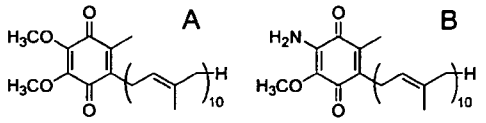

FIG. 1. Chemical structure of ubiquinone-10 (UQ_{10}) (E_m' = +110 mV) (A) and rhodoquinone-10 (RQ_{10}) (E_m' = −63 mV) (B).

minths have exploited a variety of energy-transducing systems during their adaptation to habitats in their hosts (7, 28). The parasitic nematode *Ascaris suum*, for example, resides in the host small intestine, where oxygen tensions are low, and exploits a unique anaerobic respiratory chain, called the NADH-fumarate reductase system, to adapt to its microaerobic habitat (Fig. 2) (2, 3, 14, 22; reviewed in reference 10). The NADH-fumarate reductase system is part of the unique respiratory system for parasitic helminthes and is the terminal step in the phosphoenolpyruvate carboxykinase-succinate pathway, which is found in many anaerobic organisms. Electrons from NADH are accepted by rhodoquinone (RQ) (Fig. 1B) via the NADH-RQ reductase activity of mitochondrial complex I and then transferred to fumarate through the rhodoquinol-fumarate reductase activity of mitochondrial complex II. The anaerobic electron transfer in complex I couples with proton transport across the mitochondrial inner membrane, providing ATP even in the absence of oxygen. This system, which does not normally function in mammalian mitochondria, is considered to be a good target for the development of novel anthelminthics (8, 9, 21). With regard to *Echinococcus* spp., the presence of both aerobic and anaerobic respiratory systems was previously suggested by a series of intensive studies (1, 16, 17), although the respiratory systems in this group of parasites are to be characterized in more detail.

In the present study, we prepared an enriched mitochondrial fraction from *E. multilocularis* protoscoleces and characterized the specific enzyme activities involved in mitochondrial energy metabolism as well as the quinone profile in the parasite's respiratory chain. Furthermore, based on findings reported previously by Yamashita et al. that quinazoline derivatives can inhibit the NADH-quinone reductase of mitochondria from *A. suum* (35), we tested several quinazoline-type compounds, with a view to developing novel antiechinococcal compounds.

MATERIALS AND METHODS

Isolation of *E. multilocularis* protoscoleces. We used the Nemuro strain of *E. multilocularis*, which is maintained at the Hokkaido Institute of Public Health (Sapporo, Japan). Mature larval parasites with protoscolex formation were obtained from cotton rats (*Sigmodon hispidus*) more than 4 months after oral infection with 50 parasite eggs. To isolate protoscoleces, the mature larval parasites were minced with scissors, pushed through a metal mesh, and washed repeatedly with physiological saline until host materials were thoroughly removed.

Preparation of enriched mitochondrial fractions. The enriched mitochondrial fractions of *E. multilocularis* protoscoleces were prepared essentially according to methods described previously for isolating adult *Ascaris* mitochondria (25, 26). Briefly, the isolated protoscolex sediment was suspended in 5 volumes of mitochondrial preparation buffer (210 mM mannitol, 10 mM sucrose, 1 mM disodium EDTA, and 50 mM Tris-HCl [pH 7.5]) supplemented with 10 mM sodium malonate. The parasite materials were homogenized with a motor-driven glass/glass homogenizer (six passes three to four times). The homogenate was diluted with the mitochondrial preparation buffer to 10 times the volume of the original protoscolex sediment and then centrifuged at 800 × *g* for 10 min to precipitate cell debris and nuclei. The supernatant was then centrifuged at 8,000 × *g* for 10 min to obtain the mitochondrial pellet. The pellet was resuspended in mitochondrial preparation buffer (without malonate) and centrifuged at 12,000 × *g* for 10 min. The resulting enriched mitochondrial fraction was suspended in mitochondrial preparation buffer (without malonate). The protein concentration was determined according to the method of Lowry et al. by using bovine serum albumin as a standard (15).

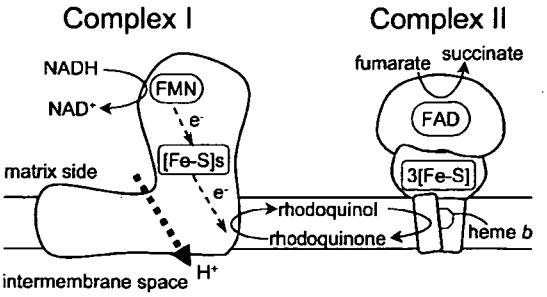

FIG. 2. Schematic representation of the NADH-fumarate reductase system in adult *A. suum*, which catalyzes the final step of the phosphoenolpyruvate carboxykinase-succinate pathway. In this system, the reducing equivalent of NADH is transferred to the low-potential RQ by the NADH-RQ reductase activity of mitochondrial complex I. This pathway ends with the production of succinate by the rhodoquinol-fumarate reductase activity of complex II. Electron transfer from NADH to fumarate is coupled to the site I phosphorylation of complex I via the generation of a proton-motive force. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; [Fe-S]s and 3[Fe-S], iron-sulfur clusters.

Western blotting. An enriched mitochondrial fraction prepared from *E. multilocularis* protoscoleces and that from the liver of a cotton rat (used as the host animal for the parasite) were analyzed by Western blotting. Reactions were performed according to a method described previously by Towbin et al. (30). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% or 15% acrylamide gel and electrophoretically transferred onto a nitrocellulose membrane. The membrane was soaked in 1:5,000 anti-cytochrome *c* oxidase subunit IV antibody (component of the ApoAlert cell fractionation kit; Clontech Laboratories) in phosphate-buffered saline containing 0.05% (wt/vol) Tween 20 and 2% (wt/vol) skim milk. The membrane was incubated for 60 min at room temperature and then washed three times for 10 min with washing buffer, which consisted of 0.05% (wt/vol) Tween 20 in phosphate-buffered saline. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G was then added as a secondary antibody, and the mixture was incubated for 30 min. After another wash with washing buffer, the membrane was soaked in reaction buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$, 500 μg/ml of 4-nitroblue tetrazolium chloride, and 165 μg/ml of 5-bromo-4-chloro-3-indolylphosphate) to initiate the development of a colored product. Finally, the membrane was washed with distilled water to stop the reaction. For Western blotting, the amounts of parasite and cotton rat mitochondrial samples were normalized by the total protein amount or cytochrome *c* oxidase activity (see below).

Enzyme assays. All enzyme assays using the enriched mitochondrial fractions were performed in a 0.7- or 1-ml reaction mixture at 25°C. The reagents used in each assay were mixed with reaction buffer containing 30 mM potassium phosphate (pH 7.4) and 1 mM $MgCl_2$. The final mitochondrial protein concentration was 80 μg per ml of reaction mixture. For all reactions performed under anaerobic conditions, the reaction medium was supplemented with 100 μg/ml glucose oxidase, 2 μg/ml catalase, and 10 mM β-D-glucose and left for 3 min to achieve anaerobiosis. NADH oxidase activity in the isolated mitochondrial fraction was determined in the presence or absence of 2 mM KCN, 100 mM malonate, or both by measuring the absorbance of NADH at 340 nm (ε = 6.2 mM^{-1} cm^{-1}). The reaction was initiated by the addition of 100 μM of NADH to the mixture. Succinate dehydrogenase (SDH) activity was determined by monitoring the absorbance change of 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide (MTT; 60 μg/ml) at 570 nm in the presence of 120 μg/ml phenazine methosulfate and 2 mM KCN. The reaction was initiated by the addition 10 mM of succinate to the mixture. Succinate-quinone reductase activity was assayed under aerobic or anaerobic conditions in the presence of 0.1%(wt/vol) sucrose monolaurate by determining the amount of decyl UQ (dUQ) or decyl RQ (dRQ)

from the absorbance change at 278 nm (ε = 12.7 mM^{-1} cm^{-1}) or 287 nm (ε = 9.2 mM^{-1} cm^{-1}), respectively. Decyl rhodoquinol-fumarate reductase activity was measured under anaerobic conditions in a reaction mixture containing 0.1% (wt/vol) sucrose monolaurate. In this reaction, 60 μM dRQ was reduced to decyl rhodoquinol in the cuvette by adding 200 μM $NaBH_4$. The reaction was started by adding 5 mM fumarate to the mixture, and the oxidation of decyl rhodoquinol was monitored at 287 nm. NADH-fumarate reductase activity was determined by monitoring the oxidation of NADH (100 μM) at 340 nm under anaerobic conditions. The reaction was initiated by the addition of 5 mM fumarate as an electron acceptor. NADH-quinone reductase activity assays were carried out under anaerobic conditions using the same reaction mixture as that used for the NADH-fumarate reductase activity assay except that 60 μM dUQ or dRQ was used as an electron acceptor instead of fumarate. The enzyme activity was determined by monitoring the absorbance change of NADH at 340 nm. Ubiquinol oxidase activity was determined by monitoring the absorbance change of ubiquinol-1 (150 μM) at 278 nm (ε = 12.7 mM^{-1} cm^{-1}) in the presence or absence of 2 mM KCN. The activity of cytochrome *c* oxidase was determined as *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) oxidase activity, which was measured by monitoring the absorbance change of TMPD (500 μM) at 610 nm (ε = 11.0 mM^{-1} cm^{-1}) in the presence or absence of 2 mM KCN.

Enzyme inhibition assays. Based on the findings of Yamashita et al. showing that that quinazoline-type compounds inhibit the NADH-quinone reductase activity of *A. suum* complex I (35), we determined 50% inhibitory concentration (IC_{50}) values of the quionazoline-type compounds against NADH-fumarate reductase activity of the parasite mitochondria and the NADH oxidase activity of bovine heart mitochondria (see "Enzyme assays"). The compounds used in the assays included quinazoline and its derivatives 6-NH_2, 6-$NHCO(CH{=}CH_2)$, 7-NH_2, 8-OH, 8-OCH_3, 8-OCH_2CH_3, and 8-$OCH(CH_3)_2$.

