration of working solution. The obtained data was used to determine the 50% inhibitory concentration (IC_{50}) by using the median effect method (135) (see Appendix 2).

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8.5 Radioisotopic method by [^{3}H] Hypoxanthine incorporation

Two tests were performed in 96-well flat-microtitration plates by using a semiautomated microdilution technique (52, 134). In each experiment, each drug concentration and control were performed in triplicate wells. Triplicate wells of drug-free or parasite-free control were also included in each experiment. Briefly stated, to each well containing 50 µl of working solution (as well as control solutions) is added 50 µl of 18 h precultured parasitized red blood cells (10% v/v suspension, 10% parasitemia) suspended in selected complete medium with serum. The microtitration plates were incubated for 6 h at 37°C in a 5%CO_2, humidified incubator. After 6 h of pre-incubation, 25 µl of [^{3}H] hypoxanthine (1.2 µM, 800 KBq/ml) (Amersham) was added on to each well of microtitration plates. After further incubation for 20 h, radioisotopically labeled cultures were harvested onto glass-fiber filter using distilled water and an automated multiple sample harvester (Skatron Combi Cell Harvester). Each filter was transferred into a small plastic tube containing 3 ml of scintillation fluid (Millipore, Bedford, USA). Each plastic tube was placed in a glass vial and ready for radioisotopic measurement. The incorporation of [^{3}H] hypoxanthine into the nucleic acids of the parasites was measured by LS 6000TA BETA counter. Data analysis was applied to the concentration-response data to determined the 50% inhibitory concentration (IC_{50}), defined as the drug concentration at which 50% decrease either in the growth parasites or in the uptake of [^{3}H] hypoxanthine was observed in comparison with the drugs-free control well (see Appendix 2).

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9. Statistical analysis

Comparison of growth and development and survival of *Theileria* parasite using mean number of parasitized cell with living and dead parasites, with multiplied piroplasm forms in Experiments 1 and 2 were done by Firedman test. In Experiment 3, comparison of growth and development and survival of *Theileria* parasite using mean number of parasitized cell with living and dead parasites, with multiplied piroplasm forms were evaluated by Independent Samples T-test. In Experiment 4, the differences of the IC_{50} values of each testing drug between the microscopic and radioisotopic methods were compared by using Independent Samples T-test. The significance of statistical test was set at $p<0.05$.

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CHAPTER IV

RESULTS

1. Growth, Development and Survival of Thai Isolate *Theileria* sp. in Four Different Types of Culture Media

Comparison of growth, development and survival of Thai isolate *Theileria* parasite in *in vitro* culture (0.4% parasitemia, 10% cell suspension) using four different types of culture media namely RPMI 1640+FCS, RPMI 1640+FBS, M199+FCS and M199+FBS were investigated for 72 hours. The results of the experiments were as follow:

1.1 RPMI 1640+FCS medium

The mean number of parasitized cells with various forms of *Theileria* counted against 5,000 erythrocytes from thin blood smears made at H0, H24, H48 and H72 after cultivation in RPMI 1640+FCS medium was summarized in Table 2. The percentage of parasitemia based on various forms inside the erythrocytes were shown as histograms in Fig. 9. The mean numbers of parasitized cells with living parasites and dead parasites as noted in Table 2 were also depicted in percentage in Fig. 10.

It was clearly demonstrated from both Table 2 and Fig. 9 that the parasites could develop and change from match form to ring form and 2 piroplasm form after 24 h of cultivation in RPMI 1640+FCS medium. The percentage of match form presented

at H0 i.e. 0.38% was remarkably decreased to only 0.11% after H24. The appearance of ring form was observed in culture at the level of 0.05%. An increase in number of 2 piroplasm form up to 0.45% was also recorded during this period of observation. However after H48, no normal or healthy living parasites were found in the culture and only abnormal or dead parasites were observed. It could be seen from Fig. 10 that the percentage of living parasites apparently started to decrease after 24 h of cultivation (from 0.41% to 0.21%) and none of living parasites was detected after H48. The percentage of dead parasites (0.15%) was sustained at during H48-H72. The pictures showing the morphology of match, ring and 2 piroplasm forms were shown in Fig. 11.

1.2 RPMI 1640+FBS medium

The mean number of parasitized cells with various forms of *Theileria* parasite counted against 5,000 erythrocytes from thin blood smears made at H0, H24, H48 and H72 after cultivation in RPMI 1640+FBS medium was summarized in Table 3. The percentage of parasitemia based on various forms inside the erythrocytes were shown as histograms in Fig. 12. The mean numbers of parasitized cells with living parasites and dead parasites as noted in Table 3 were also expressed in percentage in Fig. 13.

Growth and development of *Theileria* parasites observed from culture using RPMI 1640+FBS medium was similar to the results obtained by using RPMI 1640+FCS. After H24, a change from match form into ring form was clearly recognized (Fig. 12 and Table 3). As shown in Fig. 12, ring form was firstly observed at 0.19% after H24. A slight increase in percentage number of 2 piroplasm form from 0.03% to 0.04% was also observed during this period of cultivation. There was a

remarked decrease in percentage of living parasites after H48; however, with this type of medium, a small number of living parasites (match form) were still present in the culture at the level of 0.02%. Similar to the result obtained with RPMI 1640+FCS, no living parasites were found in the culture after 72 h of cultivation.

1.3 M199+FCS medium

The mean number of parasitized cells with various forms of *Theileria* counted against 5,000 erythrocytes from thin blood smears made at H0, H24, H48 and H72 after cultivation in M199+FCS medium was summarized in Table 4. The percentage of parasitemia based on various forms inside the erythrocytes were shown as histograms in Fig. 14. The mean numbers of parasitized cell with living parasites and dead parasites as noted in Table 4 were also presented in percentage in Fig. 15.

As shown in Fig. 14, dramatic changes from match form into ring form, 2 piroplasm and 3 piroplasm of *Theileria* parasites was observed by using M199+FCS medium. It should be noted that 3 piroplasm form (0.02%) was found for the first time with this type of medium after 24 h of incubation. However, this form disappeared after 48 h of incubation. With this type of medium ring form was continually detected until H72. A gradual decrease of living parasites was observed at H24 through H72 (Fig. 15). However, the presence of a small number of ring form at H72 could maintain the percentage of living parasites at the level of 0.02%. Fig. 11 also included the picture of the morphology of 3 piroplasm form.

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1.4 M199+FBS medium

The mean number of parasitized cells with various forms of *Theileria* parasite counted against 5,000 erythrocytes from thin blood smears made at H0, H24, H48 and H72 after cultivation in M199+FBS medium was summarized in Table 5. The percentage of parasitemia based on various forms inside the erythrocytes were shown as histograms in Fig. 16. The mean numbers of parasitized cell with living parasites and dead parasites as noted in Table 5 were also illustrated in percentage in Fig. 17.

A pronounced change in developmental stages of *Theileria* parasites was observed after H24 in M199+FBS medium (Table 5 and Fig.16). All forms of *Theileria* parasites were observed with this type of medium after 24 h of cultivation (Fig. 16). Match form, ring form, 2 piroplasm form, 3 piroplasm form and 4 piroplasm form were found at 0.05%, 0.15%, 0.07%, 0.05% and 0.02% respectively (Fig. 16). The first finding of 4 piroplasm form or quadruplet form with this type of medium was observed at H24. All five forms of *Theileria* parasites were present at significant amount in the culture throughout 72 h of observation (Table 5 and Fig. 16). Although the percentage of living parasites slightly decreased after H24 (from 0.41% to 0.34%), it was brought up and sustained at 0.35%-0.37% during H48-H72. No dead parasites were observed in the culture during 72 h of incubation (Fig. 17). The picture showing the morphology of 4 piroplasm form (quadruplet form) was shown in Fig. 11. The extracellular theilerial parasites which were observed with all types of culture media were also illustrated in Fig. 11.

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The results (1.1-1.4), clearly demonstrated that the medium M199+FBS was proved to be the most appropriate medium for culturing Thai isolate *Theileria* parasites. By using this type of medium, the parasites could develop and change from match form to ring, 2 piroplasm, 3 piroplasm and 4 piroplasm forms (quadruplet form) after H24. Although the percentage of parasitemia (percentage of total forms of living parasites) did not increase during 72 h of cultivation, all forms of parasites were apparently healthy by showing normal appearance in their morphology. In addition, statistical analysis revealed that the mean number of living parasites against 5,000 erythrocytes in M199+FBS medium was significantly higher than in the other working culture media ($p<0.05$). Therefore, the M199+FBS medium was selected to be used in the following assays in this study. Since it took more than one hour to complete counting for each slide according to the low percentage of starting parasitemia, i.e., 0.4%. Therefore, the following experiment was performed by starting the culture with higher percentage of parasitemia, i.e., 6% and the incubation period was prolonged up to 120 hours.

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Table 2 The number of parasitized cells ($\bar{X}\pm SD$) with various forms of *Theileria* counted against 5,000 erythrocytes during 72 h of cultivation in RPMI 1640+FCS medium

Hour of culture	Parasitized cells against 5,000 erythrocytes					Parasitized cells against 5,000 erythrocytes	
	match	ring	2 piroplasm	3 piroplasm	4 piroplasm	Living parasite	Dead parasite
H0	18.8±0.5	0	1.5±0.6	0	0	20.3±1.0	0
H24	5.5±0.6	2.5±0.6	2.3±0.5	0	0	10.3±0.5	0
H48	0	0	0	0	0	0	7.3±0.5
H72	0	0	0	0	0	0	7.3±0.5

Table 3 The number of parasitized cells ($\bar{X}\pm SD$) with various forms of *Theileria* counted against 5,000 erythrocytes during 72 h of cultivation in RPMI 1640+FBS medium

Hour of culture	Parasitized cells against 5,000 erythrocytes					Parasitized cells against 5,000 erythrocytes	
	match	ring	2 piroplasm	3 piroplasm	4 piroplasm	Living parasite	Dead parasite
H0	18.8±0.5	0	1.5±0.6	0	0	20.3±1.0	0
H24	1.5±0.6	9.3±0.5	2.0±0.0	0	0	12.8±0.5	0
H48	1.0±0.8	0	0	0	0	1.0±0.8	6.8±0.5
H72	0	0	0	0	0	0	8.5±0.6

Table 4 The number of parasitized cells ($\bar{X}\pm SD$) with various forms of *Theileria* counted against 5,000 erythrocytes during 72 h of cultivation in M199+FCS medium

Hour of culture	Parasitized cells against 5,000 erythrocytes					Parasitized cells against 5,000 erythrocytes	
	match	ring	2 piroplasm	3 piroplasm	4 piroplasm	Living parasite	Dead parasite
H0	18.8±0.5	0	1.5±0.6	0	0	20.3±1.0	0
H24	4.3±0.5	5.5±0.6	2.5±0.6	0.8±0.5	0	13.0±0.8	0
H48	0	2.3±1.0	0	0	0	2.3±1.0	4.3±0.5
H72	0	1.0±0.0	0	0	0	1.0±0.0	4.3±0.5

Table 5 The number of parasitized cells ($\bar{X}\pm SD$) with various forms of *Theileria* counted against 5,000 erythrocytes during 72 h of cultivation in M199+FBS medium

Hour of culture	Parasitized cells against 5,000 erythrocytes					Parasitized cells against 5,000 erythrocytes	
	match	ring	2 piroplasm	3 piroplasm	4 piroplasm	Living parasite	Dead parasite
H0	18.8±0.5	0	1.5±0.6	0	0	20.3±1.0	0
H24	2.5±0.6	7.5±0.6	3.5±0.6	2.5±0.6	0.8±0.5	16.8±0.5	0
H48	2.5±0.6	2.5±0.6	3.5±0.6	4.5±0.6	4.8±0.5	17.8±0.5	0
H72	3.5±0.6	2.3±0.5	2.5±0.6	4.8±0.5	5.3±0.5	18.3±1.0	0

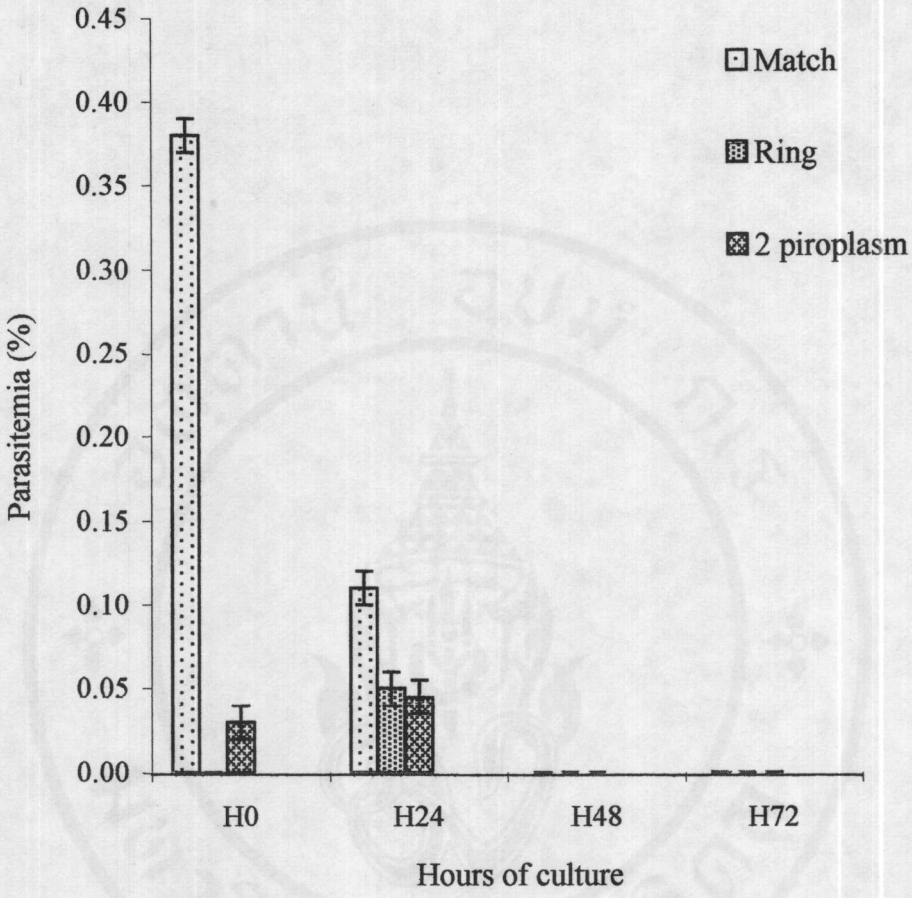

Figure 9 Histograms of the percentage ($\bar{X}\pm SD$) of parasitized cells with various forms of *Theileria* parasite during 72 h of cultivation in RPMI 1640+FCS medium

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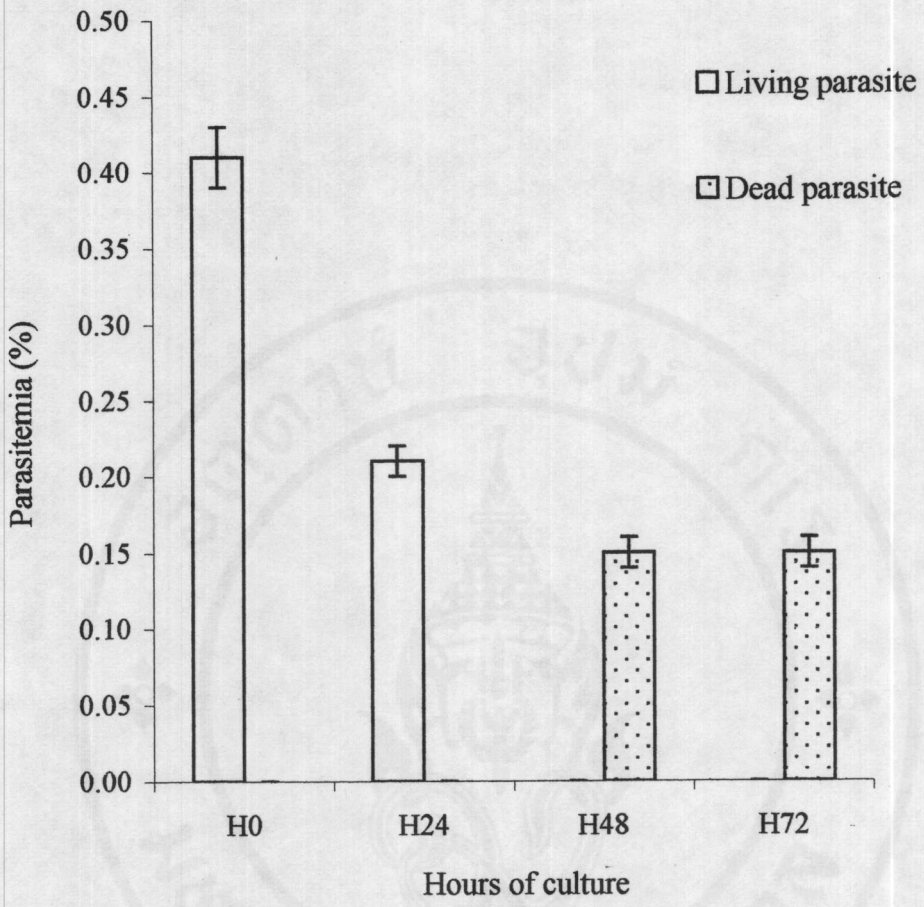

Figure 10 Histograms of the percentage ($\overline{X}\pm SD$) of parasitized cells with living and dead parasites during 72 h of cultivation in RPMI 1640+FCS medium

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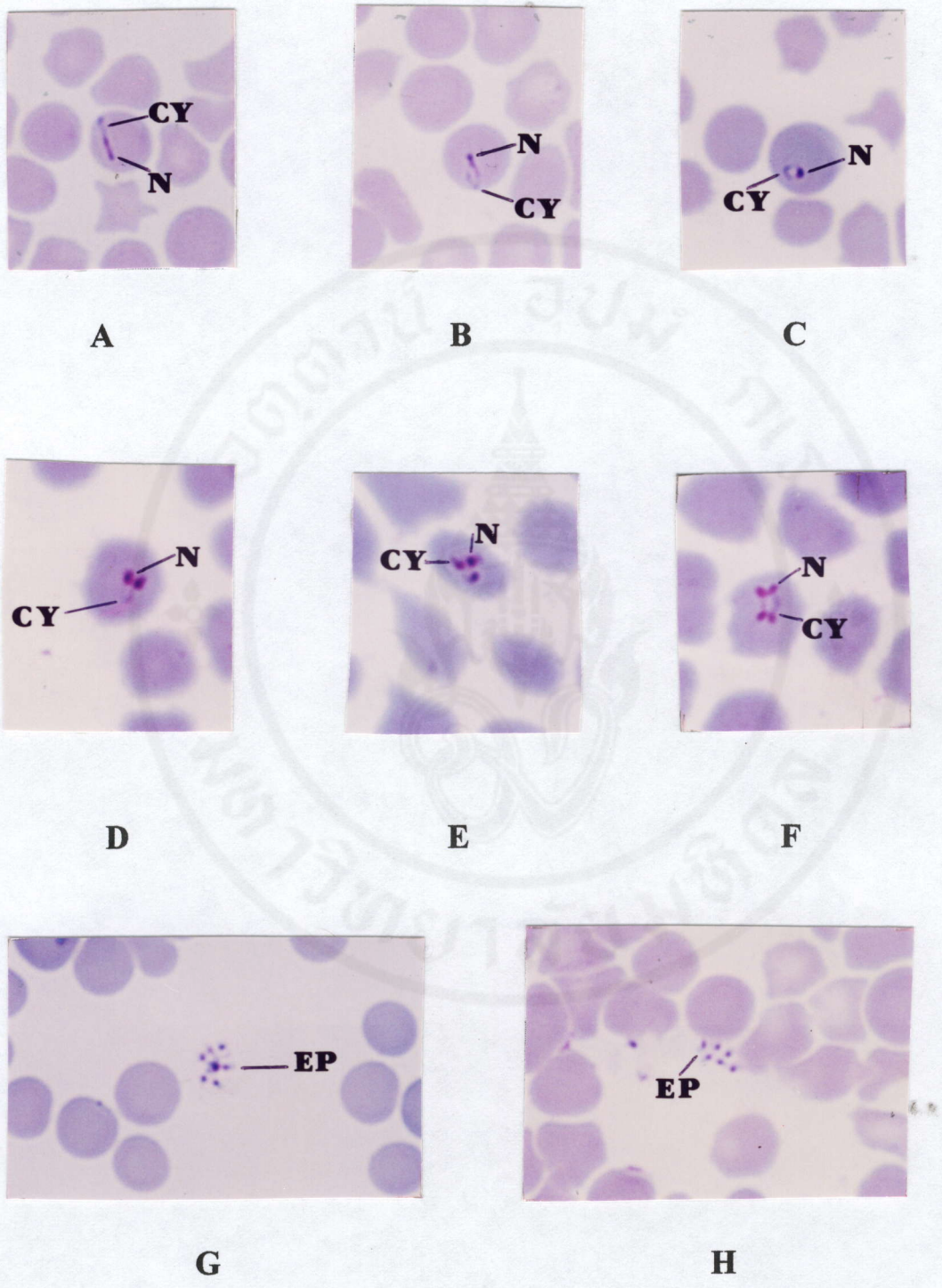

Figure 11 Intraerythrocytic forms (piroplasms) of *Theileria* sp. from the culture. Match form (A); Ring (B and C); 2 piroplasm form (D); 3 piroplasm form (E); 4 piroplasm form (F) and the extracellular piroplasm (G and H) (N = nucleus; Cy = cytoplasm; EP = extracellular piroplasm)

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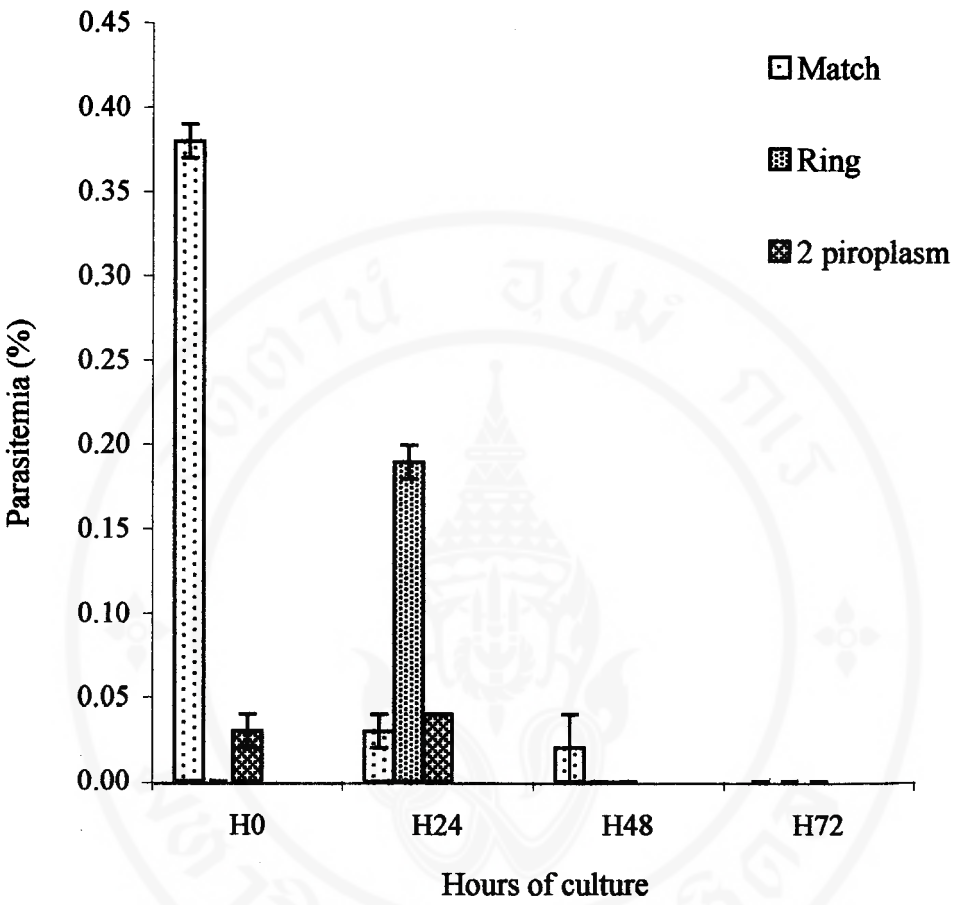

Figure 12 Histograms of the percentage ($\bar{X}\pm SD$) of parasitized cells with various forms of *Theileria* parasite during 72 h of cultivation in RPMI 1640+FBS medium

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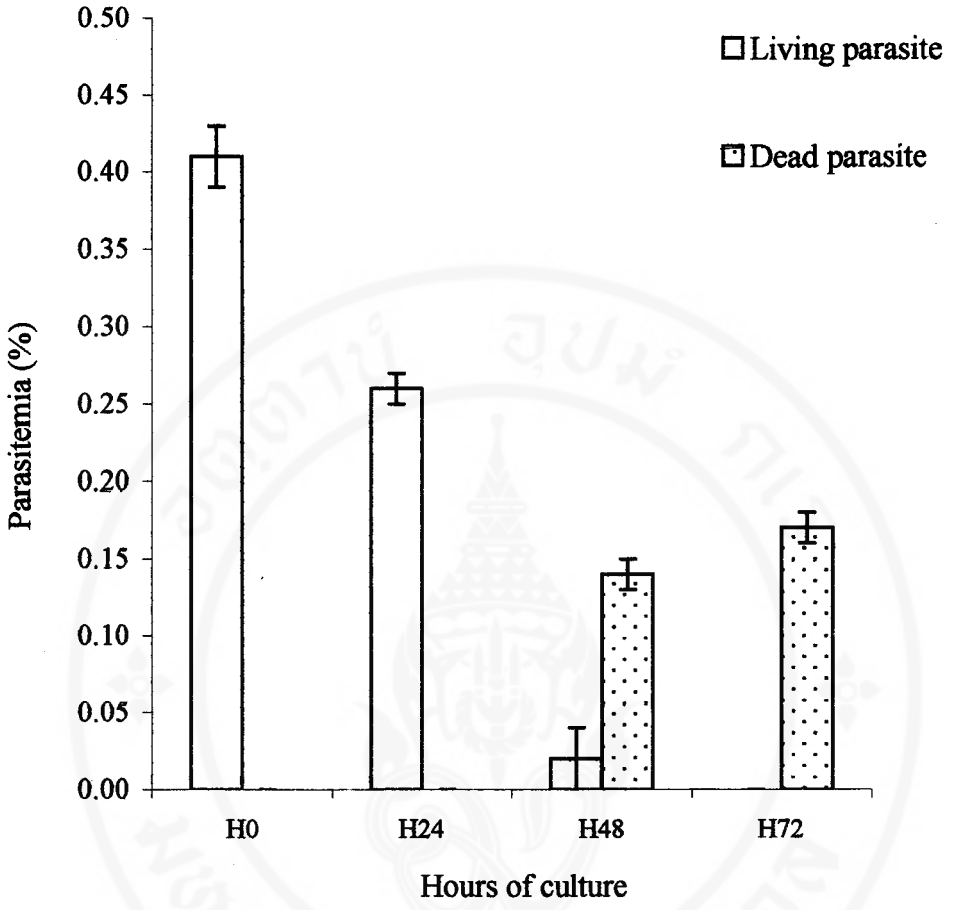

Figure 13 Histograms of the percentage ($\bar{X}\pm SD$) of parasitized cells with living and dead parasites during 72 h of cultivation in RPMI 1640+FBS medium

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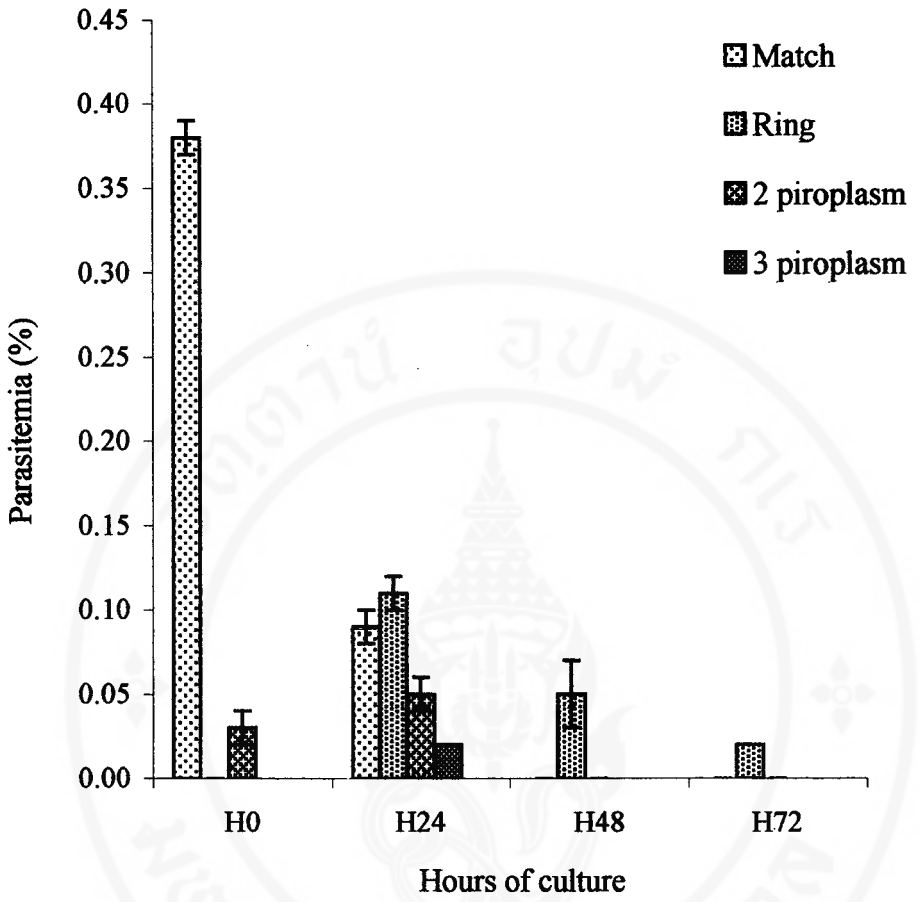

Figure 14 Histograms of the percentage ($\bar{X}\pm SD$) of parasitized cells with various forms of *Theileria* parasite during 72 h of cultivation in M199+FCS medium

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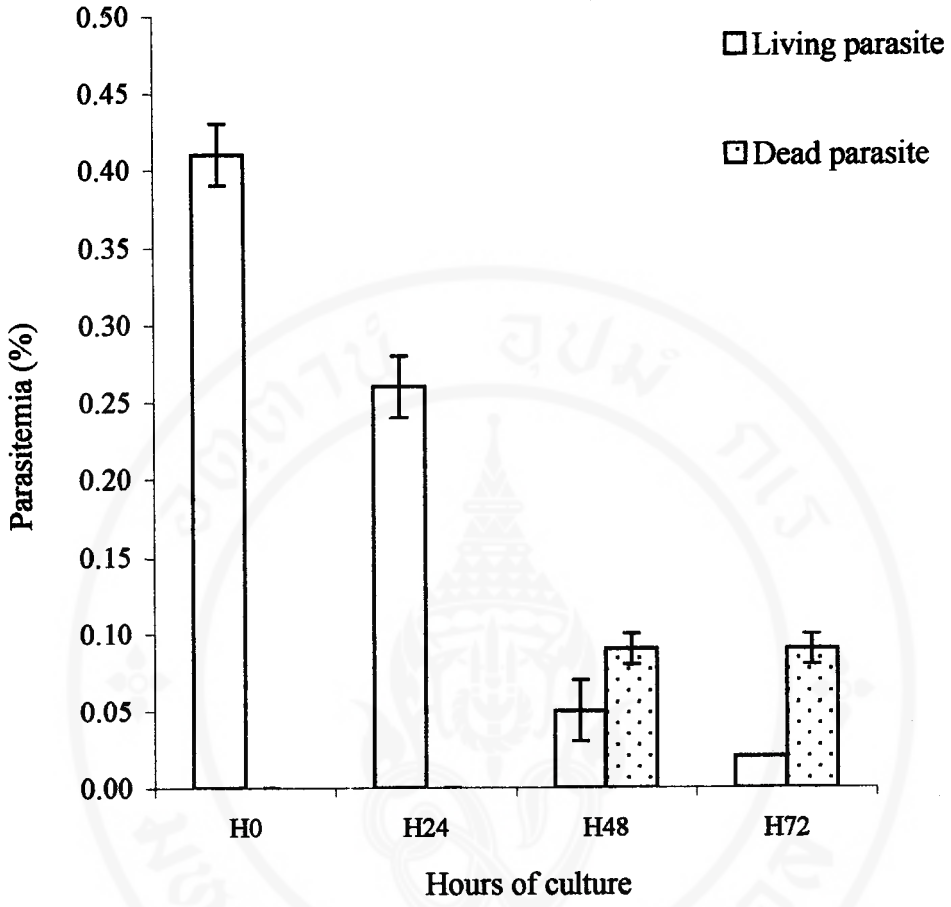

Figure 15 Histograms of the percentage ($\bar{X}\pm SD$) of parasitized cells with living and dead parasites during 72 h of cultivation in M199+FCS medium

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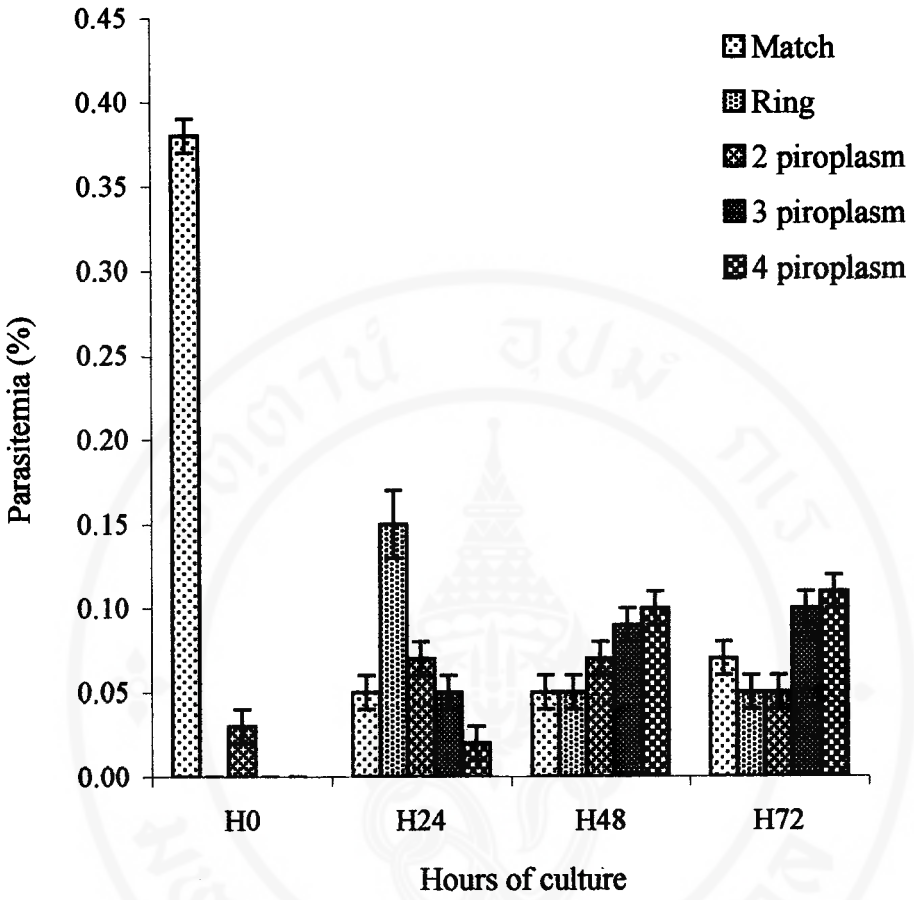

Figure 16 Histograms of the percentage ($\bar{X}$±SD) of parasitized cells with various forms of *Theileria* parasite during 72 h of cultivation in M199+FBS medium

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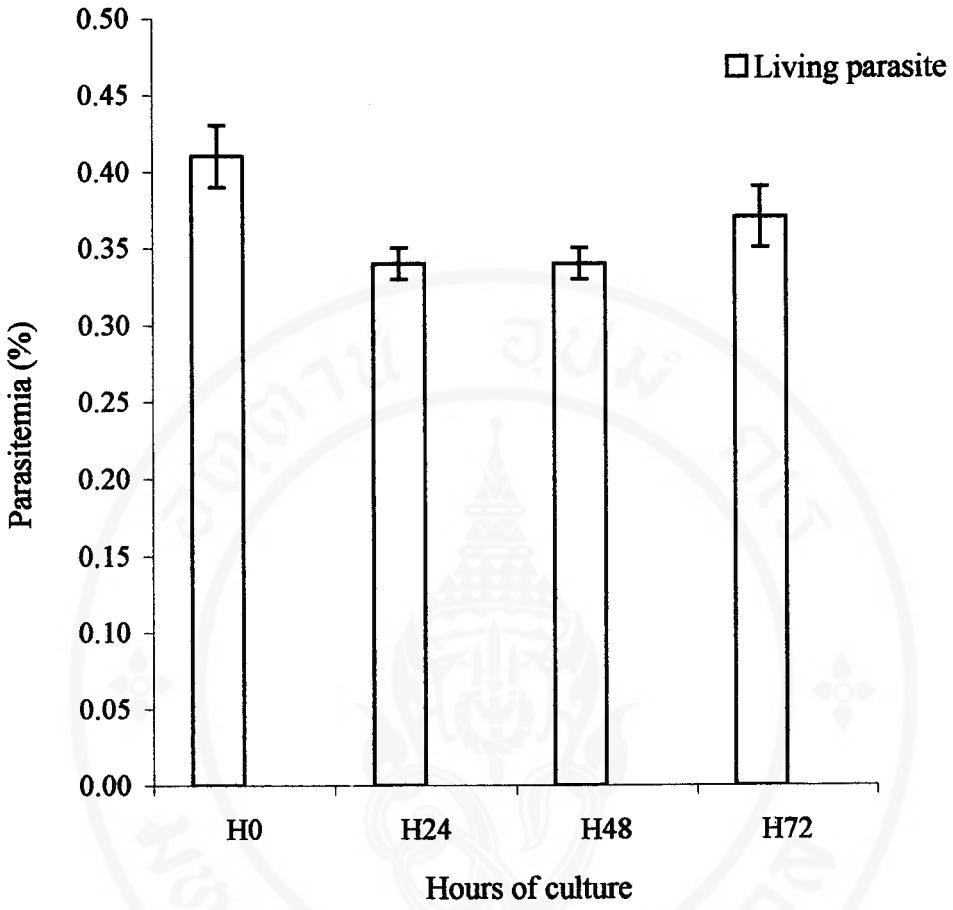

Figure 17 Histograms of the percentage ($\bar{X}\pm SD$) of parasitized cells with living parasite during 72 h of cultivation in M199+FBS medium

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2. Growth, Development and Survival of Thai isolate *Theileria* sp. in M199+FBS medium during 120 h of Cultivation

The observation of growth, development and survival of *Theileria* parasite (6% parasitemia, 10% cell suspension) was conducted in M199+FBS medium for 24 h, 48 h, 72 h, 96 h and 120 h of cultivation. The results of the experiment were as follow:

The mean numbers of parasitized cells with single piroplasm and multiplied piroplasm forms (2 piroplasm, 3 piroplasm and quadruplet forms) against 5,000 erythrocytes was summarized in Table 6. Parasitized cells with living parasites and dead parasites against 5,000 erythrocytes were also shown in Table 6. Parasitized cells with living and dead parasites, and with single piroplasm and multiplied piroplasm forms were also expressed as percentage in Fig. 18 and Fig. 19, respectively. The mean numbers of parasitized cells with living parasites observed at H24, i.e., 166$\pm$1.29 and H48, i.e., 183.3$\pm$4.75 as shown in Table 6 and Fig. 18 were significantly higher than the other periods (p<0.05) and the mean numbers of parasitized cells with dead parasites during these periods, i.e., 9.0$\pm$0.82 and 12.3$\pm$2.22, respectively (Table 6 and Fig. 18) of cultivation were significantly lower than the other periods (p<0.05). By comparing the mean numbers of parasitized cells with multiplied piroplasm forms during different periods of cultivation, it was also found that those numbers observed at H24, i.e., 114.3$\pm$1.5 and H48, i.e., 126.5$\pm$3.42 were significantly higher than the other periods (p<0.05).