Analysis of the quinone profile of isolated mitochondria. Quinones were extracted from lyophilized mitochondria essentially according to a method described previously by Takada et al. (24). A lyophilized mitochondrial sample (2.9 mg protein) was crushed into powder before extraction, vortexed in 2:5 (vol/vol) ethanol/*n*-hexane for 10 min, and centrifuged at 20,000 × *g* for 5 min at room temperature. The supernatants were pooled, and the extraction of quinones was repeated twice. Pooled extracts were evaporated to dryness, dissolved in ethanol, and kept in the dark until high-performance liquid chromatography (HPLC) analysis. Quinones were applied to a reverse-phase HPLC column (Inertsil ODS-3 [5 μm and 4.6 by 250 mm]; GL Science) and eluted under isocratic conditions (1 ml/min) with 1:4 (vol/vol) diisopropyl ether-methanol at 25°C. The molecular species of the eluted quinones were identified by their retention times and by their spectral characteristics as measured with a UV-visible photodiode array (Shimadzu SPD-10-A). The concentration of quinones was determined spectrophotometrically. The major quinone detected was confirmed by mass spectrometry (MS) using an Applied Biosystems API-165 LC/MS system with electrospray ionization.

In vitro treatment of *E. multilocularis* protoscoleces. *E. multilocularis* protoscoleces were obtained as described above (see "Isolation of *E. multilocularis* protoscoleces"). The parasite materials were placed into culture medium suitable for the long-term maintenance of the protoscoleces in vitro (27). The parasite cultures were kept in a six-well plate at a density of approximately 500 protoscoleces per ml of culture medium, and half of the medium was replaced twice a week. This culture condition was also applied during in vitro treatment of the parasite. To examine the efficacy of chemical compounds against living *E. multilocularis* protoscoleces, the parasites were kept in the culture medium supplemented with 5 or 50 μM of each compound, including quinazoline and its 8-OH derivative, rotenone (a specific inhibitor of mitochondrial complex I) (19) and nitazoxanide (a compound with strong protoscolicidal action) (32). One control group was supplemented with 0.5% (vol/vol) dimethyl sulfoxide (vehicle) alone, and all conditions were assayed in triplicate. The viability of protoscoleces was determined by microscopic analysis of more than 170 protoscoleces per well for motile behavior and the ability to exclude trypan blue (32).

RESULTS

Preparation of enriched mitochondrial fractions. To characterize the mitochondrial respiratory chain of *E. multilocularis* protoscoleces, we prepared enriched mitochondrial fractions from the parasite. Approximately 80 g of larval *E. multilocularis* (containing approximately 10^5 protoscoleces per gram) was obtained from each cotton rat more than 4 months after oral infection with 50 parasite eggs. Approximately 20 g of the larval parasite was used per isolation of protoscoleces, yielding 2 ml of cleaned protoscolex sediment (Fig. 3). The enriched mitochondrial fractions were prepared from the protoscolex sediment as described in Materials and Methods. Each 1 ml of protoscolex sediment (containing 4.5 × 10^5 protoscoleces) yielded approximately 4 mg of mitochondria. Western blotting using an antibody to mammalian cytochrome *c* oxidase detected a specific band in the mitochondria from the liver of a cotton rat but not in mitochondria from *E. multilocularis* protoscoleces even when the amounts of both mitochondrial samples were normalized according to cytochrome *c* oxidase activity (data not shown). These results demonstrated that the enriched mitochondrial fractions from the parasite were sufficiently free of host components for use in enzyme assays and quinone analyses. In order to assess the quality of mitochondria, intactness was examined by the reactivity of NADH, which is a non-membrane-permeable substrate. NADH oxidase activity was not detected in the isotonic buffer, whereas it was fully activated in hypotonic buffer after a freeze-thaw treatment of the enriched mitochondrial fraction. Based on the results obtained, the method applied here for mitochondrial preparation seemed to be appropriate.

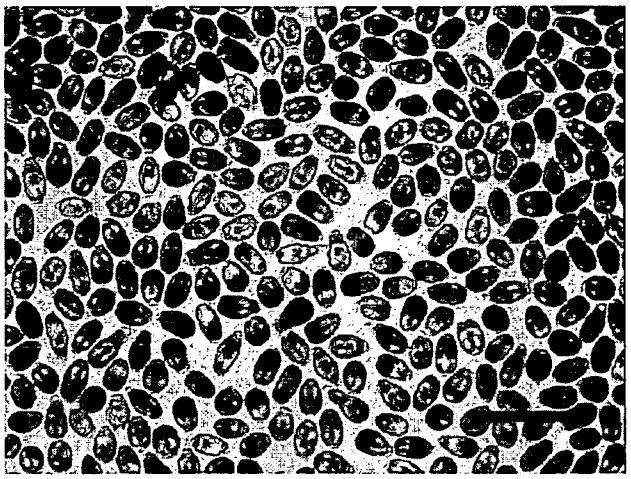

FIG. 3. Protoscoleces of *E. multilocularis* (Nemuro strain) used for the preparation of enriched mitochondrial fractions of the parasite and subsequent analyses. Bar, 500 μm.

Enzyme activities of *E. multilocularis* mitochondria. The specific enzyme activities involved in the mitochondrial respiratory chain of *E. multilocularis* protoscoleces are shown in Table 1. Parasite complex II exhibited an SDH activity of 103 nmol/min/mg. The specific activity of succinate-dUQ reductase was comparable to that of SDH activity (98.9 nmol/min/mg), whereas the succinate-dRQ reductase activity was lower (16.6 nmol/min/mg). The specific activity of decyl rhodoquinol-fumarate reductase, which is the reverse reaction of the succinate-RQ reductase activity of complex II, was determined to be 60.2 nmol/min/mg. The mitochondria of *E. multilocularis* protoscoleces exhibited NADH oxidase activity of 9.1 nmol/min/mg, which was almost eliminated by 2 mM KCN and 100 mM malonate. Ubiquinol-1 oxidase and TMPD oxidase activities were determined to be 4.4 nmol/min/mg and 12.6 nmol/

TABLE 1. Specific activities of mitochondrial respiratory enzymes in *E. multilocularis* protoscoleces

Assay	Sp act[a] (nmol/min/mg of protein) (mean ± SD)
SDH	103 ± 16
Succinate-quinone reductase	
dUQ (anaerobic)	98.9 ± 12
dRQ (anaerobic)	16.6 ± 3.5
Quinol-fumarate reductase (decyl rhodoquinol) (anaerobic)	60.2 ± 18
NADH oxidase	9.1 ± 2.1
NADH oxidase with:	
2 mM KCN	7.3 ± 1.5
100 mM malonate	4.4 ± 0.4
2 mM KCN and 100 mM malonate	1.7 ± 0.7
Ubiquinol-1 oxidase	4.4 ± 0.6
TMPD oxidase	12.6 ± 6.3
NADH-fumarate reductase (anaerobic)	45.0 ± 8.1
NADH-quinone reductase	
dUQ (anaerobic)	32.1 ± 2.7
dRQ (anaerobic)	61.3 ± 4.3

[a] Specific activities were obtained from at least three independently isolated mitochondria.

min/mg, respectively. These activities were completely inhibited by 2 mM KCN. Under anaerobic conditions, the specific activity of NADH-fumarate reductase was 45 nmol/min/mg, which was much higher than the NADH oxidase activity. The specific activity of NADH-dUQ reductase and NADH-dRQ reductase of complex I were determined to be 32.1 and 61.3 nmol/min/mg, respectively.

Quinone components in *E. multilocularis* mitochondria. To determine which quinones act as physiological electron mediators in the mitochondrial respiratory system of *E. multilocularis* protoscoleces, HPLC analyses were performed. As shown in Fig. 4A, the enriched mitochondrial fractions contained only one major quinone component at a retention time (*Rt*) of 22.4 min. The peak fraction exhibited a characteristic absorption maximum for RQs at 283 nm (Fig. 4B) (20). Subsequent MS analysis confirmed that the primary quinone of the parasite was RQ_{10} (electrospray ionization-MS *m/z* 848.8 $[M + H]^+$). The concentration of RQ_{10} was determined to be 0.73 nmol/mg of mitochondrial protein.

Effects of inhibitors on NADH-fumarate reductase in *E. multilocularis* mitochondria. To investigate the inhibitory effect of quinazoline (Fig. 5A) and its derivatives on the enzymatic activities in the anaerobic respiratory system of *E. multilocularis* mitochondria, we determined IC_{50} values against the NADH-fumarate reductase activity of the enriched mitochondrial fraction of the parasite. We found that all of the compounds inhibited the NADH-fumarate reductase activity of the parasite to some extent. Quinazoline and its derivatives including 6-NH_2, 6-NHCO(CH=CH_2), 7-NH_2, 8-OH, 8-OCH_3, 8-OCH_2CH_3, and 8-$OCH(CH_3)_2$ exhibited IC_{50} values of 2.3, 2.1, 16, 62, 71, 48, 4,100, and 910 nM, respectively. Of the compounds tested, the 8-OH derivative (Fig. 5B) exhibited relatively selective inhibition against the NADH-fumarate reductase activity of *E. multilocularis* protoscoleces compared with the NADH oxidase activities of mammalian mitochondria: the IC_{50} values of quinazoline and its 8-OH derivative for the NADH oxidase activities of mammalian (bovine heart) mitochondria were 0.40 and 230 nM, respectively.

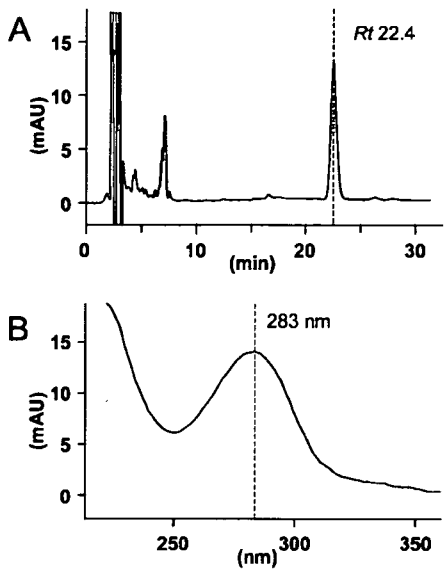

FIG. 4. (A) HPLC analysis of quinones extracted from the enriched mitochondrial fraction of *E. multilocularis* protoscoleces. Detailed experimental conditions are described in Materials and Methods. The highest peak had a retention time of 22.4 min (arrow). (B) Absorption of this peak was 283 nm, suggesting that it contained an RQ. mAU, milli-absorbance units.