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The data obtained from this experiment using starting parasitemia at 6% revealed that the period of cultivation that provided the optimal growth and development of parasites were H24 and H48 according to the higher percentage of

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parasitized cells with living parasites and multiplied piroplasm form. Therefore, an incubation period of 24-48 hours was selected for pre-cultivation of the parasites in the drug sensitivity assay.

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Table 6 The number of parasitized cells ($\bar{X}$ ±SD) with single piroplasm and multiplied piroplasm forms of *Theileria* parasite counted against 5,000 erythrocytes during 120 h of cultivation

Hours of culture	Parasitized cells against 5,000 erythrocytes		Parasitized cells against 5,000 erythrocytes	
	Single piroplasm	Multiplied piroplasm	Living parasite	Dead parasite
0	209.30±2.63	86.30±2.50	296.00±4.93	7.25±1.26
24	52.30±1.71	114.30±1.50	166.50±1.29	9.00±0.82
48	56.80±3.60	126.50±3.42	183.30±4.75	12.30±2.22
72	52.00±0.00	102.50±1.73	154.50±1.73	16.50±1.29
96	47.90±1.65	92.00±3.56	139.90±2.10	72.30±2.22
120	11.00±2.00	57.80±2.06	70.00±2.16	113.00±3.16

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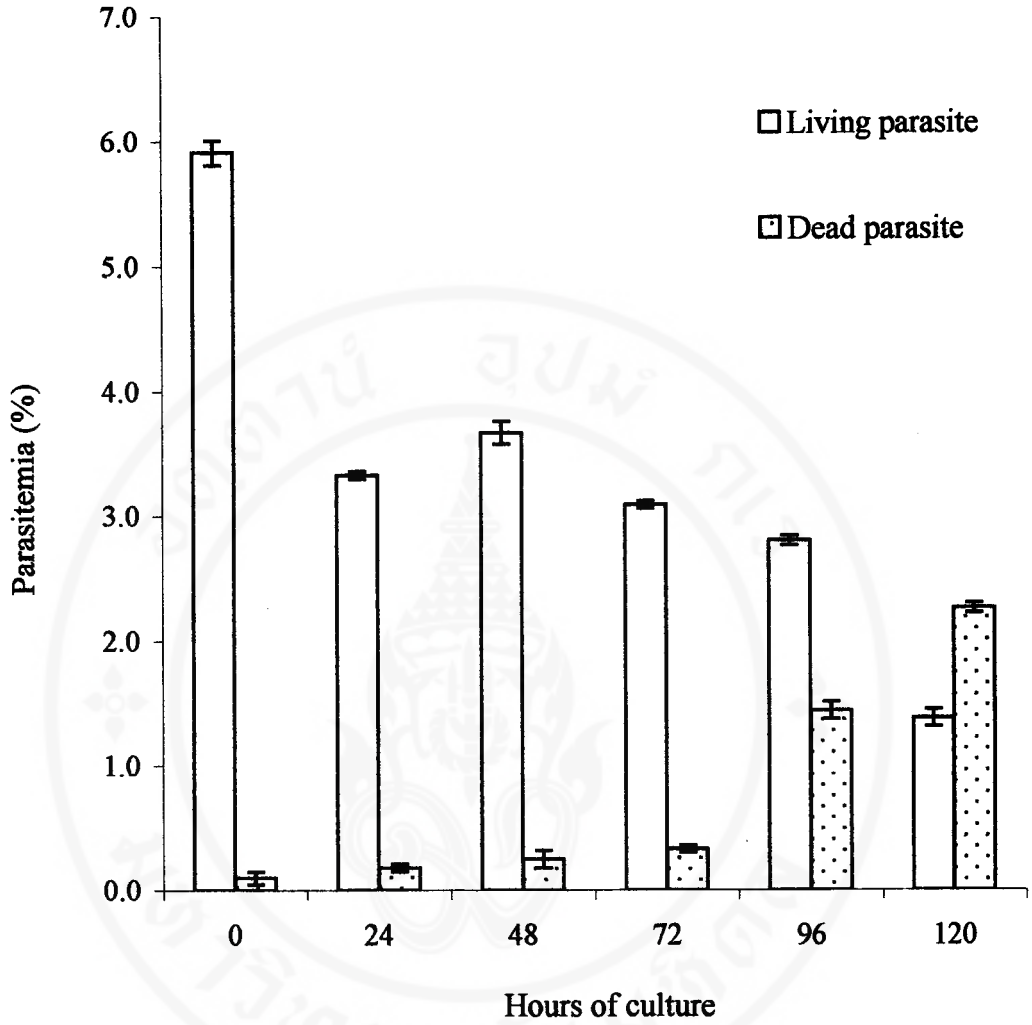

Figure 18 Histograms of the percentage ($\bar{X}\pm SD$) of parasitized cells with living and dead parasites during 120 h of cultivation

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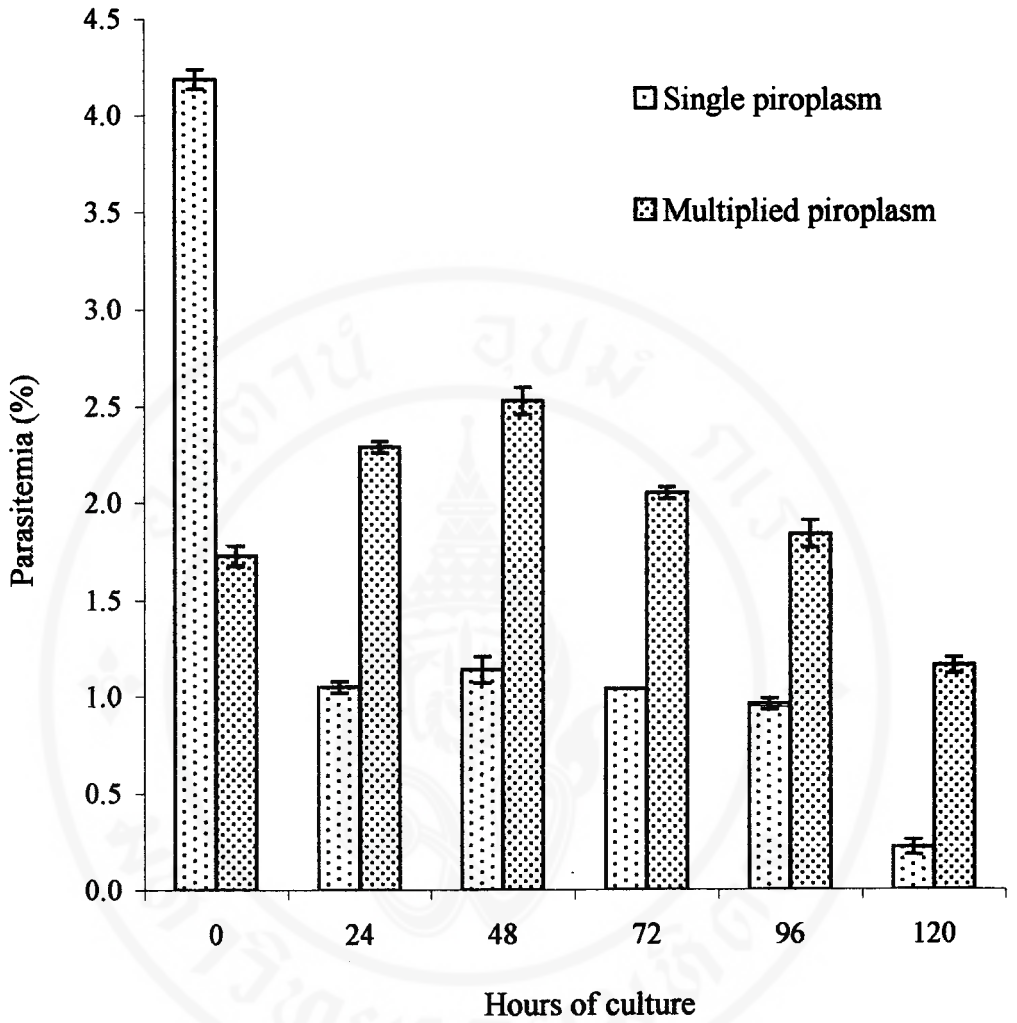

Figure 19 Histograms of the percentage ($\bar{X}\pm SD$) of parasitized cells with single piroplasm and multiplied piroplasm forms of *Theileria* parasite during 120 h of cultivation

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3. Growth, Development and Survival of Thai Isolate *Theileria* sp. Cultured in M199+FBS Medium at Varying Percentages of Parasitemia and Cell Suspension

The observation for growth, development and survival of *Theileria* parasite was conducted in M199+FBS medium with 2-fold varying percentages of starting parasitemia, i.e., 18%, 9%, 4.5% and 2.2%, respectively. At each percentage of started parasitemia, the percentage of cell suspension used for the cultivation was 2-fold diluted from 50% to 3.2%. The results were evaluated after 24 h of cultivation as follow:

The mean numbers of parasitized cells with single piroplasm and multiplied piroplasm forms (2 piroplasm, 3 piroplasm and quadruplet form), and with living parasites and dead parasites against 5,000 erythrocytes were summarized in Tables 7-10 and also expressed as percentage in Figs. 20-27.

The mean percentage of living and dead parasites and the mean percentage of single and multiplied piroplasm forms of the culture started with 2.2% parasitemia at 2-fold varying percentage of cell suspension, i.e., 50%, 25%, 12.5%, 6.3% and 3.2% are shown in Figs. 20 and 21, respectively. As shown in Fig. 20, the result clearly demonstrated that each percentage of cell suspension exhibited varying percentage of living parasites. The highest of percentage of living parasites was found in culture started at 12.5% cell suspension ($p<0.05$). Varying percentage of cell suspension also resulted in varying percentage of multiplied piroplasm forms as illustrated in Fig. 21. The highest of percentage of multiplied piroplasm forms was also found in culture started at 12.5% cell suspension.

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The results obtained from culture started with 4.5% parasitemia were similar to those of culture with 2.2% parasitemia (Figs. 22 and 23). The highest percentage of living parasites and multiplied piroplasm forms were observed at 12.5% cell suspension. However, it was interesting to record that the mean percentage of living parasites at 50% cell suspension was the lowest ($p<0.05$). Moreover, the mean percentage of multiplied piroplasm forms at 25% and 50% cell suspensions were also found to be lower than the other cell suspensions ($p<0.05$).

The results obtained by using starting parasitemia at 9% as shown in Fig. 24 revealed a remarked reduction of mean percentage of living parasites and multiplied piroplasm forms at 25% and 50% cell suspensions ($p<0.05$). Similar to the previous results, culture at 12.5% cell suspension still provided the highest percentage of living parasites and multiplied piroplasm forms ($p<0.05$) (Figs. 24 and 25). It should be noted that the mean percentage of living parasites and multiplied piroplasm forms observed at 6.3% cell suspension were more closer to 12.5% than 3.2% cell suspensions.

The results of culture started with 18% parasitemia were generally similar to culture started at 9% parasitemia (Figs. 26 and 27). Significantly higher percentage of living parasites were found in the culture started at 3.2%, 6.3% and 12.5% cell suspensions than the other cell suspensions ($p<0.05$) (Fig. 26). Same as percentage of living parasites, the percentage of multiplied piroplasm forms with culture started at these three cell suspensions were significantly higher than the others cell suspension as shown in Fig. 27 ($p<0.05$). Statistical test still indicated that at 12.5% cell suspension yielded the highest percentage of both living parasites and multiplied piroplasm forms.

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The results from this experiment clearly demonstrated that the highest percentage of living parasites and multiplied piroplasm forms were achieved from cultures started at 12.5% cell suspension. Thus, the drug sensitivity assay of theilerial parasite in the following experiments was conducted with culture started with 4.5%-18% parasitemia and with cell suspension more or less closed to 12.5%.

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Table 7 The number of parasitized cells ($\overline{X} \pm$ SD) with single and multiplied piroplasm forms of *Theileria* parasite counted against 5,000 erythrocytes after 24 h of cultivation with 2.2% parasitemia at varying cell suspension

Cell suspension (%)	Parasitized cells against 5,000 erythrocytes		Parasitized cells against 5,000 erythrocytes	
	Single piroplasm	Multiplied piroplasm	Living parasite	Dead parasite
3.2	56.00±3.60	43.75±4.50	99.75±2.06	5.00±0.82
6.3	69.50±2.88	48.75±1.89	118.25±2.63	5.75±0.50
12.5	85.25±2.62	54.50±1.91	139.75±2.63	3.25±0.96
25.0	87.00±2.00	34.75±2.06	121.75±1.50	7.25±0.96
50.0	81.50±3.87	44.00±1.82	125.50±2.52	6.00±0.82

Table 8 The number of parasitized cells ($\overline{X} \pm$ SD) with single and multiplied piroplasm forms of *Theileria* parasite counted against 5,000 erythrocytes after 24 h of cultivation with 4.5% parasitemia at varying cell suspension

Cell suspension (%)	Parasitized cells against 5,000 erythrocytes		Parasitized cells against 5,000 erythrocytes	
	Single piroplasm	Multiplied piroplasm	Living parasite	Dead parasite
3.2	38.00±2.45	53.25±1.26	121.25±1.89	4.00±0.82
6.3	71.75±2.21	59.30±2.46	131.05±2.25	1.75±1.26
12.5	71.50±2.38	96.00±1.41	167.50±1.91	10.50±1.29
25.0	95.75±1.71	35.75±0.96	131.50±1.00	6.25±0.96
50.0	43.00±2.71	38.50±1.29	81.50±2.65	13.75±0.96

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Table 9 The number of parasitized cells ($\overline{X} \pm SD$) with single and multiplied piroplasm forms of *Theileria* parasite counted against 5,000 erythrocytes after 24 h of cultivation with 9% parasitemia at varying cell suspension

Cell suspension (%)	Parasitized cells against 5,000 erythrocytes		Parasitized cells against 5,000 erythrocytes	
	Single piroplasm	Multiplied piroplasm	Living parasite	Dead parasite
3.2	172.50±0.57	103.25±2.22	275.75±1.89	12.50±1.29
6.3	170.75±0.96	107.50±3.1	278.25±2.50	16.25±1.26
12.5	218.25±0.96	119.25±2.22	337.50±2.52	6.25±0.96
25.0	104.50±0.96	66.50±1.96	171.00±1.83	8.00±0.82
50.0	70.00±1.00	58.50±1.00	128.50±1.29	17.50±2.22

Table 10 The number of parasitized cells ($\overline{X} \pm SD$) with single and multiplied piroplasm forms of *Theileria* parasite counted against 5,000 erythrocytes after 24 h of cultivation with 18% parasitemia at varying cell suspension

Cell suspension (%)	Parasitized cells against 5,000 erythrocytes		Parasitized cells against 5,000 erythrocytes	
	Single piroplasm	Multiplied piroplasm	Living parasite	Dead parasite
3.2	251.50±2.38	86.25±1.26	337.75±1.71	12.75±1.71
6.3	337.00±1.41	156.50±1.29	493.50±2.65	12.75±2.22
12.5	351.50±0.58	163.00±2.00	514.50±2.38	17.25±0.96
25.0	179.00±1.29	109.00±1.15	288.00±1.83	35.50±1.15
50.0	162.00±1.25	123.50±2.52	285.50±0.58	48.00±1.50

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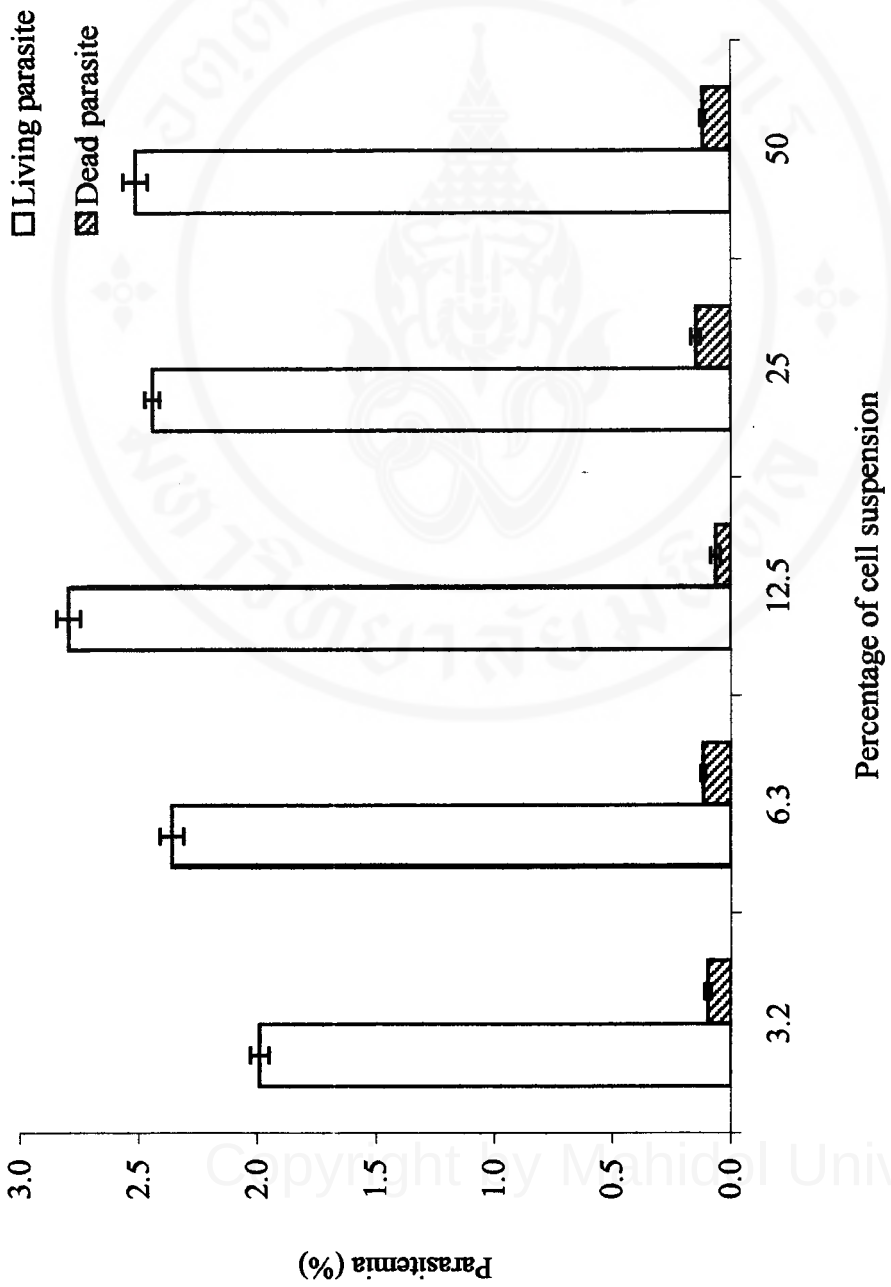

Figure 20 Histograms of percentage ($\overline{X}$±SD) of parasitized cells with living and dead parasites of *Theileria* sp. after 24 h of cultivation with 2.2% parasitemia at varying percentage of cell suspension (3.2%-50%)

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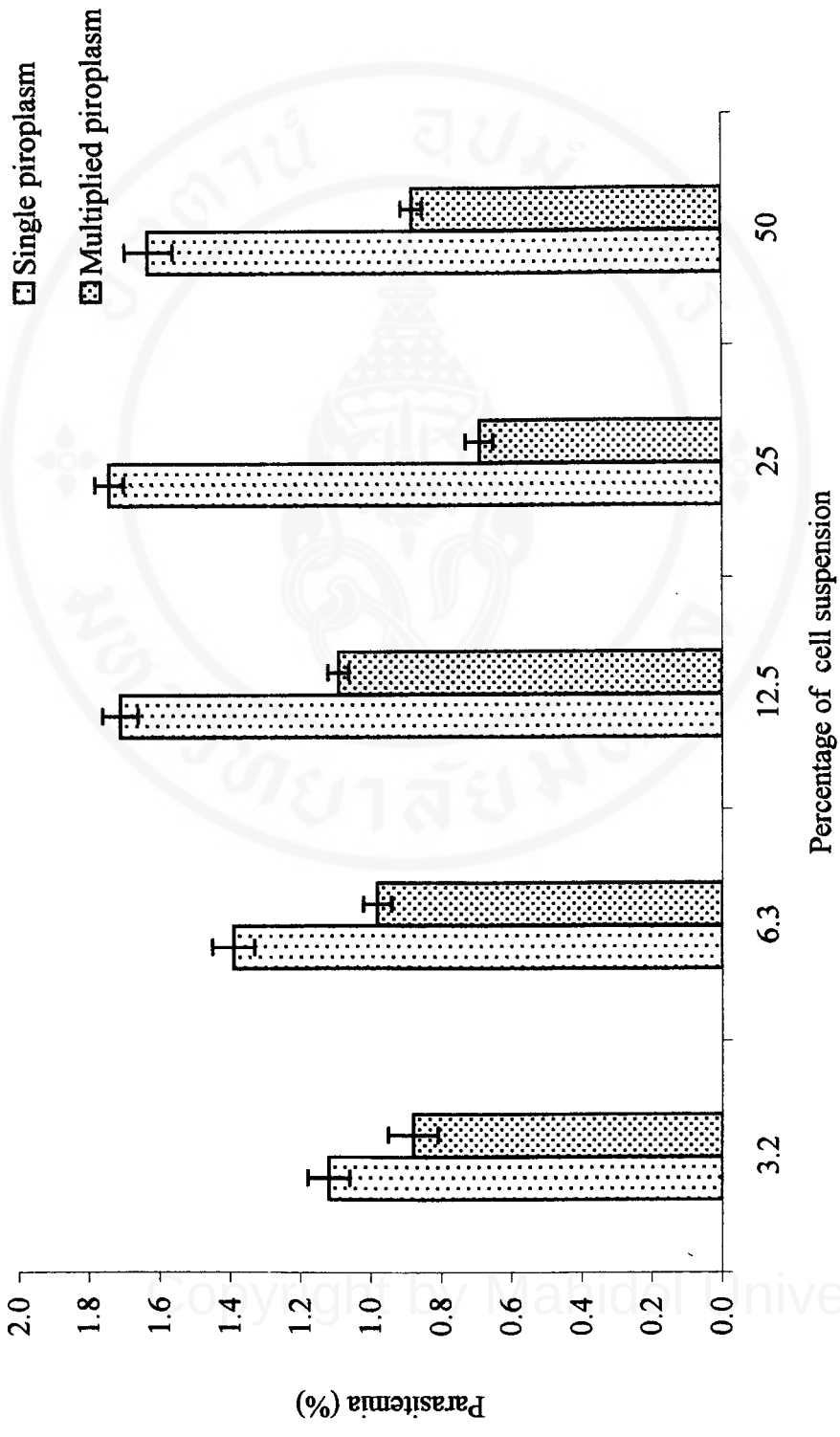

Figure 21 Histograms of percentage ($\overline{X}\pm SD$) of parasitized cells with single and multiplied piroplasm forms of *Theileria* sp. after 24 h of cultivation with 2.2% parasitemia at varying percentage of cell suspension (3.2%-50%)

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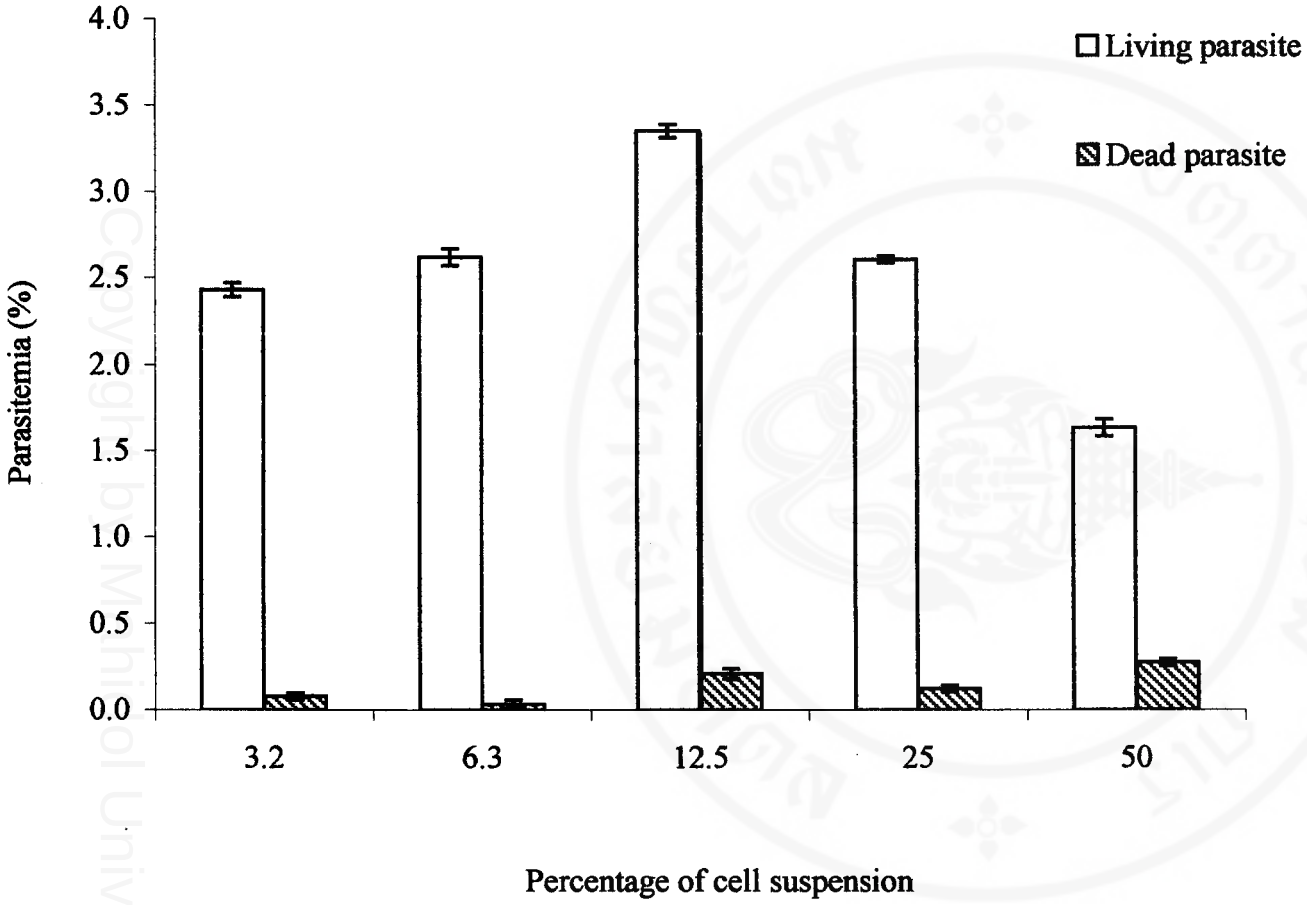

Figure 22 Histograms of percentage ($\bar{X}\pm SD$) of parasitized cells with living and dead parasites of *Theileria* sp. after 24 h of cultivation with 4.5% parasitemia at varying percentage of cell suspension (3.2%-50%)

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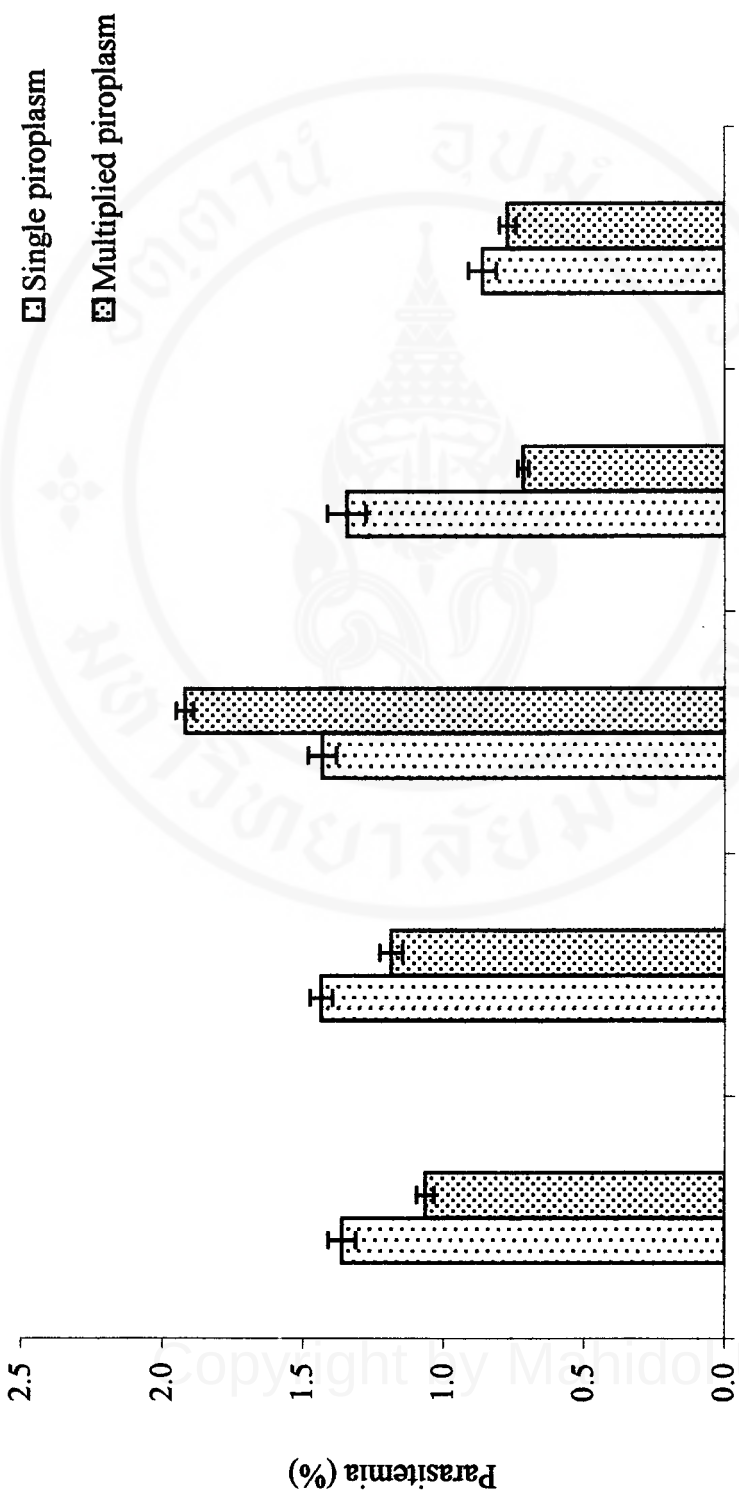

Figure 23 Histograms of percentage ($\overline{X}$±SD) of parasitized cells with single and multiplied piroplasm forms of *Theileria* sp. after 24 h of cultivation with 4.5% parasitemia at varying percentage of cell suspension (3.2%-50%)

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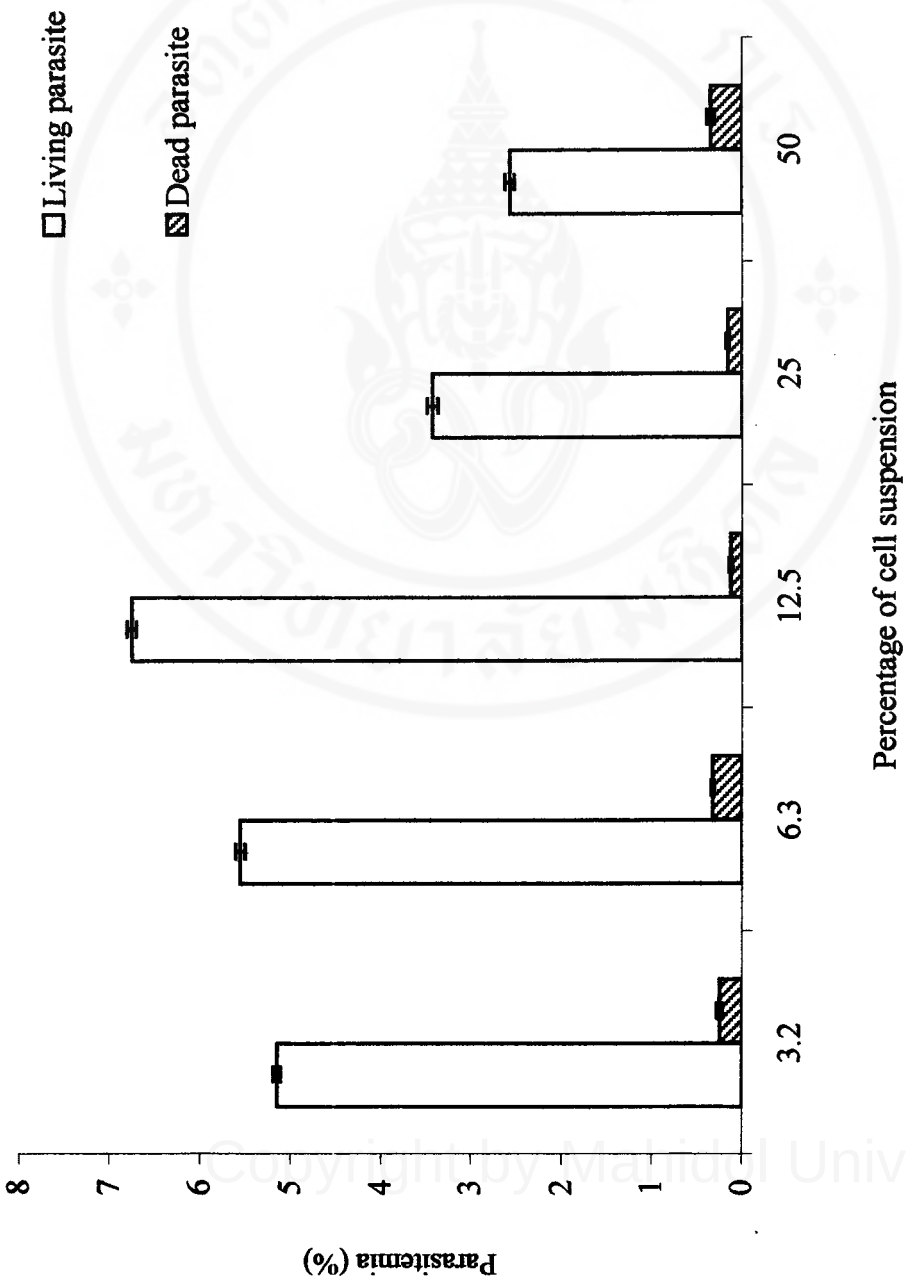

Figure 24 Histograms of percentage ($\bar{X}$±SD) of parasitized cells with living and dead parasites of *Theileria* sp. after 24 h of cultivation with 9% parasitemia at varying percentage of cell suspension (3.2%-50%)

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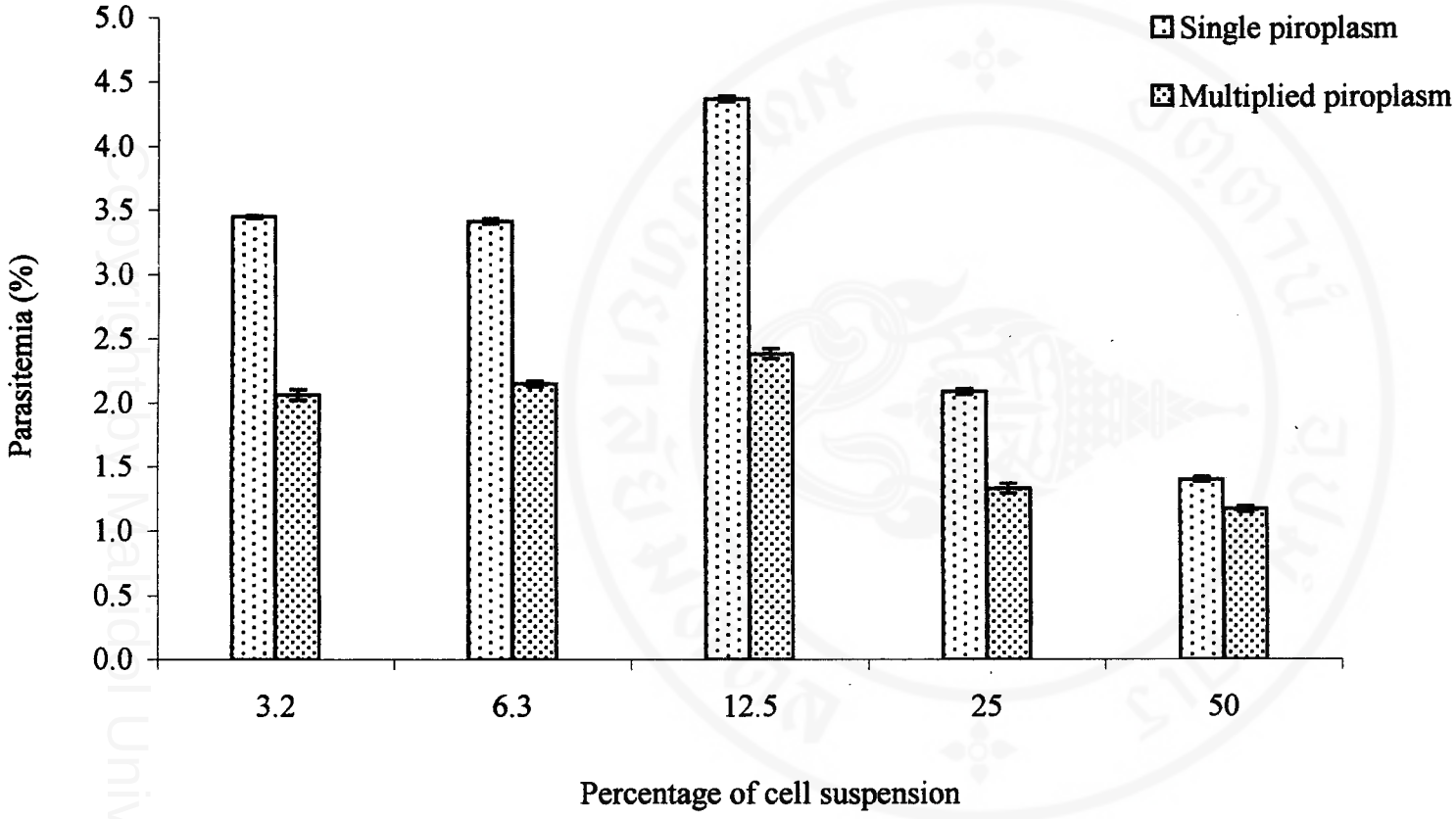

Figure 25 Histograms of percentage ($\overline{X}\pm SD$) of parasitized cells with single and multiplied piroplasm forms of *Theileria* sp. after 24 h of cultivation with 9% parasitemia at varying percentage of cell suspension (3.2%-50%)