Effects of inhibitors on living *E. multilocularis* protoscoleces. In order to examine the parasite-killing activities of the quinazoline-type compounds with different degrees of inhibitory effects against NADH-fumarate activities of *E. multilocularis* protoscoleces, we performed in vitro treatment of the parasite using quinazoline and its 8-OH derivative. The viability of the *E. multilocularis* protoscolex was progressively reduced during in vitro treatment of the parasites with 50 μM of the 8-OH derivative, and by day 5, all the parasites died (Fig. 6). The same compound did not have an obvious antiparasitic effect when used at a concentration of 5 μM. On the other hand, nonsubstituted quinazoline, which showed lower IC_{50} values with the enzymatic assay, eliminated the parasites on days 5 and 7 of in vitro treatment when used at 50 and 5 μM, respectively. Treatment with rotenone, a specific inhibitor of mitochondrial complex I (19), affected the viability of the parasite in a manner similar to that of the 8-OH derivative. The anti-echinococcal effect of nitazoxanide was relatively mild: even in

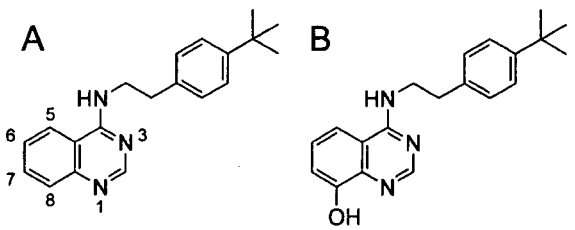

FIG. 5. Structures of quinazoline (A) and its 8-OH derivative (B) used for the enzyme inhibition assays and in vitro treatment of *E. multilocularis* protoscoleces.

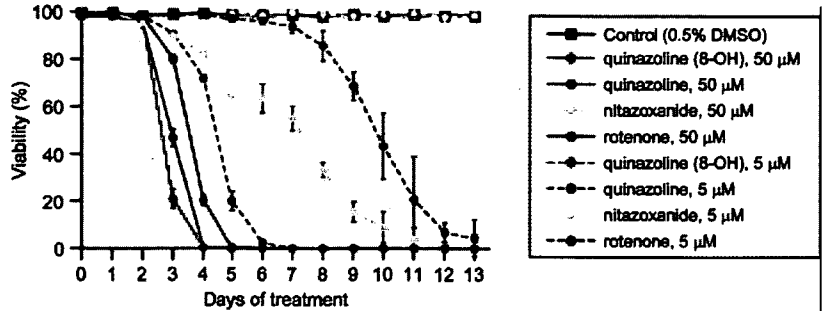

FIG. 6. Viability of *E. multilocularis* protoscoleces during in vitro treatment with quinazoline and its 8-OH derivatives, rotenone and nitazoxanide. Each compound was added to the culture medium at 5 or 50 μM. The results represent the means ± standard deviations of at least triplicate samples. DMSO, dimethyl sulfoxide.

the presence of 50 μM nitazoxanide, the viability decreased, but it did so only gradually, and it took 13 days before all the protoscoleces died. This compound did not affect parasite viability when used at 5 μM.

DISCUSSION

The most notable finding of the present study is that *E. multilocularis* protoscoleces possess a unique mitochondrial respiratory system that is highly adapted to anaerobic conditions. Specifically, the predominant enzymatic activity in the enriched mitochondrial fraction prepared from the parasite protoscoleces is the NADH-fumarate reductase system, which does not normally function in the aerobic respiratory chain of mammals. Thus, we infer that mitochondrial respiratory system of *E. multilocularis* would be a good target for the development of novel selective antiechinococcal compounds as demonstrated previously for other helminthic diseases (8, 21).

As early as 1957, Agosin found that *E. granulosus* protoscoleces have both aerobic and anaerobic respiratory systems and that glycolytic inhibitors are effective against both of them, indicating that they both depend on glycolysis (1). Subsequently, McManus and Smyth observed that protoscoleces cultured under anaerobic conditions produce more succinate than parasites kept under aerobic conditions, suggesting that the parasites survive under anaerobic conditions by utilizing the NADH-fumarate reductase system (16). Furthermore, McManus and Smyth reported that the specific activity of fumarate reductase in *Echinococcus* protoscoleces is lower than those of enzymes involved in the tricarboxylic acid cycle (17). These results, however, did not establish the importance of NADH-fumarate reductase activity in the mitochondrial respiratory system of the parasite because the other enzyme activities were not analyzed.

In the present study, we focused on the enzyme activities of the mitochondrial respiratory system of the parasite to determine whether the system is adapted to anaerobic conditions. Using the enriched mitochondrial fractions prepared from *E. multilocularis* protoscoleces, we showed that the activity of NADH-fumarate reductase in the respiratory system of the parasite is predominant compared with that of NADH oxidase, an enzyme involved in aerobic respiration in aerobic organisms such as mammals. Furthermore, direct measurements of complex II activities in both directions (i.e., succinate-RQ reductase and rhodoquinol-fumarate reductase activities) indicated that parasite complex II functions more favorably as a rhodoquinol-fumarate reductase in the presence of RQ/rhodoquinol. Thus, our results using isolated mitochondria of *E. multilocularis* protoscoleces coupled with assay systems for the determination of the parasite's enzyme activities revealed for the first time that the parasite mitochondria are highly adapted to anaerobic environments.

Analyses of the quinone components of *E. multilocularis* mitochondria revealed that RQ_{10} (Fig. 1B), whose redox potential is much more negative (E_m' [midpoint potential] = −63 mV) than that of UQ_{10} (E_m' = +110 mV) (Fig. 1A), was the primary quinone component of parasite mitochondria. In other parasitic helminths, like *A. suum* and *Hymenolepis diminuta*, RQ is an essential component of the NADH-fumarate reductase system (5, 11). In addition, van Hellemond et al. previously demonstrated that for all eukaryotes, the relative amount of RQ compared to the total amount of quinones correlates well with the importance of fumarate reduction in vivo (31). Similarly, during the development of the liver fluke *Fasciola hepatica*, there is a good correlation between the quinone composition and the importance of fumarate reduction in vivo (31). Therefore, RQ seems to be an essential component of fumarate reduction in eukaryotic respiration. Although menaquinone-related fumarate reduction in prokaryotes is well known (33, 34), there is no evidence that menaquinone serves this function in eukaryotes. In this study, enzyme assays demonstrated that the mitochondria from *E. multilocularis* possess NADH-fumarate activity as the predominant activity. In addition, the NADH-dRQ reductase activity was much higher than that of NADH-dUQ reductase, indicating that *E. multilocularis* complex I may interact preferentially with RQ rather than with UQ. Taken together, these results indicate that, as in other metazoan eukaryotes with anaerobic respiratory systems, *E. multilocularis* protoscoleces have a unique respiratory system that is highly adapted to anaerobic environments and in which RQ_{10} is used as the primary electron mediator.

Spiliotis et al. recently reported that the in vitro growth of larval *E. multilocularis* is more active under anaerobic than aerobic conditions (23). Thus, our findings for the respiratory system of *E. multilocularis* protoscoleces are consistent with the observations reported previously by Spiliotis et al. Larval *E. multilocularis* containing a large number of protoscoleces

lives in host tissues, mainly the liver, surrounded by thick connective tissues containing carbohydrate-rich laminated layers, which probably provide the parasite cells with an extremely-low-oxygen environment. Accordingly, it is not surprising that the parasite survives in the host by utilizing an anaerobic respiratory system.

Many anaerobic parasitic eukaryotes use the NADH-fumarate pathway, which is absent in mammals (2, 3, 10, 14, 22, 29). Therefore, this unique respiratory system is regarded as a promising chemotherapeutic target for the development of novel anthelminthics, as discussed in a recent review (9). In fact, Omura et al. previously found a natural compound, nafuredin, that is a potent inhibitor of the adult *A. suum* mitochondrial respiratory chain but much weaker against the mammalian mitochondrial respiratory chain (21). Yamashita et al. also found that quinazoline-type inhibitors were highly effective against adult *A. suum* complex I (35). Kinetic analyses using a series of quinazoline-type inhibitors revealed that *A. suum* complex I recognizes RQ_2 or UQ_2 in different ways, suggesting that mitochondrial complex I, which reacts preferably with RQs, could be a good target for chemotherapy. In the present study, we also tested several quinazoline-type compounds for their abilities to inhibit the anaerobic respiratory system of *E. multilocularis* protoscoleces. We found that all of the quinazoline-type compounds inhibited the NADH-fumarate reductase activity of *E. multilocularis* mitochondria to different extents. Furthermore, these compounds exhibited potent parasite-killing activities against *E. multilocularis* protoscoleces under in vitro culture conditions. Importantly, the nonsubstituted quinazoline, which has a higher inhibitory effect against NADH-fumarate oxidoreductase of the parasite mitochondria than the 8-OH derivative does, exhibited the parasite-killing activity even when used at 5 μM, whereas the 8-OH derivative did not do so at the same concentration. Such a correlation between the enzyme inhibition and the parasite-killing activities of these compounds suggests that the anaerobic NADH-fumarate reductase system of the parasite is a promising target for the development of antiechinococcal drugs.

Antiechinococcal drugs for chemotherapy of human AE should target not only protoscoleces but also the germinal layers of the *E. multilocularis* metacestode. The germinal layers in the larval parasite exhibit extremely unique characteristics. The parasite cells forming the germinal layers can differentiate into various tissues, including brood capsules and protoscoleces, and at the same time, they proliferate asexually as they remain in an undifferentiated state. This causes enlargement and, occasionally, metastasis of the lesions due to the formation of a large parasite mass. Therefore, for chemotherapy of AE, a complete cure cannot be achieved unless the germinal cells of the larval parasite are eliminated. Therefore, the mitochondrial respiratory system of germinal cells should be further characterized to aid in the development of a novel antiechinococcal compound(s) targeting the energy metabolism of larval *E. multilocularis*. However, it is presently quite difficult to obtain enough metacetode materials with homogeneous quality. Established methodologies for the in vitro cultivation of *E. multilocularis* metacestodes are now available (6, 23), and they will hopefully be applicable to large-scale preparations of metacestode materials in the near future.

During the life cycle of *E. multilocularis*, the parasite never undergoes active development and/or energy metabolism under aerobic conditions. The larval parasite lives mainly in the liver of intermediate host animals, whereas the adult worm dwells inside the small intestine of the final host, both of which are microaerobic conditions. Although the eggs of the parasite are exposed to air, they already contain a mature infective larva (oncosphere) waiting to be taken up by the next intermediate host. Therefore, the oncosphere does not develop or move under aerobic conditions. Taken together, these findings suggest that the respiratory system of *E. multilocularis* protoscoleces, as characterized in the present study, could represent the respiratory system used by the parasite throughout its developmental stages. Based on this speculation, the use of protoscolex materials in the first-step screening of candidate compounds by enzyme inhibition assays and subsequent in vitro parasite-killing assays appears to be reasonable, although it should be confirmed that the respiratory system of the *E. multilocularis* metacestode shares the same basic characteristics with that of the protoscolex stage of the parasite. We have already done preliminary experiments on the effects of the compounds used in this study, including the quinazoline derivative (8-OH), against in vitro-cultured metacestodes and found that the compounds exhibited high parasite-killing activities as evaluated by a modified MTT assay (data not shown). These results strongly suggest that our strategy is appropriate.

Highly effective chemotherapeutic compounds against human AE are not currently available despite the fact that the disease can be lethal unless the patient is appropriately treated during the early stage of the infection. Based on the findings presented here, it appears that the anaerobic respiratory system of *E. multilocularis*, which is distinct from that of host mammals, is a good target for the development of highly effective antiechinococcal drugs and, furthermore, that respiratory chain inhibitors (21, 35) are possible lead compounds for the development of antiechinococcal drugs.

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Screening of Indonesian Medicinal Plant Extracts for Antibabesial Activity and Isolation of New Quassinoids from *Brucea javanica*

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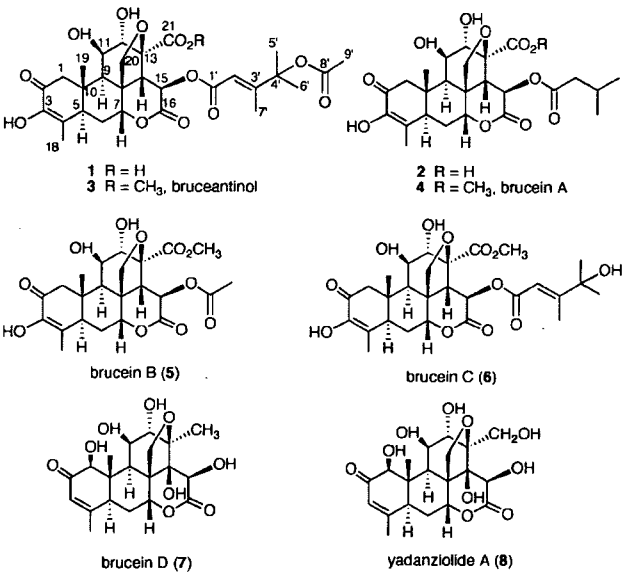
Boiled extracts derived from 28 Indonesian medicinal plants were screened for their antibabesial activity against *Babesia gibsoni* in vitro. Of these extracts, the fruit of *Brucea javanica* was the most active in inhibiting parasite growth at a concentration of 10 μg/mL. Bioassay-guided fractionation of the fruit extract of *Br. javanica* led to the isolation of two new quassinoids, bruceantinol B and bruceine J, and the structures of these compounds were elucidated on the basis of their spectroscopic data and by chemical transformation to known compounds. In addition, the known quassinoids bruceines A–D, bruceantinol, and yadanziolide A were isolated. Antibabesial activities were also examined in vitro, and bruceine A and bruceantinol were shown to be more potent than diminazene aceturate, a drug (IC_{50} = 103 ng/mL) used clinically against *B. gibsoni*, with IC_{50} values of 4 and 12 ng/mL, respectively.

Canine babesiosis is a tick-borne hemolytic disease in dogs caused by the intra-erythrocyte apicomplexan parasites, *Babesia gibsoni* and *Babesia canis*. The disease has been found to occur frequently in companion dogs and has become a serious problem clinically in various countries. Parasites of *Babesia* spp., as well as *Plasmodium* spp., invade red blood cells and induce severe clinical symptoms, such as fever, lethargy, hemolytic anemia, thrombocytopenia, lymphadenopathy, and splenomegaly.[1–3] Most dogs that recover from the acute stage of the disease become carriers and serve as potential sources of infection. These dogs are also at risk of recrudescent infection.[4] However, there is no successful chemotherapy for this disease, due to the limited number of useful drugs, their side effects, and other drawbacks of existing medication.[5,6] For this reason and to find new antibabesial drugs, we have screened several Indonesian medicinal plants for this purpose. Since promising antibabesial activity was found, further investigation of the boiled extract of the fruits of *Brucea javanica* (L.) Merr. (Simaroubaceae) was undertaken.

Brucea javanica, locally known as "buah makasar", is used in "Jamu" traditional folk medicine in Indonesia to treat malaria, dysentery, and cancer. The bitter principles of this plant are quassinoids, and some of them have been investigated extensively as antitumor agents.[7–9] These investigations have included clinical trials of the most potent of the *Br. javanica* quassinoids, bruceantin.[10,11] Bruceantin has been shown to possess high activity in vitro against *Entamoeba histolyca*.[12] Some quassinoids from this plant have also been found to exhibit antiplasmoidal activity against *Plasmodium falciparum*.[13,14] Despite the many phytochemical and pharmacological investigations, there are no reports on the antibabesial activity of this plant. We therefore investigated this plant and present herein data on two new quassinoids (bruceantinol B, **1**; bruceine J, **2**), along with six known related compounds (**3**–**8**) that possess antibabesial activity.

Boiling water extracts of the 23 selected Indonesian medicinal plants (Table S1, Supporting Information) were tested for in vitro antibabesial activity by assessing their ability to inhibit *B. gibsoni* growth. As shown in Table S2 (Supporting Information), all extracts exhibited, to different extents, dose-dependent inhibitory effects. At the lowest test concentration of 10 μg/mL, it was observed that the fruit extract of *Br. javanica* showed the most potent effect toward *B. gibsoni* growth (85.6% inhibition).



Silica gel column chromatography (MeOH–$CHCl_3$, 0:1, 3:97, 1:4, 7:3, 1:0) of the EtOAc-soluble portion of the boiled H_2O extract of *Br. javanica* fruit afforded five fractions. The MeOH–$CHCl_3$ (1:4) eluate gave, after silica gel column chromatography using hexane–EtOAc (1:1), 10 fractions. The fifth fraction gave bruceine A (**4**) on crystallization from MeOH, whereas the seventh, eighth, and ninth fractions afforded bruceantinol (**3**), bruceine B (**5**), and bruceine C (**6**), respectively,[15,16] on crystallization from hexane–EtOAc (9:1). The MeOH–$CHCl_3$ (7:3) eluate was subjected to silica gel column chromatography using MeOH–EtOAc (1:19) to give two new quassinoids (**1** and **2**), together with bruceine D (**7**) and yadanziolide A (**8**).[17,18] Identification of the known compounds was accomplished by comparing their spectroscopic data with those in the literature. The antibabesial activity of these compounds was evaluated against *B. gibsoni* quantitatively.

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Table 1. ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) Assignments for **1** and **2**

	bruceantinol B (**1**)		bruceine J (**2**)	
position	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1a	2.77 (d, 16.3)	50.2	2.76 (d, 16.2)	50.2
1b	2.46 (d, 16.0)		2.45 (d, 15.9)	
2		194.5		194.6
3		145.8		145.8
4		130.4		130.5
5	2.87 (brd, 12.8)	43.2	2.86 (brd, 12.8)	43.2
6a	2.20 (ddd, 14.5, 2.8, 2.8)	30.1	2.20 (ddd, 14.5, 2.8, 2.8)	30.1
6b	1.78 (ddd, 14.5, 14.5, 2.8)		1.78 (ddd, 14.5, 14.5, 3.0)	
7	4.76 (brs)	84.9	4.75 (brs)	85.1
8		46.3		46.0
9	2.14 (brd, 4.2)	42.8	2.10 (brd, 4.4)	42.8
10	42.2			42.1
11	4.10 (brd, 4.4)	71.1	4.09 (brd, 4.4)	70.9
12	3.85 (brs)	78.5	3.83 (brs)	78.5
13		84.0		83.4
14	3.68 (brs)	51.3	3.67 (brs)	51.6
15	6.40 (brs)	67.7	6.43 (brs)	67.4
16		169.5		169.3
18	1.73 (s)	13.4	1.73 (s)	13.4
19	1.27 (s)	16.0	1.25 (s)	16.1
20a	4.67 (d, 7.1)	74.4	4.67 (br)	74.5
	3.88 (d, 7.1)		4.01 (br)	
20b	1.73 (s)			
21		172.1		178.2
1′		166.9		173.5
2′	5.78 (s)	114.4	2.16 (d, 7.1)	43.5
3′		164.0	1.99 (m)	26.4
4′		84.0	0.90 (d, 6.2)	22.7
5′	1.48 (s)	26.5	0.89 (d, 6.4)	22.7
6′	1.47 (s)	26.7		
7′	2.01 (s)	14.9		
8′		171.8		
9′	1.93 (s)	21.7		

Compound **1** was isolated as a colorless amorphous solid. Fast atom bombardment mass spectrometry (FABMS) of **1** showed a pseudomolecular ion peak at *m/z* 591 $[M - H]^-$, indicating the molecular formula to be $C_{29}H_{35}O_{13}$. The IR spectrum displayed characteristic absorptions for hydroxyl (3407 cm^{-1}), δ-lactone and ester (1722 cm^{-1}), and α,β-unsaturated carbonyl (1685 and 1647 cm^{-1}) groups, respectively. The ^{1}H NMR spectrum showed resonances ascribable to three tertiary methyls (δ 1.48, 1.47, and 1.27), two olefinic methyls (δ 2.01, 1.73), one acetyl (δ 1.93), and one olefinic proton (δ 5.78) (Table 1). The ^{13}C NMR spectrum gave resonances of C-3 (δ 145.8), C-11 (δ 71.1), and C-12 (δ 78.5), indicating that hydroxyl groups are attached to these carbons (Table 1). Analysis of the ^{13}C NMR (δ 171.8, 166.9, 164.0, 114.4, 84.0, 26.7, 26.5, and 14.9), COSY, and HMBC spectra revealed the presence of a 4-acetoxy-3,4-dimethyl-2-pentenoyloxy group connected to C-15. Most quassinoids previously isolated from this plant possess a carbomethoxy group at C-13. However, these signals were not present in the spectra of **1**. Thus, the C-13 moiety of **1** was assigned as a free carboxylic acid. To confirm this carboxylic acid moiety, compound **1** was methylated with CH_2N_2 for 2.5 h. The usual workup was performed to give a colorless solid, which was purified by preparative thin layer chromatography (TLC) to afford **3**. Compound **3** was determined to be bruceantinol[15,16] by comparing its spectroscopic data with those reported. Therefore, compound **1** is a new quassinoid and has been named bruceantinol B.