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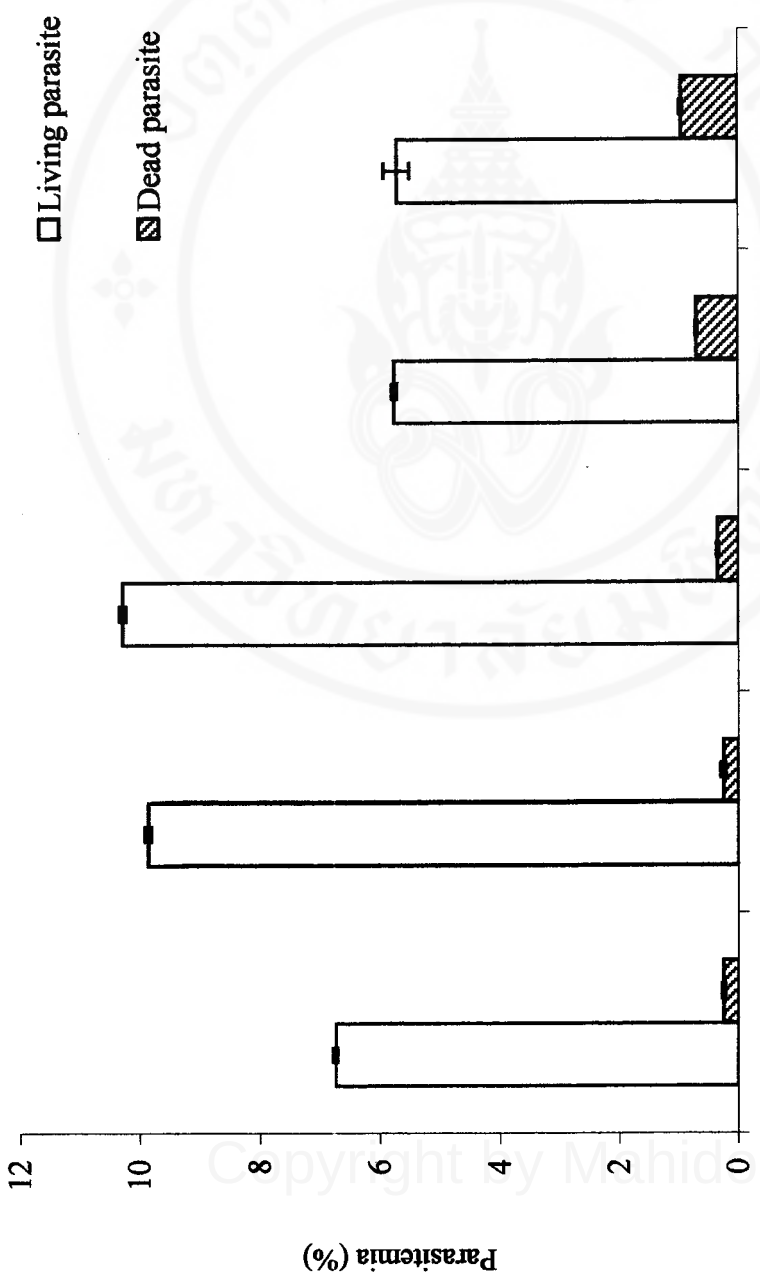

Figure 26 Histograms of percentage ($\overline{X}$±SD) of parasitized cells with living and dead parasites of *Theileria* sp. after 24 h of cultivation with 18% parasitemia at varying percentage of cell suspension (3.2%-50%)

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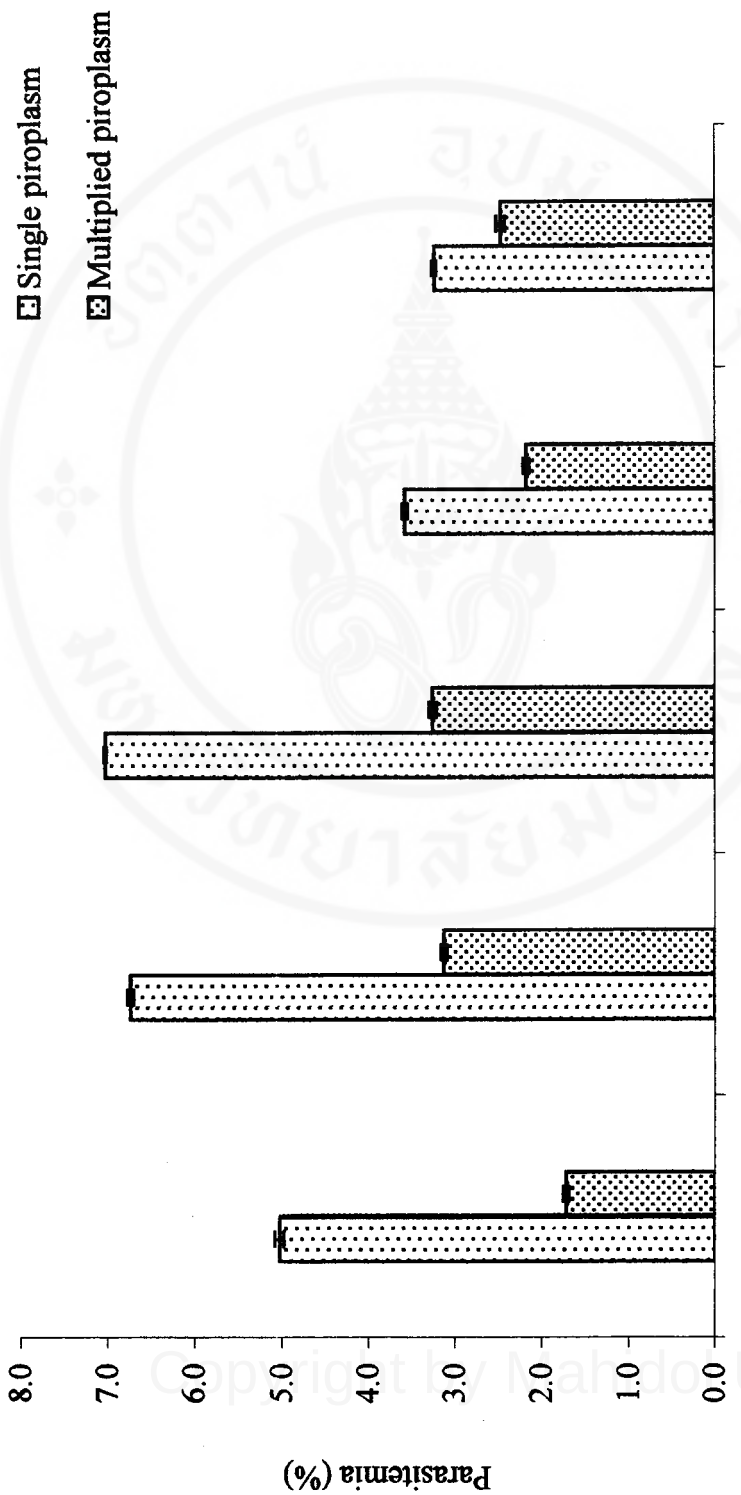

Figure 27 Histograms of percentage ($\bar{X}$±SD) of parasitized cells with single and multiplied piroplasm forms of *Theileria* sp. after 24 h of cultivation with 18% parasitemia at varying percentage of cell suspension (3.2%-50%)

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4. Assessment of 50% Inhibitory Concentration against *Theileria* parasite by Microscopic and Radioisotopic Methods

The 50% inhibitory concentration (IC_{50}) values of four drugs, i.e., chloroquine (CQ), quinine (QN), Berenil® and Imizol® and seven medicinal plant extracts, i.e., *Alyxia reinwardtii* stems, *Barringtonia acutangula* stems, *Oroxylum indicum* stems, *Alsotonia scholaris* stems and barks, *Vernonia cinerea* stems and roots against *Theileria* parasite were evaluated by both microscopic and radioisotopic methods.

4.1 Microscopic method

As shown in Fig. 28, CQ was found to be the most potent compound among the four drugs tested against *Theileria* parasite with the IC_{50} value of 13.09 μM. QN only showed moderate inhibitory effect with the IC_{50} value of 285.96 μM whereas Berenil® and Imizol® had very low effect against this parasite and it was not possible to determine their IC_{50} values. Among the seven medicinal plant extracts used in the experiment, only the crude extract from *V. cinerea* stems was shown to possess inhibitory effect on the growth and development of *Theileria* parasite with the IC_{50} value of 888.61 μg/ml (Fig. 29). The other six medicinal plant extracts were shown to have very little effect.

The morphology of *Theileria* parasite in the control wells and the testing wells observed under microscopically were as follow:

In control wells, all forms of parasites, i.e., match, ring, 2 piroplasm, 3 piroplasm and 4 piroplasm forms (quadruplet form) were normal in appearance as shown in Fig. 3. In the wells with incomplete inhibition, both normal and abnormal parasites were observed. In the wells which complete inhibition was achieved, no living parasites were found and all forms of parasites were inhibited and appeared as

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dead parasites. Based on morphology, the drugs and medicinal plant extract seemed to have the same effect on *Theileria* parasite. The majority of all forms of dead parasites showed only nuclei and no cytoplasm was seen (Fig. 30). The infected red blood cells harboring dead parasites showed no changes in size, shape, or color. In wells without inhibitory effect, all forms of normal parasites were observed. The three active compounds, i.e., CQ, QN and crude extract from *V. cinerea* stems were re-assayed for their inhibitory effect on the growth of *Theileria* parasite by using radioisotopic method and the IC_{50} values of each drug obtained from the two methods were compared.

4.2 Radioisotopic method

Two drugs, i.e., CQ, QN and one medicinal plant extract, i.e., *V. cinerea* stems were evaluated for their IC_{50} values against *Theileria* parasite by using radioisotopic method and the results were as follow:

A graphic result showing a dose-dependent inhibition of [^{3}H] hypoxantine incorporation by CQ and QN was illustrated in Fig. 31. The IC_{50} values of CQ and QN were 12.00 μM and 260.00 μM, respectively. The IC_{50} value of *V. cinerea* stems extract determined from a dose-dependent inhibition curve as shown in Fig. 32 was found at 745.00 μg/ml. There was no significant difference between the IC_{50} values of CQ, QN and *V. cinerea* stems extract from the two methods (CQ: 13.09 *vs* 12.00 μM; QN: 285.96 *vs* 260.00 μM; *V. cinerea* stems extract: 888.61 *vs* 745.00 μg/ml) (p=0.256; p=0.065; p=0.175).

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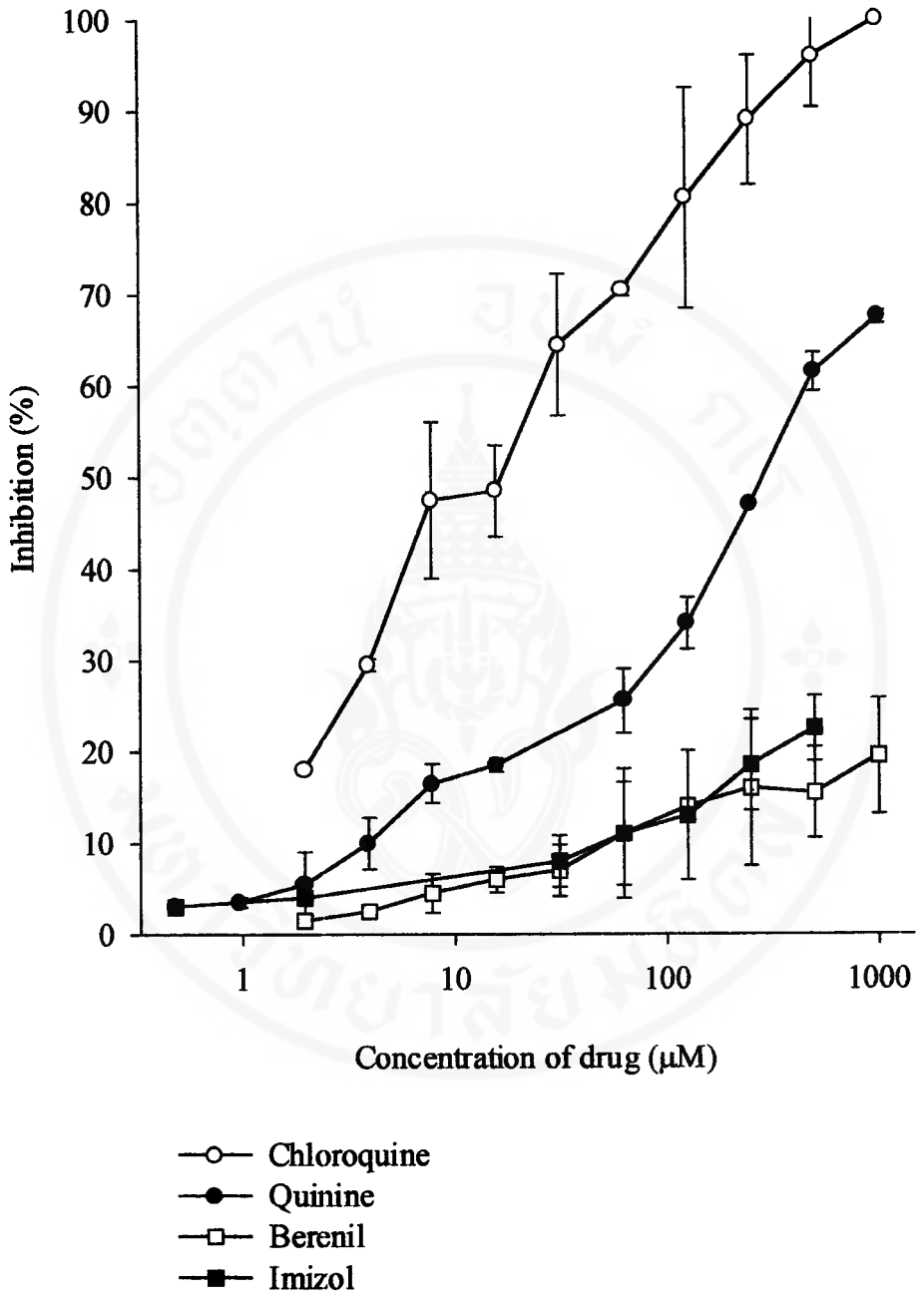

Figure 28 Plots of the percentage inhibition testing drugs on growth of *Theileria* parasite after 24 h exposure

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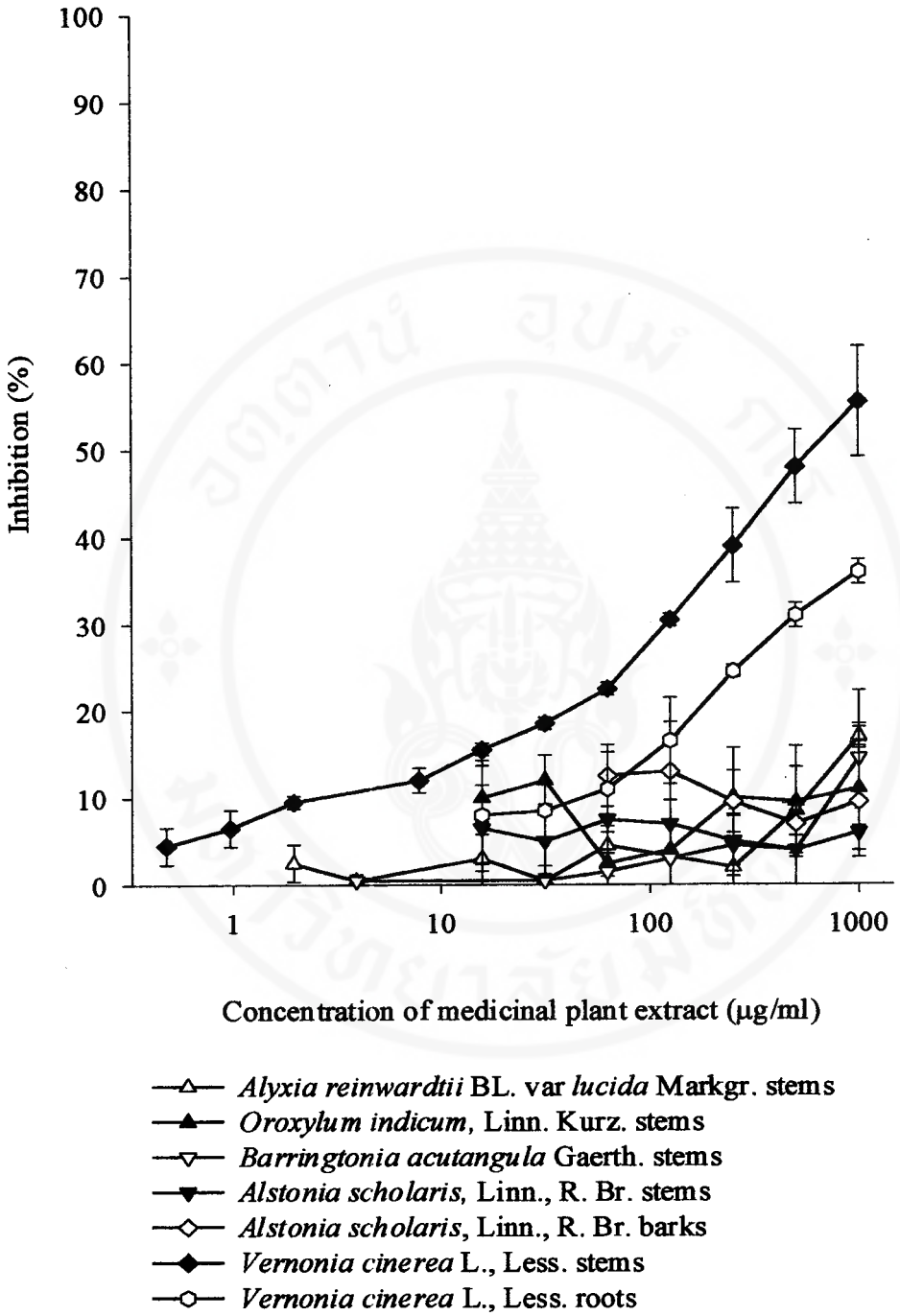

Figure 29 Plots of the percentage inhibition medicinal plant extracts on growth of *Theileria* parasite after 24 h exposure

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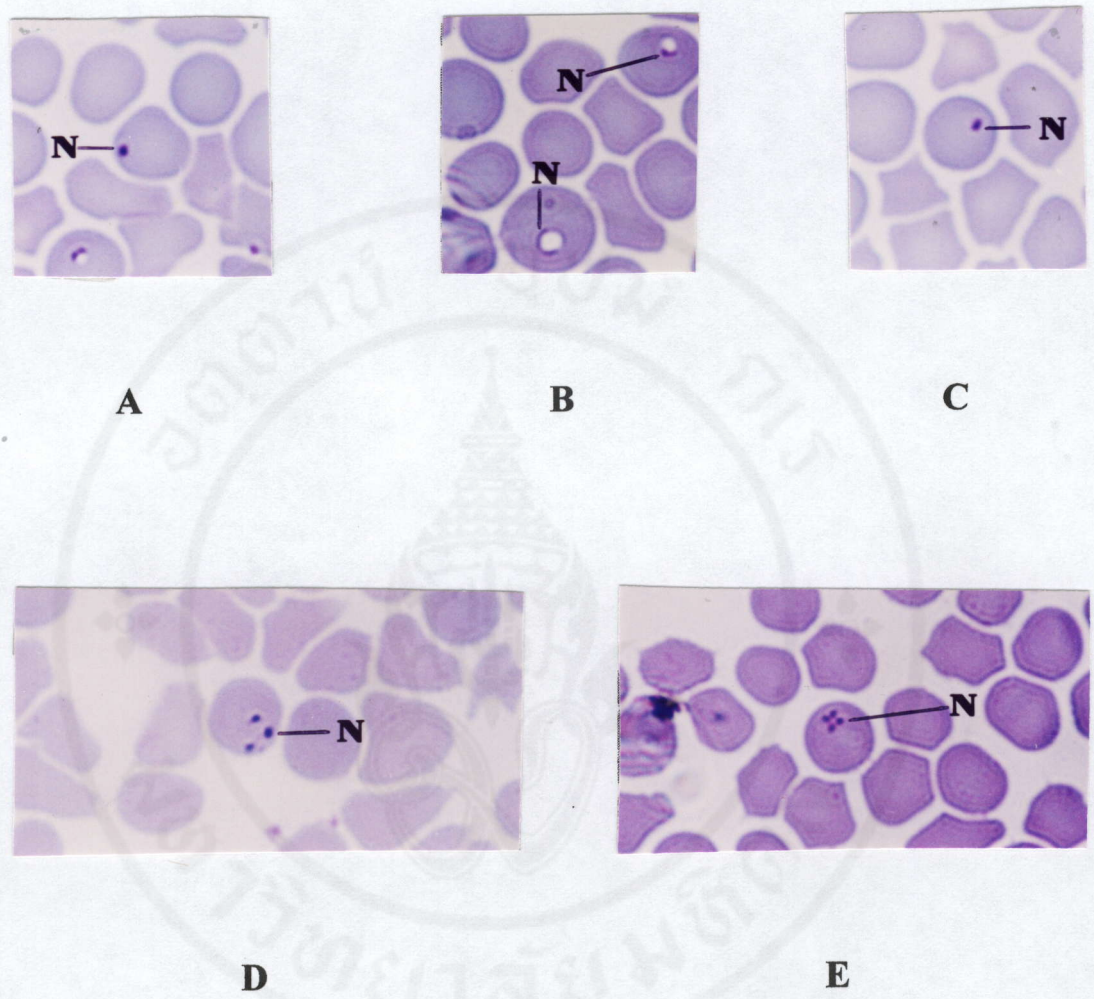