Compound **2** was isolated as a colorless amorphous solid. FABMS of **2** showed a pseudomolecular ion peak at *m/z* 507 $[M - H]^-$, indicating the molecular formula to be $C_{25}H_{31}O_{11}$. The IR spectrum displayed characteristic absorptions for hydroxyl (3411 cm^{-1}), δ-lactone and ester (1724 cm^{-1}), and α,β-unsaturated carbonyl (1686 and 1649 cm^{-1}) groups. The ^{1}H NMR spectrum showed resonances ascribable to one tertiary methyl (δ 1.25), two secondary methyls (δ 0.90, 0.89), and one olefinic methyl (δ 1.73) (Table 1). The ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1**, with a free carboxylic acid group at C-13, except for the resonances ascribable to the ester side chain at C-15. This side chain was shown to be a 3-methylbutanoyloxy group, on the basis of analysis of the ^{13}C NMR (δ 173.5, 43.5, 26.4, 22.7, and 22.7), COSY, and HMBC spectra (Table 1). Methylation of **2** with CH_2N_2 gave bruceine A (**4**).[16] Accordingly, compound **2** is a new quassinoid and has been named bruceine J.

Table 2. Antibabesial Activity of Compounds **1–8** against *Babesia gibsoni* in Vitro[a]

compound	IC_{50} (ng/mL)
bruceantinol B (**1**)	978 ± 98
bruceine J (**2**)	742 ± 180
bruceantinol (**3**)	12 ± 3
bruceine A (**4**)	4 ± 1
bruceine B (**5**)	893 ± 223
bruceine C (**6**)	107 ± 51
bruceine D (**7**)	835 ± 100
yadanziolide A (**8**)	216 ± 46
diminazene aceturate[b]	103 ± 12

[a] Data are expressed as means ± standard deviation in triplicate analysis. [b] Positive control drug.

Although compounds **1** and **2** were isolated as naturally occurring constituents, it is possible that these compounds might be artifacts due to the use of boiling water for extraction. To investigate this, we carried out multiple reaction monitoring (MRM) using an ultra-performance liquid chromatography (UPLC)/tandem mass spectrometry (MS/MS) system with the negative-ion mode employed. Although the ionization of bruceantinol B (**1**) was not successful, the ionization of bruceine J (**2**) was accomplished. An authentic sample of **2** was subjected to the UPLC/MS/MS system, equipped with a photodiode array detector, run with the conditions described in the Experimental Section, and the peak of authentic **2** was observed at t_R = 2.45 min, together with the daughter ions *m/z* 100.4, 110.4, and 422.7 derived from *m/z* 508 in the MRM analysis. The sample that was prepared without using boiling water for extraction of raw material was subjected to the UPLC/MS/MS system, and the results of the MRM analysis are given in Figure S1 (Supporting Information). Since each corresponding daughter ion peak at *m/z* 100.4, 110.4, and 422.7 derived from bruceine J (**2**) (*m/z* 508) was observed at the same retention time (2.45 min), it was confirmed that **2** is a naturally occurring substance.

Compounds **1–8** were tested for their antibabesial activity against *B. gibsoni* in vitro.[19] *B. gibsoni* parasites treated with the test compounds (**1–8** and diminazene aceturate) demonstrated stagnation in the ring forms, including size reduction of the nucleus and disappearance of the cytoplasm. On the other hand, untreated parasites demonstrated typical petaloid forms after 3 days of incubation with clear cytoplasm in the parasite cells. Bruceine A (**4**) and bruceantinol (**3**) displayed promising activities (IC_{50} = 4 and 12 ng/mL, respectively) and exhibited more potent activity than the standard drug, diminazene aceturate (IC_{50} = 103 ng/mL) (Table 2). Bruceine C (**6**) showed almost the same antibabesial potency as diminazene aceturate, and the other isolated compounds (**1**, **2**, **5**, **7**, and **8**) exhibited IC_{50} values of <1 mg/ mL. A methyl ester carbonyl group at C-21 and a hydrophobic side chain attached at *O*-C-15 seem essential for potent activity among the quassinoids. These findings may provide the basis for the further understanding of *B. gibsoni* infections and contribute toward the development of new and effective treatments against this parasite.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO DIP-370 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 2000 series FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-500 FT-NMR spectrometer. FDMS, FABMS, HREIMS, and HRFABMS were obtained on a JEOL JMS-AX500 mass spectrometer. Column chromatography was performed on silica gel 60 (Spherical, 70–140 mesh, Kanto Chemical). Silica gel 60 F_{254} precoated plates (Merck) were used.

UPLC was performed on a Waters Acquity UPLC system, which was equipped with a binary solvent delivery system and a sample delivery system. MS was performed on a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer. The LC/MS system control was achieved by using MassLynx 4.0.

UPLC Conditions. The UPLC separation was performed on a Waters Acquity ethylene-bridged (BEH) C_{18} column (1.7 μm, 2.1 × 100 mm) at 38 °C with a flow rate of 0.3 mL/min. The analytes were eluted from the column with a mixed solvent of 20% aqueous MeOH with 0.1% AcOH (solvent A) and MeOH with 0.1% AcOH (solvent B) using a linear gradient mode. From 0 s to 0.2 min, the combination of solvents A and B was 20:80, and from 0.2 min to 3.1 min, the combination of solvents A and B was linearly converted from 20:80 to 0:100. The column was finally eluted with solvent A:B (0:100) from 3.1 min to 4.0 min.

MS Conditions. All MS optimization experiments were performed in both the MS scan mode and the product scan mode. All quantifications were performed in the multiple reaction monitoring (MRM) mode. The tune page parameters and conditions for each of the MRM transitions were optimized by infusing the neat standard solution into the mass spectrometer at 10 μg/mL. To ensure that the tune page parameters were compatible with the UPLC flow during the tuning, an UPLC flow of 0.6 mL/min at solvent A:B (1:1) was introduced into the mass spectrometer at the same time by utilizing a T unit (Upchurch Scientific, Oak Harbor, WA). For MRM data collection during the LC experiments, the capillary voltage was 3.0 kV, the source temperature was 120 °C, the desolvation temperature was 350 °C, the desolvation gas flow was 800 L/h, and the cone gas flow was 50L/h. During each LC injection, the mass spectrometer was set to collect data in MRM mode using electrospray ionization (ESI) in the negative-ion mode. The MRM transitions for the analytes for bruceine J (**2**) in ESI^- were as follow: 506.90 → 100.4 using cone voltage 46.00 and collision energy 46.00, 506.90 → 110.4 using cone voltage 46.00 and collision energy 36.00, and 506.90 → 422.7 using cone voltage 46.00 and collision energy 28.00.

Plant Material. The plant materials were purchased from the Bandar Jaya traditional market, Lampung, Indonesia, in April 2005. The plants were identified by Aris Winarso at the Herbal Medicine Research and Education Centre of "Karya Tama", Lampung, Indonesia. Voucher specimens are deposited at the Laboratory of Bioorganic Chemistry, Graduate School of Agriculture, Hokkaido University, Japan. The names of these medicinal plants, their families, local name, parts used, and voucher specimen numbers for the experiments are listed in Table S1 (Supporting Information).

Plant Extract Preparation. For preliminary testing, 10 g samples of dried material of each plant part were boiled twice in 200 mL of H_2O for 30 min. The boiling solution was cooled, filtered, and freeze-dried to give a powder. The powdered extract was then tested for antibabesial activity against *B. gibsoni* in vitro.

Extraction and Isolation. Extraction and isolation of the active compounds were monitored by the assay of antibabesial activity. The dried fruits of *Br. javanica* (1 kg) were boiled twice in 5 L of H_2O for 30 min. The boiling solution was filtered and extracted with EtOAc to give aqueous and EtOAc layers. The EtOAc layer (60.6 g) was chromatographed on a silica gel column, eluted successively with $CHCl_3$ (2 L), MeOH–$CHCl_3$ (3:97, 2 L), MeOH–$CHCl_3$ (1:4, 2 L), MeOH–$CHCl_3$ (7:3, 2 L), and MeOH (2 L). The MeOH–$CHCl_3$ (1:4) eluate was evaporated to yield a residue (10.8 g), which was subjected to column chromatography on silica gel, eluted with hexane–EtOAc (1:1), to give 10 fractions (A–J). Fraction E was recrystallized from methanol to give bruceine A (**4**, 362 mg). Recrystallization of fractions G, H, and I from hexane–EtOAc (9:1) gave bruceantinol (**3**, 247 mg), bruceine B (**5**, 533 mg), and bruceine C (**6**, 362 mg), respectively. The MeOH–$CHCl_3$ (7:3) eluate was evaporated to yield a residue (4.9 g), which was subjected to column chromatography on silica gel, eluted with MeOH–EtOAc (1:19), to give six fractions (K–P). Fraction M was purified by preparative TLC, eluted with MeOH–EtOAc (1:19), to yield bruceine D (**7**, 159 mg). Fraction O was purified by preparative TLC using MeOH–EtOAc (1:9) to yield yadanziolide A (**8**, 44 mg). After removal of the solvent, fraction P (832 mg) was further subjected to column chromatography on silica gel using $CHCl_3$–MeOH–H_2O (60:36:4) to give four subfractions (K–N). When rechromatograped on a silica gel column using $CHCl_3$–MeOH–H_2O (60:36:4), fractions L (67 mg) and M (48 mg) afforded bruceantinol B (**1**, 41 mg) and bruceine J (**2**, 36 mg), respectively.