Figure 30 The abnormal or dead *Theileria* parasites after exposure to testing drugs and *Vernonia cinerea* stems. Dead match form (A); dead ring form (B); dead 2 piroplasm form (C); dead 3 piroplasm form (D) and dead 4 piroplasm form (E) (N = nucleus)

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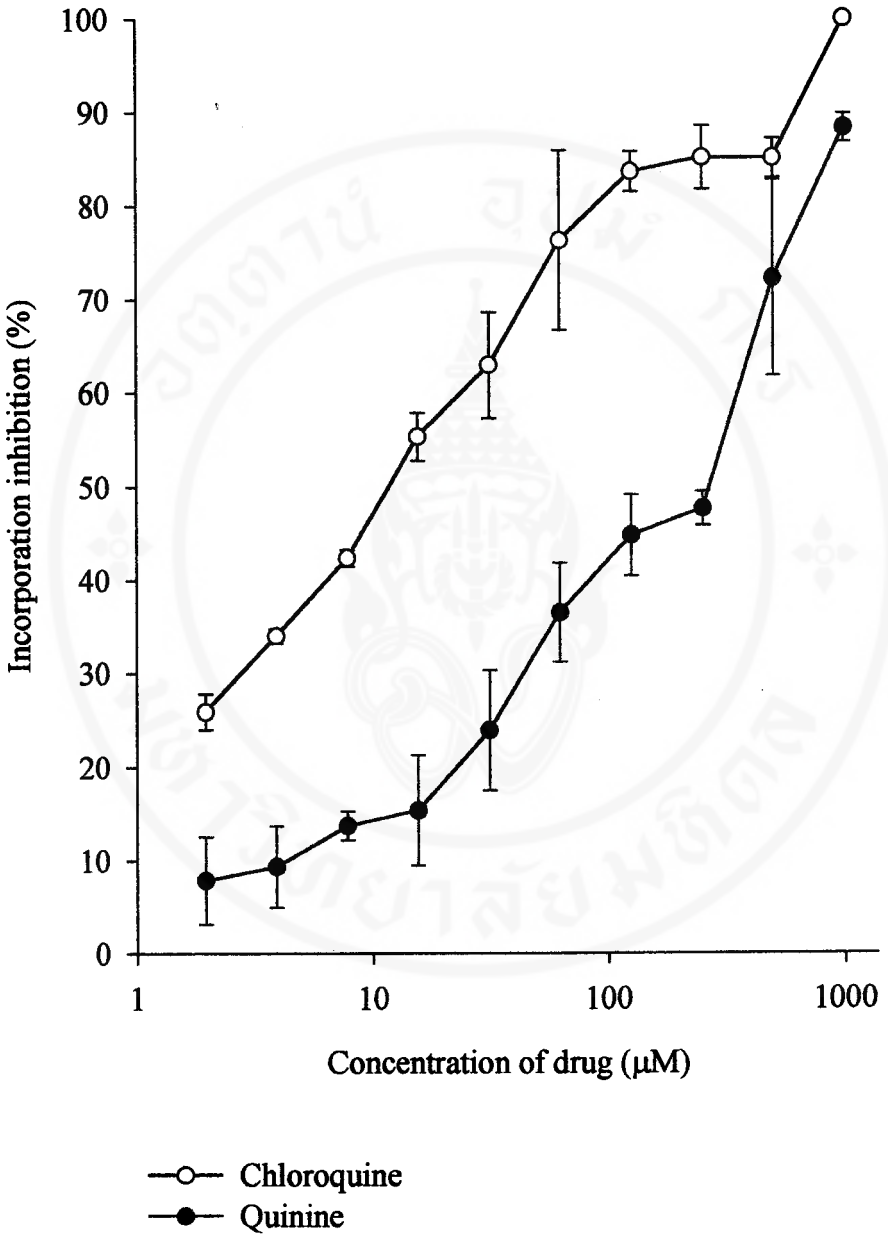

Figure 31 Plots of the percentage inhibition of [^{3}H] hypoxantine incorporation by testing drugs after 24 h exposure

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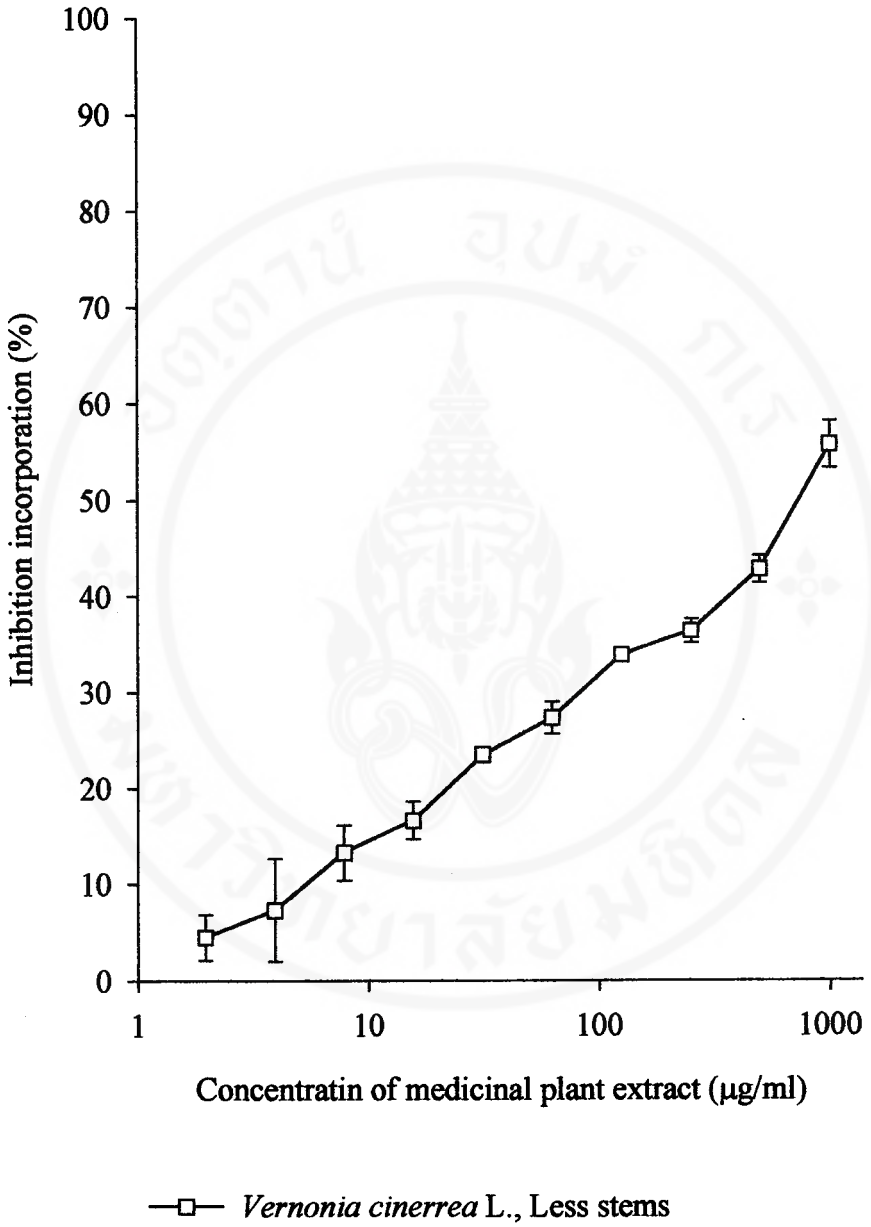

Figure 32 Plots of the percentage inhibition of [^{3}H] hypoxantine incorporation by medicinal plant extract drugs after 24 h exposure

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CHAPTER V

DISCUSSION

1. Growth, Development and Survival of Thai Isolate *Theileria* sp. in Four Different Types of Culture Medium during 72 h of Cultivation

In this experiment, growth, development and survival of Thai isolate *Theileria* parasites *in vitro* culture (0.4% parasitemia and 10% cell suspension) using four different types of culture medium namely RPMI 1640+FCS, RPMI 1640+FBS, M199+FCS and M199+FBS medium of cultivation were compared. The results of 72 h of cultivation clearly demonstrated that the M199+FBS was the most appropriate medium. By using this medium, all forms of *Theileria* were observed after 24 h of incubation (Fig. 16). The first finding of the 4 piroplasm or quadruplet forms with this type of media was also found at H24 and no dead parasites were observed in the culture during 72 h of incubation. These results are consistent with the finding by Conrad *et al* (15). In their study, a dramatic change of erythrocytic stage of *T. parva* was observed in the culture after 24-48 h. The increased number of erythrocytes containing quadruplet form was clearly evident during this period. Results from Conrad *et al.*, (15) and this study apparently indicated that M199+FBS medium should be one of the appropriate medium for cultivation of erythrocytic stage of *Theileria* spp. M199 has been used for tissue culture due to their enrichment of the ingredients. It has

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higher inorganic salts, amino acids, vitamins and accessory growth factors when compared with RPMI 1640. Generally, a culture medium must contain an inorganic salt solution to provide the correct ionic environment for the cultured cells, and buffering to maintain physiological pH. The inorganic salts solution in the M199 medium incorporates a bicarbonate buffer, which maintains the medium at pH of 7.4. Inorganic salts in M199 also included the trace metal, i.e., ferric nitrate, known for their catalytic action on certain enzyme reactions. (129). The amino acid requirements of tissue cell (the fibroblast cells) have been investigated extensively by Fisher (130). They demonstrated that amino acids have been regarded as being able either to prolong the life of cells or to accelerate cell growth in tissue cultures. The M199 accessory growth factor contained adenine sulphate, adenosine-triphosphate (ATP), denosine-5-phosphate, cholesterol, deoxyribose, guanine HCl, ribose, sodium acetate, thiamin and Tween 80 which are not present in RPMI 1640. It was found that these ingredients might increase very greatly the survival time of the synthesis of protoplasm (129). ATP is known to activate many metabolic processes (131). It is possible that stimulating by ATP may occur only in the presence of phosphorylated intermediates of the glycolytic cycle. In addition, Fisher *et al* (132) found that whereas ATP not stimulate growth, fructose diphosphate was definitely beneficial. Tweens is the surface-active agent that acts as possible means of supplying the tissue with a water-soluble source of fatty acid (organic carbon) that involved in lipid biosynthesis of the parasite. The cell might require tweens as a source of unsaturated fatty acid. Thus, Morgan *et al* (129) decided to incorporate Tween 80 (containing oleic acid) in M199 medium. This compound was also utilized to dissolve fat-soluble compounds. It

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was found that if vitamin A, vitamin D and cholesterol were first dissolved in a minimal amount of 95% ethylalcohol, and Tween 80 was added subsequently, these substances remained in solution and did not precipitate even in high dilution with distilled water. Another ingredient present in M199 but not in RPMI 1640 is nucleic acid. The addition of nucleic acid constituents, the purines, pyrimidines and pentose sugars, to the basal solution was found to be beneficial. The incorporation of these compounds in to M199 would seem to constitute an improvement over other synthetic mixtures that have been devised. The important role that has been assigned to the nucleic acid in cellular metabolism (133,134) would suggest that any synthetic medium for animal tissue cells should contain either the nucleic acids themselves or substances from which they may be synthesized by the cells.

The results using RPMI 1640 medium supplemented with either FBS or FCS serum were similar (Figs. 9,10,12 and 13). The parasites could only develop from ring to 2 piroplasm forms and the percentage of living parasites remarkably decreased throughout the period of cultivation. Though, RPMI 1640 plus FCS culture medium was used for cultivation of *T. sergenti* by Kamiyama *et al* (50). However, the morphology of developmental stage was not examined and the culture period was only 18 hours. The data from this study likely indicates that RPMI 1640 medium is not suitable for cultivation of Thai isolate *Theileria* parasite. This may be due to inadequate nutritional supply that it would be resulted in gradual retardation of parasite growth and hampered multiplication of erythrocytic stage of *Theileria* parasite. Primarily, RPMI 1640 formulated for growing the lymphocytes in suspension cultures (134) have been adapted for *Theileria* suspension cultures and for establishment of primary cultures from infected animal (136-138). To date, RPMI

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1640 medium supplemented with 20% fetal bovine serum has shown to be the standard growth medium for long-term *in vitro* cultivation of schizont stage of *T. annulata* (122). On the other hand, the M199 medium based on Hank's salts has been appeared to be less suitable for mass propagation of *T. annulata* (139). Thus, RPMI 1640 medium was preferable for the long-term cultivation of schizont stage within the lymphoid cell but not for erythrocytic stage.

It is well accepted that serum is an essential nutritional component for growth and multiplication of *Theileria* infected cells (137,140). At present, fetal bovine serum (FBS) is one of the most universally applicable cell culture additives for the stimulation of cellular proliferation and biological production (141). This study demonstrated that growth and development of Thai isolate *Theileria* parasite in M199 supplemented with FBS were better than that supplemented with FCS (Figs. 14-17). This finding accorded well with the report of Conrad *et al* (14,15). The parasite in M199 supplemented with FCS developed from ring to 2 piroplasm and 3 piroplasm forms only after 72 h of cultivation. A gradual decrease of living parasites was observed throughout the period of cultivation. Moreover, a significant number of the dead parasites was also found in the culture. Supplementation of M199 with FBS was previously proved to provide satisfactory result of growth of *T. annulata* and *T. parva* (14,15). These finding revealed that the fetal calf serum could not support the growth and multiplication of the erythrocytic stage of *Theileria* parasites. Olmted *et al* (142) reported that the use of fetal calf serum did not always insure adequate maintenance of tissue cultured cells. Because of this, it seemed of importance to look for other possible variations, such as chemical differences, which might explain occasional

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toxicity in fetal calf serum used in tissue culture media, e.g., high protein content, low acid soluble phosphorus content, and high phospholipid and total cholesterol content. Many *in vitro* cell culture systems are known to be sensitive to lipid constituents of the medium, and to vary fatty acid chain length of the triglycerides (143). Bailey (144) has demonstrated that cholesterol uptake by fibroblasts from the medium was followed by cholesterol excretion unless other changes promote an accumulation of lipid droplets in the cytoplasm resulting in altered functional capacity and lysis of the cell. Therefore, the 40% supplementation of fetal calf serum added to both the M199 and RPMI 1640 used in this study might lead to the toxicity for culturing Thai isolate *Theileria* parasites.

Morphology of erythrocytic stage of Thai isolate *Theileria* parasite observed in this study was similar to that of *T. sergenti*, which has been described by Kawamoto *et al* (4). Various shapes of parasites were found in the culture; such as, large and small rod- and comma-shaped, spherical or ovoid, two, three and tetrad forms (quadruplet form) (Fig. 11). The match (rod and comma shaped) should be the trophozoite stage as confirmed by electron microscopic observation (4). Ultrastructural observation revealed that this stage consisted of many ribosomes, acistate mitochodria, cytostome, and food vacuole. After 24 h of incubation, the multiplied form, i.e., 2 piroplasm, 3 piroplasm and 4 piroplasm forms (quadruplet form) was observed in the culture with M199+FBS medium (Fig. 16). The 2 piroplasm should be the Y-like shaped or paired piriform organism which has been demonstrated under electron microscope (4). This form is proved to be a binary fission of intraerythrocytic multiplication of *Theileria* parasite. Ultrastructural study confirmed that the progenies of this dividing form are merozoites. Clusters of four piroplasms (quadruplet form) observed in this study were

in fact previously demonstrated in erythrocytes of cattle infected with *T. parva* by Nuttall *et al* (145), Cowdry & Ham (146) and Cowdry & Danks (147). This form was considered by some investigators to be the result of another type of intraerythrocytic multiplication, i.e., schizogony (4,148,149). This was later proved by Conrad *et al* (14,15) who found that each progeny of the 4 piroplasm form had the same morphological characteristic features as of merozoites which were resulted from schizogony. Therefore, it is reasonable to conclude those two types of asexual multiplication, i.e., binary fission and schizogony were observed in the culture of Thai isolate *Theileria* parasite from this study. However, confirmation by electron microscope should be carried out in order to prove the above hypothesis. Since, it has been suggested that only merozoite has ability to penetrate the erythrocyte cell, therefore, the medium that could sustain the viability of extracellular merozoites should be discovered in order to ensure the successful reinvasion of merozoites which is necessary for the continuous *in vitro* culture of *Theileria* parasite.

2. Growth, Development and Survival of Thai isolate *Theileria* sp. in M199+FBS Medium during 120 h of Cultivation

Reinvasion of growth and development of Thai isolate *Theileria* parasite in M199+FBS medium was performed by increasing parasitemia, i.e., 6% to observe their growth and development. The results were evaluated at 24 h, 48 h, 72 h and 120 h of cultivation. The periods of cultivation that provided optimal growth and development of parasites were found at H24 and H48 according to the higher

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percentage of parasitized cells with living parasites and multiplied piroplasm form (Figs 18 and 19). Increased percentage of multiplied piroplasm form, i.e., 2,3 and 4 piroplasm forms was found during these periods of observation. This finding confirms the previous result (Experiment 1). It should be noted that decreasing of the percentage of parasitized cells with living parasites and multiplied piroplasm form were observed after 48 h of cultivation. Though, the highest percentage of multiplied piroplasm form was recognized at H48 but the evidence of reinvasion indicated by single piroplasm infected cell erythrocyte was not seen at H72 (Fig. 19). This could due to the failure of reinvasion of merozoites resulting from either binary fission or schizogony (5). Since, extracellular parasites were found in the culture mostly as aggregated form (probably a mixture of quadruplet and with some other forms). These extracellular parasites were also mentioned with *T. parva* culture by Conrad *et al* (15). Reinvasion of *Theileria* merozoites into the erythrocyte cell is required for continuous *in vitro* cultivation (long-term cultivation). It is necessary to find out the *in vitro* condition that could maintain the survival of released merozoites (extracellular merozoites) and allow them to reinvade the erythrocyte cells. The problem associated with the long-term cultivation of the erythrocytic stages of *Theileria* parasites could be attributed to three interrelated factors; the viability of the merozoite, the viability of the erythrocyte and the receptivity of the host erythrocyte membrane to the merozoite based on the studies of malaria parasite.

The importance of parasite viability has been demonstrated by Trigg & Gutteride (150), who showed that a progressive decrease in RNA synthesis of *P. knowlesi in vitro* paralleled the progressive decrease in multiplication rates during each successive subculture. The decreased multiplication of parasites grown *in vitro*

could be due to a reduction in the number of merozoites produced per schizont, to a decrease in the viability the merozoite or a combination of both. Segments with a reduced number of merozoites have been observed in malaria parasites growing *in vitro* (Trigg, unpublished observations). However, these were not investigated in this study. Possibly, the 3 piroplasm found in this experiment may be the incomplete or abnormal forms of schizogony and these can not reach maturity and no merozoites will be produced from this form. On the other hand, this 3 piroplasm form may be the mature schizont which will produce only 3 merozoites instead of 4 merozoites as quadruplet form did.

The importance of maintaining erythrocyte viability during the serial cultivation of malarial parasites has been stressed by Trigg (151) and Bertagna *et al* (152). Brewer & Powell (153) and Powell *et al* (154) have suggested that a low level of ATP in the host erythrocytes might result in lysis before the parasite has matured to the schizont stage. Moreover, the destruction of ATP has been described by Gompertz (155) in cultures of human erythrocytes incubated at 37°C.

In addition, mechanical damage resulting from the methods of cultivation, i.e., passing through CF11 used in this study (see Materialas and Methods) could possibly be responsible for the change in the erythrocyte membrane of both infected and non infected cells. Irrespective of the cause, change in the structure of the erythrocyte membrane could result in alteration in the permeability, which might be reflected in the slower growth of the parasite after invasion. A study on invasion of bovine erythrocytes by *T. sergenti* piroplasm *in vitro* was conducted by Kawamoto *et al* (5). In their study, the isolated viable parasites were incubated with erythrocytes in

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minimum essential medium supplemented with 10% heat-activated fetal calf serum. The majority of parasites attach themselves to erythrocytes, and varied in shape, such as rod and comma shaped, spherical, and ovoid. However, most of parasites that invaded blood cells were the form with the ubiquitous crescent basophillic portion and lucent cytoplasm (5). It was apparent that *Theileria* parasite firstly attaches to the host cell membrane by the basal end (nuclear hemisphere) which acts as the specific receptor site as previously reported by Jura *et al* (156) with *T. annulata* and later confirmed by *T. sergenti* by Kawamoto *et al* (5). Electron microscopic observation of the invading stage by Kawamoto *et al* (4) revealed that this stage was similar to what was described for merozoite. Therefore, it was suggested that only merozoite has ability for penetrating into the erythrocytes. It is interesting to find out *in vitro* conditions that provide both good multiplication and reinvasion of the parasites. Moreover, these also appropriate conditions should be for sustaining the merozoite infectivity in order to reach the ultimate goal that is the continuous culture of *Theileria* parasite.

3. Growth, Development and Survival of Thai Isolate *Theileria* sp. Cultured in M199+FBS Medium at Varying Percentages of Parasitemia and Cell Suspension

The results from this experiment clearly demonstrated that the cultures started with 4.5% to 18% parasitemia with cell suspension varying from 3.2% to 12.5% were the optimal conditions for culturing parasite for being used in assessment of their sensitivity to anti-theilerial drugs. The high percentage of parasitized cells with living

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parasites and multiplied piroplasm forms were observed under these conditions (Figs. 22-27). Since, the cultures started with higher percentages of cell suspension, i.e., 25% and 50% regardless of percentages of starting parasitemia (4.5%, 9% and 18%) were found with a remarkable decrease in the percentage of parasitized cells with living parasites and multiplied piroplasm forms. This might be due to the high density of infected cells which leads to an inadequate nutritional supply and results in gradual retardation of parasite growth and hampered multiplication of parasite. In addition, the parasites might produce high concentrations of waste product or inhibitory metabolites that can negatively influence parasite growth and development. Furthermore, the spent medium would have the accumulation of toxic products from parasites and erythrocyte cells which affected to change environmental conditions for culturing parasite that could not support parasite growth and development. Therefore, the cultures started with high percentage of both parasitemia and cell suspension are not suitable for cultivation. The results from this study suggest that the cultures started with higher percentages parasitemia (9%,18%) should be adjusted to have a lower percentages of cell suspension (3.2%, 6.3% and 12.5%) in order to achieve successful cultivation (Figs. 22-27). Results from this experiment revealed that cultures started with low percentage of parasitemia (2.2%), showed the correlation between the percentage of parasitized cells with living parasites and multiplied piroplasm form and percentage of cell suspension (Figs. 20 and 21). However, the percentage of parasitized cells with living parasites and multiplied piroplasm form obtained in these cultures were not enough amounts of infected cells with multiplied form for following use in the drug sensitivity assay. Therefore, higher percentages of starting parasitemia (4.5% to 18%)

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and appropriately 10% to 12.5% cell suspensions should be used for cultivation in order to achieve enough number of infected cells with multiplied piroplasm form for further use in drug sensitivity assay.

4. Assessment of 50% Inhibitory Concentration against *Theileria* Parasite by Microscopic and Radioisotopic Methods

A comparison of the inhibitory effect of the four anti-hemosporozoal drugs and seven medicinal plant extracts on growth and multiplication of parasite was assessed by both microscopic and radioisotopic methods. The microscopic method revealed that chloroquine (CQ) and quinine (QN) exerted an inhibitory against *Theileria* parasite. The 50% inhibitory concentration (IC_{50}) of CQ and QN against the erythrocytic stage of Thai isolate *Theileria* parasite were found at 13.09 μM and 285.96 μM, respectively (Fig. 28). Anti-hemosporozoal drugs, i.e., Imizol®and Berenil® for babesia infection were shown to have no activity against Thai isolate *Theileria* parasite in this study. The observation is similar to the previous report using *T. sergenti* by Kamiyama *et al* (50). Only, *V. cinerea* was found to have an effect with the IC_{50} value of 888.61 μg/ml (Fig. 29). The other six medicinal plant extracts did not show any activity. *V. cinerea* extract was previously reported to have an anti-malarial activity in Thai folklore medicine (80). However, the investigation of their active ingredients of *V. cinerea* has not been worked out. Due to little information about effective drug against *Theileria* parasites, it is interesting to find out whether this plant can be actually used for treatment of theileriosis or not. In parallel experiment, the *in vitro* drug sensitivity assay was determined by radioisotopic method. The IC_{50} values of CQ, QN and *V.*

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cinerea stems from this method and from the microscopic method were not significantly different (CQ: 12.00 μM *vs* 13.09 μM; QN 260 μM *vs* 285.96 μM; *V. cinerea* 745.00 μg/ml *vs* 888.61 μg/ml) (p=0.256; p=0.065; p=0.175). This finding suggests that both methods can be used for assessment of the IC_{50} values of anti-theilerial drugs. However, the IC_{50} values of each compound observed from the radioisotopic method seemed to be slightly lower than those observed with microscopic method (Figs 31 and 32). This could be explained by the fact that the radioisotopic method is more sensitive than the microscopic method (157,158). The error of counting of parasites may occur in the microscopic method due to the difficulty of distinguishing the living and the dead parasites since their sizes are quite small. Though the radioisotopic method is proved to be more rapid, more accurate and highly reproducible technic, however, it requires the special equipment. Regarding to microscopic method, though it does not require the special equipment but this technic relies on skilled researchers and is time-consuming. According to the above conditions, it is thus depends on each laboratory for selecting the method to determine the anti-theilerial drugs. The IC_{50} values of CQ and QN observed in this study were much higher compared with the report of Kamiyama *et al* (52) (CQ: 12.00 μM *vs* 25.00 nM; QN 260.00 μM *vs* 150.00 nM). It is possible that Thai isolate *Theileria* parasites were more tolerant to these drugs than *T. sergenti* Japanese strain. However, more isolates of *Theileria* Thai strain should be evaluated for their sensitivity to these anti-malarial drugs before the final conclusion can be made. In addition, variety of Thai medicinal plant extracts should be screened for their anti-theilerial activity in order to find out the effective compounds for controlling theileriosis in Thailand.

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CHAPTER VI

CONCLUSION

In this study, the attempt of establishment of short-term cultivation of *Theileria* parasite was carried out with Thai isolate. A number of parameters for optimization of culture growth condition for *Theileria* including types of culture medium and serum supplemented the culture medium, the period of incubation and the percentage of initial parasitemia and cell suspension were studied. The established short-term *in vitro* culture method was applied for assessing the susceptibility of the parasite to four anti-hemosporozoal drugs, i.e., chloroquine (CQ), quinine (QN), Berenil® and Imizol®, and seven medicinal plant extracts, i.e., *Alyxia reinwardtii* stems, *Barringtonia acutangula* stems, *Oroxylum indicum* stems, *Alsotonia scholaris* stems and barks, *Vernonia cinerea* stems and roots. The *in vitro* drug sensitivity assay of Thai isolate *Theileria* parasite was evaluated by both microscopic and radioisotopic methods. The results of the study are summarized as follow:

1. The medium M199+FBS was proved to be the most appropriate medium for culturing Thai isolate *Theileria* parasites. By using this type of medium, though no reinvasion was evident but the parasites could develop and change from match form to ring, 2 piroplasm, 3 piroplasm, 4 piroplasm forms (quadruplet form) after H24. All forms of parasites were apparently healthy by showing normal appearance in their morphology. In addition, statistical analysis revealed that the mean number of living

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parasites against 5,000 erythrocytes in M199+FBS medium was significantly higher than in the other working culture media ($p<0.05$).

2. The optimal periods of cultivation which provided the best result, i.e., growth and development indicated by the percentage of parasitized cells with living parasites and multiplied piroplasm form was during H24 and H48.

3. The optimal percentage of starting parasitemia and cell suspensions for the short-term cultivation of *Theileria* parasite were found to be ranged from 4.5%-18% and 10%-12.5%, respectively.

4. Among the four anti-hemosporozoal drugs and seven medicinal plant extracts used in the experiment, only CQ, QN and *V. cinerea* stem extract were shown to exert an inhibitory effect against Thai isolate *Theileria* parasite assessed by microscopic and radioisotopic methods.

5. Both microscopic and radioisotopic methods can be used for assessment of the IC_{50} values of anti-theilerial drugs against Thai-isolate *Theileria* parasite since the IC_{50} values of CQ, QN and *V. cinerea* stems from both methods were not significantly different (CQ: 13.09 *vs* 12.00 μM; QN: 285.96 *vs* 260.00 μM; *V. cinerea* stems extract: 888.61 *vs* 745.00 μg/ml) ($p=0.256$; $p=0.065$; $p=0.175$).

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APPENDICES

Appendix 1

1. Complete RPMI 1640 medium

RPMI 1640 power medium with L-glutamine and without sodium bicarbonate (GIBCO)	10.4	gm
25 mM HEPES (Sigma) (N-2-Hydroxyethylpiperazine-n-2 ethanesulphonic acid)	1.50	gm
L-glutamine (Sigma)	0.30	gm
$NaHCO_3$ (Sigma)	1.50	gm
Reduced glutathione (Sigma)	1	mg
Gentamicin sulfate (Sigma)	50	mg
Hypoxanthine (Sigma)	20	mg
Double distilled water	960	ml

Adjusted to pH 7.2-7.4 by using 1.0 N NaOH and 1.0 N HCl and sterilized by 0.45 μM membrane filtration and kept at 4°C (must be used in one month) or frozen at -20°C

2. Complete RPMI 1640 medium with fetal calf serum

Complete RPMI 1640 medium	60	ml
Fetal calf serum (heat-inactivated) (Bio◆ Whittaker)	40	ml

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3. Complete RPMI 1640 medium with fetal bovine serum

Complete RPMI 1640 medium	60	ml
Fetal bovine serum (heat-inactivated) (Biochrome KG seromed®)	40	ml

4. Complete M199 medium with fetal calf serum

Medium 199 (1x), liquid contains Hanks' salts,	60	ml
25 mM HEPES buffer, and L-glutamine (GIBCO)		
Fetal calf serum (heat-inactivated) (Bio◆ Whittaker)	40	ml

5. Complete M199 medium with fetal bovine serum

Medium 199 (1x), liquid contains Hanks' salts,	60	ml
25 mM HEPES buffer, and L-glutamine (GIBCO)		
Fetal bovine serum (heat-inactivated) (Biochrome KG seromed®)	40	ml

6. Vym's solution (10x)

$Na_2HPO_4.7H_2O$	14.50	gm
KH_2PO_4	14.154	gm
NaCl	70.77	gm
$CaCl_2.2H_2O$	0.16	gm
KCl	4.00	gm
$MgSO_4.7H_2O$	1.54	gm
D-glucose (Dextrose)	205.00	gm
Adenine HCl	0.42	gm
Guanosine	0.708	gm
Double distilled water	1,000	ml

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Sterilized by 0.45 μM membrane filtration and kept at 4°C . This stock solution will be diluted with double distilled water to 1:10 before use.

7. Field strain solution

Solution A

Methylene blue	0.80	gm
Azure B	0.50	gm
Na_2HPO_4	5.00	gm
KH_2PO_2	6.25	gm
Double distilled water	500	ml

Stored at 4°C for 24 h before use

Solution B

Eosin	1.00	gm
Na_2HPO_4	5.00	gm
KH_2PO_2	6.25	gm
Double distilled water	500	ml

Stored at 4°C for 24 h before use

8. Testing drugs and medicinal plant extracts

Microscopic method

8.1 10 mM Chloroquine

Chloroquine (Sigma)	0.03	gm
M199 supplemented with 40% FBS medium	3.00	ml

Dissolve chloroquine and filter with 0.45 μM filter membrane.

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8.2 10 mM Quinine

Quinine (Sigma)	0.02	gm
50% ethanol (J.T. Baker)	1.50	ml
M199 supplemented with 40% FBS medium	1.50	ml

Dissolve quinine and filter with 0.45 μM filter membrane.

8.3 10 mM Berenil®

Berenil® (Hoechst)	0.07	gm
M199 supplemented with 40% FBS medium	3.00	ml

Dissolve Berenil® and filter with 0.45 μM filter membrane.

8.4 10 mM Imizol®

Imidocarb dipropinate (Cooper Animal Health., Ltd)	0.26	ml
M199 supplemented with 40% FBS medium	2.74	ml

Dissolve imidocarb dipropinate and filter with 0.45 μM filter membrane.

8.5 0.01 g/ml medicinal plant extracts

Medicinal plant crude extract	0.06	gm
M199 supplemented with 40% FBS medium	3.00	ml

Dissolve medicinal plant extract and filter with 0.45 μM filter membrane

Radioisotopic method

8.6 10 mM Chloroquine

Chloroquine (Sigma)	0.04	gm
M199 supplemented with 40% FBS medium	3.00	ml

Dissolve chloroquine and filter with 0.45 μM filter membrane.

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8.7 10 mM Quinine

Quinine (Sigma)	0.03	gm
50% ethanol (J.T. Baker)	1.50	ml
M199 supplemented with 40% FBS medium	1.50	ml

Dissolve quinine and filter with 0.45 μM filter membrane.

8.8 10 mM Berenil®

Berenil® (Hoechst)	0.09	gm
M199 supplemented with 40% FBS medium	3.00	ml

Dissolve Berenil® and filter with 0.45 μM filter membrane.

8.9 10 mM Imizol®

Imidocarb dipropinate (Cooper Animal Health., Ltd)	0.33	ml
M199 supplemented with 40% FBS medium	2.67	ml

Dissolve imidocarb dipropinate and filter with 0.45 μM filter membrane.

8.10 0.01 g/ml medicinal plant extracts

Medicinal plant crude extract	0.08	gm
M199 supplemented with 40% FBS medium	3.00	ml

Dissolve medicinal plant extract and filter with 0.45 μM filter membrane

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Appendix 2

Assessment of 50% inhibition concentration against *Theileria* parasite by microscopic method

Two drugs, i.e., chloroquine, quinine and medicinal plant extract, i.e., *Vernonia cinerea* stems were examined for their inhibitory effect on the growth *Theileria* parasite and the results were determined for the 50% inhibition concentration (IC_{50}) by using the median effect equation plot as described by Chou and Talalay (127). The number of parasitized cells with living parasite and various forms of treated and control wells after 24 h exposure were counted against 100 parasitized cells and the IC_{50} value of each compound was calculated.

From the equation $y = mx + C$

According to Chou and Talay: $y = \log (fa/fu)$

(*fa* or % inhibition = number of parasitized cells with living parasite in control well - number of parasitized cells with living parasite in treated well ; *fu* or % survival = number of parasitized cells with living parasite in treated well)

Thus, y = log [% inhibition / % survival]

m = slope

x = log Dose (D) of extract

C = y intercept

Dm is the median effect dose or 50% inhibition concentration (IC_{50}); the intercept on the x axis when $y = 0$ will give the *Dm*, because when $fa + fu = 1.0$, $fa = fu$ and $fa/fu = 1$. Hence, *Dm* or IC_{50} can be calculated from the antilog of (-) intercept/m.

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Table 11 The number of parasitized cells ($\bar{X}$±SD) with various forms of *Theileria* parasite counted against 100 erythrocytes after 24 h of exposure with various concentrations of chloroquine

Chloroquine (I)

Dose (μM)	Parasitized cells per 100 erythrocytes						Parasitized cells per 100 erythrocytes	
	ring	match	2 piroplasm	3 piroplasm	4 piroplasm	>4 piroplasm	Living parasite	Dead parasite
Control	38 ±1.2	2 ± 3.1	0	1±0.5	59±2.2	0	100±3.5	0
1000.0	0	0	0	0	0	0	0	100
500.0	0	0	0	0	0	0	0	100
250.0	1 ± 0.5	0	0	0	0	0	1±0.5	99±0.5
125.0	5 ± 1.7	1 ± 0.5	0	0	5±2.1	0	11±2.5	89±1.6
62.5	12 ± 1.4	3 ± 1.2	0	1±0.5	14±2.6	0	30±1.5	70±2.9
31.3	20 ± 0.6	0	0	0	10±1.7	0	30±1.9	70±2.1
15.6	29 ± 1.1	3 ± 1.1	0	0	16±2.6	0	48±2.9	52±3.1
7.8	29 ± 3.1	2 ± 1.3	0	0	16±2.9	0	47±2.7	53±2.9
3.9	20 ± 1.8	9 ± 1.1	1±0.5	4±1.2	33±2.5	4±1.2	71±3.2	29±2.7
1.9	17 ± 2.7	13 ±1.5	0	8±2.9	40±1.8	4±1.2	82±2.5	18±1.7

Table 11 The number of parasitized cells ($\bar{X}$±SD) with various forms of *Theileria* parasite counted against 100 erythrocytes after 24 h of exposure with various concentrations of chloroquine (continued)

Chloroquine (II)

Dose (μM)	Parasitized cells per 100 erythrocytes						Parasitized cells per 100 erythrocytes	
	ring	match	2 piroplasm	3 piroplasm	4 piroplasm	>4 piroplasm	Living parasite	Dead parasite
Control	38±1.2	2±3.1	0	1±0.5	59±2.2	0	100±3.5	0
1000.0	0	0	0	0	0	0	0	100
500.0	3±2.3	0	0	0	5±1.2	0	8±1.6	92±0.5
250.0	2±1.5	1±0.5	0	0	13±2.3	0	16±2.2	84±1.5
125.0	5±1.2	2±0.5	0	1±0.8	20±3.2	0	28±1.8	72±2.8
62.5	3±2.3	0	1±0.5	0	25±1.8	0	29±2.1	71±3.1
31.3	18±3.6	2±1.3	0	0	21±1.6	0	41±2.8	59±2.2
15.6	21±3.6	0	0	0	27±1.3	0	55±3.3	45±3.1
7.8	10±2.3	0	0	1±0.5	38±3.1	0	59±4.5	41±2.8
3.9	21±1.3	2±0.5	2±0.5	5±2.2	30±2.4	3± 0.5	70±2.6	30±1.3
1.9	10±1.8	1±0.5	1±0.5	2±1.8	45±2.6	5± 0.5	82±4.6	18±0.5

Table 12 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of chloroquine

Chloroquine (I)

Dose (μM)	x = log dose	% inhibition (*fa*)	% survival (*fu*)	*fa/fu*	y = log *fa/fu*
1000.0	3.00000	100	0	ND*	ND
500.0	2.69897	100	0	ND	ND
250.0	2.39794	94	6	15.66667	1.19498
125.0	2.09691	89	11	8.09091	0.90800
62.5	1.79588	70	30	2.33333	0.36798
15.6	1.19312	52	48	1.08333	0.03476
1.9	0.27875	18	82	0.21951	-0.65854

*ND = not determined

Chloroquine (II)

Dose (μM)	*x* = log dose	% inhibition (*fa*)	% survival (*fu*)	*fa/fu*	y = log *(fa/fu)*
1000.0	3.00000	100	0	ND	ND
500.0	2.69897	92	8	11.5000	1.06070
250.0	2.39794	84	16	5.2500	0.72016
62.5	1.79588	71	29	2.4483	0.38886
31.3	1.49485	59	41	1.4390	0.15807
3.9	0.59176	30	70	0.4286	-0.36798
0.2	-0.61236	10	90	0.1111	-0.95424

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Then, the relationship between x and y can be calculated.

For chloroquine (I), $y = 0.8577\,x + -0.9622$

$x = -(-0.9622)/0.8577$

$= 1.12$

Since anti-log of 1.12 = 13.23, thus, the IC_{50} of chloroquine (I) is equal to 13.23 μM.

For chloroquine (II), $y = 0.5944\,x + -0.6613$

$x = -(-0.6613)/0.5944$

$= 1.11$

Since anti-log of 1.11 = 12.96, thus, the IC_{50} of chloroquine (II) is equal to 12.96 μM.