Bruceantinol B (1): amorphous solid; $[\alpha]^{20}_D$ −41.0 (*c* 0.60, pyridine); IR (KBr) ν_{max} 3407 (OH), 1722 (δ-lactone and ester C=O), 1685, 1647 (α,β-unsaturated C=O) cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) data, see Table 1; FABMS (negative) *m/z* 591 $[M - H]^-$; HRFABMS *m/z* 591.2073 $[M - H]^-$ (calcd for $C_{29}H_{35}O_{13}$, 591.2077).

Bruceine J (2): amorphous solid; $[\alpha]^{20}_D$ −73.0 (*c* 0.6, pyridine); IR (KBr) ν_{max} 3411 (OH), 1724 (δ-lactone and ester C=O), 1686, 1649 (α,β-unsaturated C=O) cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) data, see Table 1; FABMS (negative) *m/z* 507 $[M - H]^-$; HRFABMS *m/z* 507.1864 $[M - H]^-$ (calcd for $C_{25}H_{31}O_{11}$, 507.1866).

Methylation of 1 and 2. An excess of an Et_2O solution of CH_2N_2, which was prepared from *N*-methyl-*N*-nitrosourea, was added to a MeOH (1 mL) solution of **1** (10 mg, 0.0169 mmol) at 0 °C. After the reaction mixture was stirred at 0 °C for 2.5 h, the solvent was evaporated to give a crude product. Purification of the crude product by preparative TLC (hexane–EtOAc, 1:4) afforded **3** (8.4 mg, amorphous solid, $[\alpha]^{20}_D$ −7.3 (*c* 1.2, pyridine). Methylation of **2** (10 mg, 0.0197 mmol) was carried out in the same manner as described above and afforded **4** (8.0 mg, amorphous solid, $[\alpha]^{20}_D$ −80.3 (*c* 0.8, pyridine). The physical and spectroscopic data of **3** and **4** were identical with those reported for bruceantinol and bruceine A, respectively.[15,16]

Extraction for MRM Experiment. The dried fruits of *Br. javanica* (0.1 kg) were extracted with 70% aqueous MeOH. The 70% aqueous MeOH layer was filtered to give a dark blown solution, which was evaporated and extracted with EtOAc to give aqueous and EtOAc layers. The volatile components of the EtOAc layer were removed under reduced pressure, and the residue was dissolved with MeOH (2 mL) and placed on a Bond Elut DEA cartridge column. The column was successively washed with MeOH (2 mL × 2) and 1 mol/L AcOH–MeOH (2 mL × 3). The volatile components of the AcOH–MeOH eluents were removed, and a portion of the residue was subjected to the UPLC/MS/MS experiment.

Antibabesial Assay. The in vitro assay against *B. gibsoni* was described in detail in a previous paper.[19] In this study, diminazene aceturate (Ganaseg) was used as control.

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Supporting Information Available: Tables listing plants collected and preliminary screening data and figure showing UPLC/photodiode array and MS/MS MRM chromatograms for bruceine J. This information is available free of charge via the Internet at http://pubs.acs.org.

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Anti-Babesial Compounds from *Rosa damascena* Mill.

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Bioassay-guided investigation of extracts of the flowers of *Rosa damascena* Mill. led to the isolation of four anti-babesial compounds, 3,4-dihydroxy benzoic acid (**1**), gallic acid (**2**), 2-phenylethyl 6-*O*-galloyl-β-D-glucopyranoside (**3**), and quercetin 3-*O*-β-D-(6-*O*-acetyl)-glucopyranosyl-(1→4)-α-L-rhamnopyranoside (**9**), in addition to five flavonoid compounds which possessed weak anti-babesial activity, kaempferol 3-*O*-β-D-glucopyranoside (**4**), kaempferol 3-*O*-β-D-xylopyranoside (**5**), kaempferol 3-*O*-α-L-rhamnopyranoside (**6**), quercetin 3-*O*-β-D-glucopyranoside (**7**), and quercetin 3-*O*-α-L-rhamnopyranoside (**8**). Among the isolated compounds, **3** possessed the greatest anti-babesial activity with an IC_{50} value of 11.78 μg/mL. Although compound **3** is known, no published reports list detailed spectroscopic data for the molecule. Therefore, the first complete structural elucidation of **3** using 1D and 2D NMR spectroscopic analysis is reported here. This is the first report of the isolation of compounds **1**, **5**, and **9** from *R. damascena*.

Keywords: *Rosa damascena*, Rosaceae, anti-babesial activity, diminazene aceturate, *Babesia gibsoni*.

Canine babesiosis is a tick-borne disease caused by the protozoal parasites *Babesia gibsoni* and *B. canis*. They infect the red blood cells of dogs and typically cause hemolytic anemia. Infection with *B. gibsoni* usually results in more severe clinical manifestations than infection with *B. canis*, and may cause multiple organ dysfunctions. Therefore, *B. gibsoni* is clinically more important than *B. canis* in Japan [1]. *B. gibsoni* is distributed in many regions throughout the world, including Asia, Africa, Europe, America, and Australia [2,3]. Treatment of or spontaneous recovery from an acute infection frequently fails to clear the organism from the host, resulting in a carrier stage. The animals that recover are a reservoir for tick-transmitted infections and are at risk for recrudescent infection [2]. No drugs have been proven effective for the elimination of *B. gibsoni* organisms from infected dogs. Some anti-babesial drugs reduce the severity of clinical signs and the mortality associated with the disease. These drugs include diminazene aceturate, imidocarb diproprionate, phenamidine isethionate, pentamidine isethionate, parvaquone, and niridazone [2]. However, these drugs usually cause pronounced and severe side effects [4]. Therefore, an alternative chemotherapeutic agent with fewer side effects is urgently needed for the treatment of *B. gibsoni* infection. We have reported anti-babesial activity of North African medicinal plants and found promising activity in extracts of *Berberis vulgaris* and *Rosa damascena* [5]. The aim of this study was to isolate and characterize the anti-babesial components of *Rosa damascena*.

R. damascena Mill., Rosaceae, is a perennial shrub indigenous to countries of Europe and the Middle East [6]. The essential oils of the plant are used in fine perfumery applications and cosmetic preparations [7]. In addition to antioxidant activities, water extracts of rose flowers possess anti-inflammatory and analgesic, antibacterial, antifungal, and anti-HIV effects [8-10]. In the last two studies, the active compound was identified as gallic acid. Water extracts of rose flowers are used also in traditional medicines such as antidepressants,

diuretics, and laxatives [6, 11]. Various terpenes and their glycosides, flavonoids and their glycosides, and anthocyanins have been isolated from the flowers of *R. damascena* [12-14].

An extract of *R. damascena* flowers, obtained using boiling water, was partitioned into H_2O and EtOAc layers. The EtOAc-soluble layer exhibited anti-babesial activity, and successive column chromatography on silica gel, followed by HPLC led to the isolation of nine compounds (**1-9**; Figure 1). The compounds' structures were deduced based on 1D and 2D NMR spectroscopy as 3, 4-dihydroxy benzoic acid (**1**), gallic acid (**2**), 2-phenylethyl 6-*O*-galloyl-β-D-glucopyranoside (**3**), and six flavonoid compounds, kaempferol 3-*O*-β-D-glucopyranoside (**4**), kaempferol 3-*O*-β-D-xylopyranoside (**5**), kaempferol 3-*O*-α-L-rhamnopyranoside (**6**), quercetin 3-*O*-β-D-glucopyranoside (**7**), quercetin 3-*O*-α-L-rhamnopyranoside (**8**), and quercetin 3-*O*-β-D-(6-*O*-acetyl)-glucopyranosyl-(1→4)-α-L-rhamnopyranoside (**9**). The spectroscopic data of compounds (**1**, **2**, and **4-9**) agreed with previously published data [15-20]; however, no detailed spectroscopic data have been published for compound **3**.

Compound **3** was obtained as a colorless amorphous powder. Its FAB-MS showed a $[M-H]^-$ peak at *m/z* 435, which indicated a molecular formula of $C_{21}H_{23}O_{10}$ by HR-FABMS. The ^1H NMR spectrum of **3** indicated the presence of a galloyl group (δ 7.00, 2H, s), a monosubstituted benzene ring (δ 7.10, 5H, m), three methylene groups (δ 4.44, 1H, br d, J=10.5 Hz, H-6'a; 4.36, 1H, dd, J= 5.4, 11.6 Hz, H-6'b; 3.92, 1H, dd, J= 7.6, 16.7 Hz, H-8a; 3.70, 1H, dd, J= 7.6, 16.7 Hz, H-8b; 2.81, 2H, t, J= 7.0 Hz, H-7), and five methine protons in the sugar regions. The connectivity of each proton was established by COSY spectra. Acetylation of **3** using acetic anhydride in pyridine afforded compound **3a**. The ^1H NMR spectrum of **3a** is similar to that of **3**, except for six singlet signals (δ 2.29, 2.28, 2.20, 2.00, 1.98, 1.87) corresponding to the six acetyl groups. The HR-FDMS of **3a** at *m/z* 688.2016 indicated a molecular formula of $C_{33}H_{36}O_{16}$, which supported the original structure of **3**. From coupling constant calculations and the COSY spectra of **3a**, the sugar moiety was unambiguously identified as β-glucose. The location of the galloyl unit at O-6 of the glucopyranose unit of **3** was established from low field shifts of the H-6' signals (δ 4.44, 4.36) and through the observation of long-range correlation between glucose H-6' protons (δ 4.44, 4.36) and the galloyl carbonyl group (C-7", δ 168.3) in the HMBC spectrum. Other correlations were observed between the methylene protons of H-8 (δ 3.92, 3.70) and the anomeric carbon C-1' (δ 104.5) and C-1 (δ 139.8) of the phenyl group. Based on the above evidence, the structure of **3** was elucidated as 2-phenylethyl 6-*O*-galloyl-β-D-glucopyranoside, which was reported previously without detailed NMR spectroscopic data [9]. The full ^1H and ^{13}C NMR spectroscopic data of **3** are given in the Experimental section.