The mean IC_{50} value of chloroquine $= \dfrac{IC_{50} \text{ of chloroquine (I)} + IC_{50} \text{ of chloroquine(II)}}{2}$

$= (13.23 + 12.96)/2$

IC_{50} value of chloroquine = 13.09 μM

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Table 13 The number of parasitized cells ($\bar{X}$±SD) with various forms of *Theileria* parasite counted against 100 erythrocytes after 24 h of exposure with various concentrations of quinine

Quinine (I)

Dose (μM)	Parasitized cells per 100 erythrocytes						Parasitized cells per 100 erythrocytes	
	ring	match	2 piroplasm	3 piroplasm	4 piroplasm	>4 piroplasm	Living parasite	Dead parasite
Control	38±1.2	2±3.1	0	1±0.5	59±2.2	0	100±3.5	0
1000.0	14±0.4	8±2.1	2±0.3	1±0.5	8±0.5	0	33±2.4	67±3.3
500.0	15±0.8	7±1.3	0	0	18±1.2	0	40±2.1	60±3.4
250.0	28±2.6	9±0.9	0	0	15±2.6	1±0.5	53±3.1	47±2.5
125.0	21±2.1	9±1.6	0	2±1.2	35±3.2	0	67±2.8	33±2.8
62.5	25±1.8	14±2.8	2±0.5	4±1.2	26±2.4	1±0.5	72±2.5	28±0.5
15.6	21±1.3	18±1.8	1±0.2	9±0.5	37±1.6	0	86±2.1	14±0.5
7.8	17±0.8	15±0.4	2±0.5	5±1.8	40±0.5	6±0.5	85±3.3	15±0.3
3.9	11±0.6	22±1.3	1±0.2	6±2.4	47±2.6	1±0.5	88±3.8	12±0.3
1.9	28±1.7	11±2.1	0	3±2.1	50±1.2	1±0.5	93±3.5	7±0.5
0.9	27±1.3	27±0.6	1±0.5	3±0.8	39±1.5	0	97±2.4	3±0.3

Table 13 The number of parasitized cells ($\bar{X}\pm SD$) with various forms of *Theileria* parasite counted against 100 erythrocytes after 24 h of exposure with various concentrations of quinine (continued)

Quinine (II)

Dose (μM)	Parasitized cells per 100 erythrocytes						Parasitized cells per 100 erythrocytes	
	ring	Match	2 piroplasm	3 piroplasm	4 piroplasm	>4 piroplasm	Living parasite	Dead parasite
Control	38±1.2	2±3.1	0	1±0.5	59±2.2	0	100±3.5	0
1000.0	18±2.4	7±0.8	1±0.5	1±0.5	5±0.8	0	32±2.4	68±2.5
500.0	18±0.5	5±0.8	0	1±0.5	13±0.5	0	37±0.5	63±0.8
250.0	26±0.8	9±0.4	0	0	30±1.6	0	65±2.1	35±2.4
125.0	32±1.5	7±0.5	0	8±0.6	30±0.7	0	77±3.8	23±1.5
62.5	33±1.9	5±0.3	0	8±0.8	31±2.4	6±0.5	83±3.4	19±0.6
31.3	39±2.5	8±0.5	1± 0.5	4±0.5	28±2.6	2±0.5	82±3.1	18±0.1
15.6	41±2.8	20±0.4	1± 0.5	0	23±3.4	7±1.6	92±2.6	8±0.5
7.8	53±3.4	8±0.5	1± 0.5	3±0.5	23±3.2	4±0.8	92±3.5	8±0.5
3.9	45±2.9	10±0.5	1±0.5	6±0.5	28±3.6	5±0.5	95±2.7	5±0.5
1.9	41±1.5	14±0.4	0	4±0.3	36±2.6	1±0.4	96±3.1	4±0.8

Table 14 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of quinine

Quinine (I)

Dose (μM)	x = log dose	% inhibition (*fa*)	% survival (*fu*)	*fa/fu*	y = log *fa/fu*
1000.0	3.0000	67	33	2.03030	0.30756
500.0	2.6990	60	40	1.67500	0.22401
250.0	2.3979	47	53	1.13208	0.05388
125.0	2.0969	33	67	0.70149	-0.15398
62.5	1.7959	28	72	0.45833	-0.33882
7.8	0.8921	15	85	0.32941	-0.48226
3.9	0.5911	12	88	0.17045	-0.76839

Quinine (II)

Dose (μM)	*x* = log dose	% inhibition (*fa*)	% survival (*fu*)	*fa/fu*	y = log (*fa/fu*)
1000.0	3.00000	68	32	2.12500	0.32736
500.0	2.69897	63	37	1.70270	0.23114
125.0	2.09691	35	65	0.53846	-0.26885
62.5	1.79588	23	77	0.29870	-0.52476
15.6	1.19382	19	81	0.23457	-0.62973
7.8	0.89279	18	82	0.21951	-0.65854
3.9	0.59176	8	92	0.08696	-1.06070

Then, the relationship between x and y can be calculated.

For quinine (I), $y = 0.5728\,x + -1.4026$

$x = -(-1.4026)/0.5728$

$= 2.45$

Since anti-log of 2.45 = 280.99, thus, the IC_{50} of quinine (I) is equal to 280.99 μM.

For quinine (II), $y = 0.5052\,x + -1.2443$

$x = -(-1.2443)/0.5052$

$= 2.46$

Since anti-log of 2.46 = 290.93, thus, the IC_{50} of quinine (II) is equal to 290.93 μM.

The mean IC_{50} value of quinine $= \dfrac{IC_{50} \text{ of quinine (I)} + IC_{50} \text{ of quinine (II)}}{2}$

$= (280.99+290.93)/2$

IC_{50} value of quinine $= 285.96$ μM

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Table 15 The number of parasitized cells ($\bar{X}$±SD) with various forms of *Theileria* parasite counted against 100 erythrocytes after 24 h of exposure with various concentrations of *Vernonia cinerea* stems

V. cinerea L., Less stems (I)

Dose (µg/ml)	Parasitized cells per 100 erythrocytes						Parasitized cells per 100 erythrocytes	
	Ring	Match	2 piroplasm	3 piroplasm	4 piroplasm	>4 piroplasm	Living parasite	Dead parasite
Control	3.8±1.2	2±0.5	0	1±0.5	59±2.2	0	100±3.5	0
1000.0	46±2.7	3±0.2	0	0	0	0	49±1.3	51±2.9
500.0	43±2.1	5±0.5	0	0	0	0	48±2.5	45±2.4
250.0	46±3.1	5±0.5	0	0	1±0.5	0	52±3.1	36±1.9
125.0	43±2.4	3±0.5	0	0	3±1.1	0	49±1.8	30±1.5
62.5	41±2.1	5±0.2	0	0	3±0.8	0	49±2.2	22±0.9
7.8	40±1.9	4±0.9	0	0	2±0.3	0	46±1.7	11±1.3
1.9	39±1.9	5±0.5	0	0	2±0.1	0	46±1.9	9±0.5
0.9	45±2.4	3±0.2	0	0	4±0.3	0	52±2.9	5±0.5
0.5	58±3.2	4±0.5	0	0	6±0.5	0	68±3.2	3±0.5

Table 15 The number of parasitized cells ($\bar{X}\pm SD$) with various forms of *Theileria* parasite counted against 100 erythrocytes after 24 h of exposure with various concentrations of *Vernonia cinerea* stems (continued)

V. cinerea L., Less stems (II)

Dose (µg/ml)	Parasitized cells per 100 erythrocytes						Parasitized cells per 100 erythrocytes	
	Ring	Match	2 piroplasm	3 piroplasm	4 piroplasm	>4 piroplasm	Living parasite	Dead parasite
Control	38±1.2	2±3.1	0	1±0.3	59±2.2	0	100±3.5	0
1000.0	35±2.4	13±0.8	0	0	16±1.4	0	40±2.8	60±3.2
500.0	29±1.4	18±1.9	0	1±0.2	24±1.7	1±0.1	49±1.8	51±2.6
250.0	24±1.2	16±1.4	0	0	30±2.3	0	58±3.0	42±2.2
125.0	24±0.5	12±0.7	0	1±0.3	34±1.8	0	69±1.4	31±2.6
62.5	24±2.3	14±0.8	0	1±0.4	34±1.2	0	73±3.4	27±0.5
31.3	25±2.4	14±1.0	1±0.4	2±0.6	35±2.0	0	77±2.8	23±1.1
15.6	23±2.1	18±1.2	1±0.3	0	36±2.5	3±0.3	81±3.2	19±0.6
7.8	28±2.5	17±1.9	0	1±0.2	36±1.6	4±0.5	84±2.7	16±0.5
3.9	27±3.0	14±0.9	0	3±0.3	38±3.2	5±0.2	87±3.1	13±0.5

Table 16 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of *V. cinerea* stems

V. cinerea L., Less stems (I)

Dose (µg/ml)	x = log dose	% inhibition (*fa*)	% survival (*fu*)	*fa/fu*	y = log *fa/fu*
1000.0	3.00000	51	49	1.04082	0.01737
500.0	2.69897	45	55	0.81818	-0.08715
250.0	2.39794	36	64	0.56250	-0.24988
125.0	2.09691	30	70	0.42857	-0.36798
62.5	1.79588	22	78	0.28205	-0.54967
7.8	0.89209	11	89	0.12360	-0.90800
1.9	0.27875	9	91	0.09890	-1.00480

V. cinerea L., Less stems (II)

Dose (µg/ml)	*x* = log dose	% inhibition (*fa*)	% survival (*fu*)	*fa/fu*	y = log (*fa/fu*)
1000.0	3.00000	60	40	1.50000	0.17609
500.0	2.69897	51	49	1.04082	0.01737
250.0	2.39794	42	58	0.72414	-0.14018
125.0	2.09691	31	69	0.44928	-0.34749
62.5	1.79588	27	73	0.36986	-0.43196
31.3	1.49554	23	77	0.29870	-0.52476
15.6	1.19312	19	81	0.23457	-0.62973
7.8	0.89209	16	84	0.19048	-0.72016

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Then, the relationship between x and y can be calculated.

For *V. cinerea* (I), $y = 0.4311x + -1.2736$

$x = -(-1.2736)/0.4311$

$= 2.95$

Since anti-log of 2.95 = 900.20, thus, the IC_{50} of *V. cinerea* (I) is equal to 900.20 µg/ml.

For *V. cinerea* (II), $y = 0.4080x + -1.1875$

$x = -(-1.1875)/0.4080$

$= 2.91$

Since anti-log of 2.91 = 877.02, thus, the IC_{50} of *V. cinerea* (II) is equal to 877.02 µg/ml.

The mean IC_{50} value of *V. cinerea* = $\frac{IC_{50} \text{ of } V. \text{ cinerea (I)} + IC_{50} \text{ } V. \text{ cinerea of (II)}}{2}$

$= (900.20+877.02)/2$

IC_{50} value of *V. cinerea* extract stems = 888.61 µg/ml

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Assessment of 50% inhibition concentration against *Theileria* parasite by radioisotopic method

Table 17 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of chloroquine

Chloroquine (I)

Dose (μM)	[^{3}H] hypoxantine incorporated (c.p.m.)*	[^{3}H] hypoxantine incorporated (c.p.m.)**	% Inhibition incorporation (I)***
RBC+CM199	69 ± 255	-	-
PRBC+CM199	3473 ± 745	3404	-
1000.0	69 ± 21	0	100.00
500.0	625 ± 180	556	83.64
250.0	655 ± 154	586	82.77
125.0	675 ± 83	606	82.17
62.5	1106 ± 179	1037	69.51
31.3	1466 ± 182	1397	58.95
15.6	1650 ± 139	1581	53.54
7.8	2009 ± 385	1940	42.99
3.9	2296 ± 421	2227	34.55
1.9	2636 ± 287	2567	24.56

* These results are expressed as the mean of triplicates (±) standard deviations.

** [^{3}H] hypoxantine incorporated are expressed as counts per minute (c.p.m.) in parasitized erythrocytes well minus counts per minute in parasite-free control well.

*** % inhibition incorporations were calculated using the following formula,

$$= \frac{\{\text{c.p.m. in the drug-free control well}^{**} - \text{c.p.m. in the treated well}^{**}\} \times 100}{\text{c.p.m. in the drug-free control well}^{**}}$$

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Table 17 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of chloroquine (continued)

Chloroquine (II)

Dose (μM)	[^{3}H] hypoxantine incorporated (c.p.m.)*	[^{3}H] hypoxantine incorporated (c.p.m.)**	% Inhibition incorporation (I)***
RBC+CM199	69 ± 255	-	-
PRBC+CM199	3473 ± 745	3404	-
1000.0	69 ± 47	0	100.00
500.0	525 ± 215	455	86.60
250.0	492 ± 110	423	87.56
125.0	573 ± 228	504	85.18
62.5	643 ± 121	574	83.13
31.3	1194 ± 206	1125	66.94
15.6	1559 ± 201	1490	56.20
7.8	2052 ± 710	1983	41.72
3.9	2335 ± 672	2266	33.41
1.9	2545 ± 432	2476	27.25

* These results are expressed as the mean of triplicates (±) standard deviations.

** [^{3}H] hypoxantine incorporated are expressed as counts per minute (c.p.m.) in parasitized erythrocytes well minus counts per minute in parasite-free control well.

*** % inhibition incorporations were calculated using the following formula,

$$= \frac{\{\text{c.p.m. in the drug-free control well}^{**} - \text{c.p.m. in the treated well}^{**}\} \times 100}{\text{c.p.m. in the drug-free control well}^{**}}$$

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Table 17 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of chloroquine (continued)

Dose (μM)	% Inhibition incoporation (I)	% Inhibition incoporation (II)	Mean
1000.0	100.00	100.00	100.00
500.0	83.64	86.60	85.12
250.0	82.77	87.55	85.16
125.0	82.17	85.18	83.67
62.5	69.52	83.13	76.33
31.3	58.95	66.94	62.94
15.6	53.54	57.13	55.34
7.8	42.99	41.72	42.36
3.9	34.55	33.41	33.98
1.9	24.57	27.23	25.90

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Table 18 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of quinine

Quinine (I)

Dose (μM)	[^{3}H] hypoxantine incorporated (c.p.m.)*	[^{3}H] hypoxantine incorporated (c.p.m.)**	% Inhibition incorporation (I)***
RBC+CM199	69 ± 255	-	-
PRBC+CM199	3473 ± 745	3404	-
1000.0	502 ± 108	433	87.28
500.0	761 ± 214	692	79.67
250.0	1805 ± 370	1736	48.99
125.0	1843 ± 270	1774	47.87
62.5	2102 ± 330	2033	40.26
31.3	2504 ± 426	2435	28.44
15.6	3092 ± 486	3023	11.16
7.8	2967 ± 599	2898	14.83
3.9	3260 ± 554	3191	6.24
1.9	3319 ± 486	3250	4.49

* These results are expressed as the mean of triplicates (±) standard deviations.

** [^{3}H] hypoxantine incorporated are expressed as counts per minute (c.p.m.) in parasitized erythrocytes well minus counts per minute in parasite-free control well.

*** % inhibition incorporations were calculated using the following formula,

$$= \frac{\{\text{c.p.m. in the drug-free control well}^{**} - \text{c.p.m. in the treated well}^{**}\} \times 100}{\text{c.p.m. in the drug-free control well}^{**}}$$

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Table 18 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of quinine (continued)

Quinine (II)

Dose (μM)	[^{3}H] hypoxantine incorporated (c.p.m.)*	[^{3}H] hypoxantine incorporated (c.p.m.)**	% Inhibition incorporation (I)***
RBC+CM199	69 ± 255	-	-
PRBC+CM199	3473 ± 745	3404	-
1000.0	429 ± 80	360	89.41
500.0	1265 ± 62	1196	64.85
250.0	1895 ± 236	1826	46.34
125.0	2052 ± 273	1983	41.72
62.5	2358 ± 521	2289	32.74
31.3	2812 ± 656	2743	19.39
15.6	2807 ± 113	2738	19.55
7.8	3043 ± 492	2974	12.63
3.9	3049 ± 237	2980	12.43
1.9	3092 ± 745	3023	11.16

* These results are expressed as the mean of triplicates (±) standard deviations.

** [^{3}H] hypoxantine incorporated are expressed as counts per minute (c.p.m.) in parasitized erythrocytes well minus counts per minute in parasite-free control well.

*** % inhibition incorporations were calculated using the following formula,

$$= \frac{\{\text{c.p.m. in the drug-free control well}^{**} - \text{c.p.m. in the treated well}^{**}\} \times 100}{\text{c.p.m. in the drug-free control well}^{**}}$$

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Table 18 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of quinine (continued)

Dose (μM)	% Inhibition incoporation (I)	% Inhibition incoporation (II)	Mean
1000.0	87.28	89.41	88.34
500.0	79.67	64.85	72.26
250.0	48.99	46.34	47.66
125.0	47.87	41.72	44.79
62.5	40.26	32.74	36.50
31.3	28.44	19.39	23.92
15.6	11.16	19.55	15.36
7.8	14.83	12.63	13.73
3.9	6.24	12.43	9.33
1.9	4.49	11.16	7.83

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Table 19 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of *V. cinerea* stems

V. cinerea L., Less stems (I)

Dose (μg/ml)	[^{3}H] hypoxantine incorporated (c.p.m.)*	[^{3}H] hypoxantine incorporated (c.p.m.)**	% Inhibition incorporation (I)***
RBC+CM199	69 ± 255	-	-
PRBC+CM199	3473 ± 745	3404	-
1000.0	1515 ± 300	1446	57.51
500.0	1989 ± 353	1920	43.58
250.0	2204 ± 121	2135	37.27
125.0	2323 ± 172	2254	33.77
62.5	2583 ± 153	2514	26.13
31.3	2689 ± 234	2620	23.02
15.6	2859 ± 171	2790	18.02
7.8	2951 ± 204	2882	15.32
3.9	3095 ± 231	3026	11.09
1.9	3376 ± 132	3307	2.84

* These results are expressed as the mean of triplicates (±) standard deviations.

** [^{3}H] hypoxantine incorporated are expressed as counts per minute (c.p.m.) in parasitized erythrocytes well minus counts per minute in parasite-free control well.

*** % inhibition incorporations were calculated using the following formula,

$$= \frac{\{\text{c.p.m. in the drug-free control well}^{**} - \text{c.p.m. in the treated well}^{**}\} \times 100}{\text{c.p.m. in the drug-free control well}^{**}}$$

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Table 19 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of *V. cinerea* stems (continued)

V. cinerea L., Less stems (II)

Dose (μg/ml)	[^{3}H] hypoxantine incorporated (c.p.m.)*	[^{3}H] hypoxantine incorporated (c.p.m.)**	% Inhibition incorporation (I)***
RBC+CM199	69 ± 255	-	-
PRBC+CM199	3473 ± 745	3404	-
1000.0	1632 ± 687	1563	54.07
500.0	2042 ± 100	1973	42.00
250.0	2264 ± 219	2195	35.50
125.0	2315 ± 269	2246	34.00
62.5	2501 ± 164	2432	28.54
31.3	2654 ± 125	2585	24.05
15.6	2953 ± 224	2884	15.26
7.8	3089 ± 421	3020	11.26
3.9	3351 ± 550	3282	3.55
1.9	3261 ± 247	3192	6.20

* These results are expressed as the mean of triplicates (±) standard deviations.

** [^{3}H] hypoxantine incorporated are expressed as counts per minute (c.p.m.) in parasitized erythrocytes well minus counts per minute in parasite-free control well.

*** % inhibition incorporations were calculated using the following formula,

$$= \frac{\{\text{c.p.m. in the drug-free control well}^{**} - \text{c.p.m. in the treated well}^{**}\} \times 100}{\text{c.p.m. in the drug-free control well}^{**}}$$

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Table 19 The arrangement of data for calculation of 50% inhibitory concentration (IC_{50}) of *V. cinerea* stems (continued)

Dose (μg/ml)	% Inhibition incoporation (I)	% Inhibition incoporation (II)	Mean
1000.0	57.51	54.06	55.79
500.0	43.58	45.06	44.59
250.0	37.27	35.51	36.39
125.0	33.76	34.01	33.89
62.5	26.13	28.52	27.33
31.3	23.01	24.05	23.53
15.6	18.02	15.21	16.62
7.8	15.32	11.26	13.29
3.9	11.09	3.55	7.32
1.9	2.08	6.20	4.51

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