Although DMSO-d_6 is typically used as a solvent for NMR spectroscopic analysis of flavonoids, methanol-d_4 was used because it provided better signal dispersion, especially for ^1H NMR spectra of glycosides [19]. Since no published NMR spectroscopic data on compounds **5** and **9** measured in methanol-d_4 exist, the ^1H NMR and ^{13}C NMR spectral characteristics of **5** and **9** in this solvent are given in the Experimental section. The assignments of carbon and proton signals for **5** and **9** were confirmed by HMBC, HMQC, and COSY experiments.

The anti-babesial activity of compounds **1-9** (Figure 1) is given in Table 1. Compound **3** exhibited the greatest *in vitro* activity against *B. gibsoni*, followed by gallic acid (**2**). The anti-babesial activity of other gallic acid derivatives [21] suggests that the activity of **3** is due to the presence of the galloyl moiety.

Although a variety of biological activities have been reported for flavonoids, those isolated in this study showed weak anti-babesial activity compared with the standard drug diminazene aceturate (Ganaseg).

Considering the potentially fatal side-effects of diminazene aceturate, it can be seen that gallic acid and its derivatives are promising new candidates for the treatment of *B. gibsoni* infection.

Table 1: Anti-babesial activity of compounds **1-9** against *B. gibsoni in vitro*.

Compound	IC_{50} (μg/mL)	IC_{50} (mol/L)×10^{-5}
1	78.00	50.06
2	15.74	9.25
3	11.78	2.67
4	>100	>22.30
5	>100	>23.90
6	>100	>23.13
7	>100	>21.53
8	>100	>22.30
9	90.88	13.92
Diminazene aceturate (Ganaseg)[a]	0.60	0.21

[a] Standard drug for the treatment of the symptom caused by *Babesia gibsoni*.

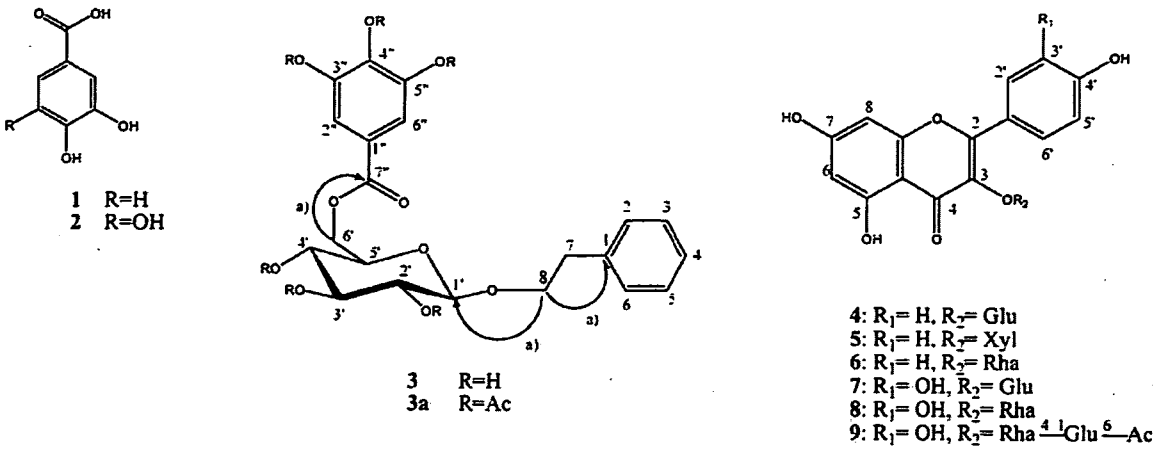

Figure 1: Chemical structures of compounds (1-9)
[a] Important HMBC correlations of compound 3 (500 MHz, CD_3OD).

Experimental

General: Optical rotations were measured with a Jasco DIP-370 digital polarimeter. Mass spectra were recorded on JEOL JMS-SX102A and JMS-AX500 spectrometers. NMR spectra were recorded on JEOL JNM-EX 270 FT-NMR and Bruker AMX-500 FT-NMR spectrometers. Column chromatography was conducted with silica gel 60 (Kanto Chemical). Analytical thin-layer chromatography was performed with silica gel 60 F254 (Merck).

Plant material: Air-dried flowers of *Rosa damascena* were purchased from a local shop in Cairo, Egypt, in October 2005.

In vitro test for anti-babesial activity: The anti-babesial assay was performed against *Babesia gibsoni in vitro* according to the reported methods [22].

Extraction and isolation: Activities of each fraction and compound were monitored by the bioassay test mentioned earlier. Air-dried flowers of *Rosa damascena* (240 g) were extracted with boiling water (4.8 L) for 30 min, and then re-extracted in the same manner. The combined extract was filtered and concentrated to 500 mL under reduced pressure and then partitioned with EtOAc (500 mL ×4). The combined EtOAc extract was dried over Na_2SO_4 and then concentrated under reduced pressure. The residue (11.8 g) was chromatographed on a silica gel column (300 g) and eluted with $CHCl_3$ (1 L), MeOH-$CHCl_3$ (3:97, 1 L), MeOH-$CHCl_3$ (20:80, 1 L), MeOH-$CHCl_3$ (50:50, 1 L), and MeOH (1 L), successively. The MeOH-$CHCl_3$ (50:50, 3.5 g) extract was re-chromatographed on a silica gel column (250 g) and eluted with MeOH-$CHCl_3$ (20:80) to give two fractions, Fr. I and II. Fr. I (375 mg) was further purified by Sephadex LH-20 and eluted with MeOH-$CHCl_3$ (20:80) to give four fractions, Fr. I-1-Fr. I-4. Fr. I-1 was further purified by HPLC (Capcell Pak C18, 5 µm, 15 mm × 250 mm, Shiseido) using CH_3OH-H_2O-AcOH (1000:1000:10, *v/v/v*) as solvent at a flow rate of 3 mL/min, to yield **3** (20 mg, *t*R: 17.51 min), **4** (8 mg, *t*R: 22.87 min), **5** (2 mg, *t*R: 26.35 min) and **6** (20 mg, *t*R: 31.98 min). Fr. I-4 was found to contain compound (**2**, 280 mg). Fr. II (476 mg) was further purified by HPLC as for Fr. I-1 to give **2** (108 mg, *t*R: 9.72 min), **1** (4 mg, *t*R: 10.86 min), **7** (27.6 mg, *t*R: 17.28 min), **8** (57.6 mg, *t*R: 21.92 min), and **9** (16 mg, *t*R: 26.56 min).

2-Phenylethyl 6-*O*-galloyl-β-D-glucopyranoside (3)

$[\alpha]_D^{22}$: -31.8° (*c* 0.94; MeOH).
^{1}H NMR (270 MHz, CD_3OD) δ: 7.10 (5H, m, phenyl), 7.00 (2H, s, H-2", 6"), 4.44 (1H, br d, *J* = 10.5 Hz H-6'a), 4.36 (1H, dd, *J* = 5.4, 11.6 Hz, H-6'b), 4.25 (1H, d, *J* = 7.6 Hz H-1'), 3.92 (1H, dd, *J* = 7.6, 16.7 Hz, H-8a), 3.70 (1H, dd, *J* = 7.6, 16.7 Hz, H-8b), 3.46 (1H, m, H-3'), 3.31 (2H, m, H-4',5'), 3.15 (1H, m, H-2'), 2.81 (2H, t, *J* = 7.0 Hz, H-7).
^{13}C NMR (67.5 MHz, CD_3OD) δ: 168.3 (C-7"), 146.5 (C-3", 5"), 139.8 (C-1, 4"), 129.9 (C-3, 5), 129.3 (C-2, 6), 127.1 (C-4), 121.4 (C-1"), 110.2 (C-2", 6"), 104.5 (C-1'), 77.9 (C-3'), 75.4 (C-5'), 75.0 (C-2'), 71.8 (C-8), 71.7 (C-4'), 64.7 (C-6'), 37.3 (C-7).
FAB-MS: *m/z* 435 $[M-H]^-$, HR-FABMS: 435.1301 (calc. for $C_{21}H_{23}O_{10}$: 435.1291).

Compound 3a

^{1}H NMR (270 MHz, $CDCl_3$) δ: 7.77 (2H, s, H-2", 6"), 7.20 (5H, m, phenyl), 5.21 (1H, t, *J* = 9.7 Hz, H-3'), 5.10 (1H, t, *J* = 9.7 Hz, H-4'), 5.01 (1H, dd, *J* = 8.0, 9.7 Hz, H-2'), 4.48 (1H, d, *J* = 8.0 Hz, H-1'), 4.45 (1H, dd, *J* = 2.7, 12.2 Hz, H-6'a), 4.37 (1H, dd, *J* = 5.1, 12.2 Hz, H-6'b), 4.14 (1H, dt, *J* = 6.2 Hz, H-8a), 3.80 (1H, ddd, *J* = 2.7, 5.1, 9.7 Hz, H-5'), 3.69 (1H, dt, *J* = 7.0 Hz, H-8b), 2.88 (2H, t, *J* = 7.0 Hz, H-7), 2.29 (3H, s, -COC*H*$_3$), 2.28 (3H, s, -COC*H*$_3$), 2.20 (3H, s, -COC*H*$_3$), 2.00 (3H, s, -COC*H*$_3$) , 1.98 (3H, s, -COC*H*$_3$), 1.87 (3H, s, -COC*H*$_3$) .
FD-MS: *m/z* 688 [M]+, HR-FDMS: 688.2016 (calc. for $C_{33}H_{36}O_{16}$: 688.2003).

Kaempferol 3-*O*-β-D-xylopyranoside (5)

$[\alpha]_D^{22}$: -56.3° (*c* 0.16; MeOH).
^{1}H NMR (270 MHz, CD_3OD) δ: 7.95 (2H, d, *J* = 8.9 Hz, H-2', 6'), 6.79 (2H, d, *J* = 8.9 Hz, H-3', 5'), 6.31 (1H, br s, H-8), 6.11 (1H, br s, H-6), 5.10 (1H, d, *J* = 7.0 Hz, H-1"), 3.69 (1H, dd, *J* = 11.6, 5.4 Hz, H-5"a), 3.41 (2H, m, H-2", H-4"), 3.35 (1H, m, H-3"), 3.03 (H-5"b, dd, *J* = 11.6, 9.6 Hz).
^{13}C NMR (67.5 MHz, CD_3OD) δ: 158.5 (C-2), 135.3 (C-3), 179.4 (C-4), 163.1 (C-5), 100.0 (C-6), 166.3 (C-7), 94.8 (C-8), 158.9 (C-9), 105.6 (C-10), 122.7 (C-1'), 132.2 (C-2', 6'), 116.2 (C-3', 5'), 161.7 (C-4'), 104.6 (C-1"), 75.3 (C-2"), 77.5 (C-3"), 71.0 (C-4"), 67.2 (C-5").
FAB-MS: *m/z* 417 $[M-H]^-$, HR-FABMS: 417.0834 (calc. for $C_{20}H_{17}O_{10}$: 417.0821).

Quercetin 3-*O*-β-D-(6-*O*-acetyl)-glucopyranosyl-(1→4)-α-L-rhamnopyranoside (9)

$[\alpha]_D^{22}$: -80.3° (*c* 0.38; MeOH).
^{1}H NMR (270 MHz, CD_3OD) δ: 7.23 (1H, d, *J* = 1.9 Hz, H-2'), 7.19 (1H, dd, *J* = 1.9, 8.1 Hz, H- 6'), 6.83 (1H, d, *J* = 8.1 Hz, H-5'), 6.28 (1H, d, *J* = 1.9 Hz, H-8), 6.11 (1H, d, *J* = 1.9 Hz, H-6), 5.27 (1H, d, *J* = 1.6 Hz, H-1"), 4.40 (1H, d, *J* = 7.6 Hz, H-1'"), 4.27 (1H, dd, *J* = 2.4, 12.0 Hz, H-6'"a), 4.14 (1H, dd, *J* = 1.6, 2.7 Hz, H-2"), 4.11 (1H, dd, *J* = 6.3, 12.0 Hz, H-6'"b), 3.88 (1H, dd, *J* = 3.1, 9.0 Hz, H-3"), 3.43 (1H, t, *J* = 9.0 Hz, H-4"), 3.31 (1H, m, H-5'"), 3.29 (1H, m, H-5"), 3.26 (1H, m, H-3'"), 3.17 (1H, m, H-4'"), 3.12 (1H, m, H-2'"), 1.92 (3H, s, -COC*H*$_3$), 0.86 (1H, d, *J* = 5.9 Hz, H-6").
^{13}C NMR (67.5 MHz, CD_3OD) δ: 158.6 (C-2), 136.0 (C-3), 179.6 (C-4), 163.2 (C-5), 99.9 (C-6), 166.0 (C-7), 94.7 (C-8), 159.4 (C-9), 105.9 (C-10), 122.7 (C-1'), 116.4 (C-2'), 149.8 (C-3'), 146.5 (C-4'), 117.0 (C-5'), 122.9 (C-6'), 103.1 (C-1"), 71.7 (C-2"), 71.7 (C-3"), 83.0 (C-4"), 70.5 (C-5"), 17.8 (C-6"), 105.4 (C-1'"), 75.8 (C-2'"), 78.0 (C-3'"), 72 (C-4'"), 75.3 (C-5'"), 64.8 (C-6'"), 20.70 (COC*H*$_3$), 172.67 (-*C*OCH3).
FAB-MS: *m/z* 651 $[M-H]^-$, HR-FABMS: 651.1574 (calc. for $C_{29}H_{31}O_{17}$: 651.1560).

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Anti-Babesial Compounds from *Berberis vulgaris*

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Extracts of nine North African medicinal plants have been evaluated for their anti-babesial properties *in vitro*. The extracts of *Berberis vulgaris* and *Rosa damascena* showed more than 90% inhibition of the parasite growth at a test concentration of 100 μg/mL. In addition to berberine alkaloids, we isolated nine active ingredients from *B. vulgaris*: *E*-coniferyl alcohol, (–)-simulanol, *p*-hydroxybenzaldehyde, 3-hydroxy-4,5-dimethoxybenzoic acid, *trans*-ferulic acid, syringic acid, vanillic acid, *cis*-ferulic acid, and syringaresinol-β-D-glucoside. These compounds are all known, but are newly reported for *B. vulgaris*. Among them, *trans*-ferulic acid showed the lowest IC_{50} value (7.33 μg/mL), not much above that of the standard drug, diminazene aceturate (0.60 μg/mL).

Keywords: *Berberis vulgaris*, Berberidaceae, anti-babesial activity, diminazene aceturate, *Babesia gibsoni*.

The infection of dogs with the parasite *Babesia gibsoni* is a worldwide problem, and in recent years the geographic range of the infection has spread. *B. gibsoni* proliferates within erythrocytes by lysing the cells, resulting in anemia in infected animals. The drug diminazene aceturate (Ganaseg) is effective against *B. gibsoni* infection [1], but causes side effects such as weakness, irritability, paralysis, non-responsiveness to stimuli, and fatal central nervous system hemorrhage [2]. Because of these side effects, alternative chemotherapeutic agents with fewer side effects are urgently needed for the treatment of *B. gibsoni* infection. We have already reported active ingredients from Indonesian medicinal plants used for the treatment of malaria. Here we examined nine North African medicinal plants and found promising activity in extracts of *Berberis vulgaris* and *Rosa damascena*. *B. vulgaris* (Berberidaceae) is a bush with yellow to brown colored bark. The plant has obovate leaves, bearing pendulous yellow flowers in spring succeeded by oblong red colored fruits (barberry) [3]. In folk medicine, the bark of *B. vulgaris* has been used for various conditions, including liver dysfunction, gallbladder disease, diarrhea, indigestion, malaria, and urinary tract disease [4–7]. In Egypt, it is still used for fever associated with pestilence [8]. Here we report the isolation and structural elucidation of anti-babesial compounds from *B. vulgaris*.

Based on our hypothesis [9] in which medicinal plants used to treat malaria might have anti-babesial activity, we tested nine North African medicinal plants. Some of these are used for the treatment of malaria and others as traditional antifebritics [10]. Anti-babesial activities of extracts of the nine plants against the growth of *B. gibsoni in vitro* are shown in Figure 1. Extracts prepared from *B. vulgaris* and *R. damascena* showed more than 90% inhibition at a concentration of 100 μg/mL, and extracts prepared from *Marrubium vulgare* and *Taraxacum officinale* showed more than 85% inhibition at 1000 μg/mL. The remaining five plants extracts showed more than 50% inhibition at 1000 μg/mL.

The active ingredients of *B. vulgaris* were isolated, and their structures deduced by spectroscopic analysis, including 1D- and 2D-NMR spectroscopy,

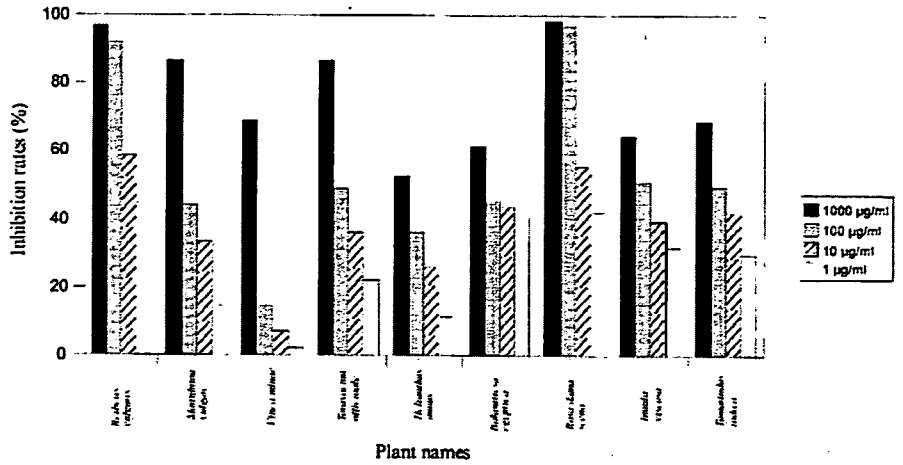

Figure 1: Inhibition of *Babesia gibsoni in vitro* by plant extracts.

to be berberine, palmatine, jatrorrhizine, E-coniferyl alcohol (**1**), (–)-simulanol (**2**), *p*-hydroxybenzaldehyde (**3**), 3-hydroxy-4,5-dimethoxybenzoic acid (**4**), *trans*-ferulic acid (**5**), syringic acid (**6**), vanillic acid (**7**), *cis*-ferulic acid (**8**), and syringaresinol- -D-glucoside (**9**). The spectroscopic data agreed with previously published data [11–13].

Compound **5** showed the strongest activity, with an IC_{50} value of 7.33 µg/mL, followed by **8** and **9**, with IC_{50} values of 134.84 and 51.60 µg/mL, respectively. Compounds **1–4**, **6**, and **7** showed very weak activities against *B. gibsoni in vitro*, with IC_{50} values > 250 µg/mL, compared with the standard drug diminazene aceturate (Ganaseg, IC_{50} = 0.60 µg/mL). Interestingly, *trans*-ferulic acid (**5**) has 20 times the activity of its *cis* isomer (**8**).

Although these nine compounds are all known, this report describes the first isolation of them from *B. vulgaris*. Berberine, palmatine, and jatrorrhizine, have already been isolated from other plant species [14] as anti-babesial compounds.

Experimental

General: Optical rotations were measured with a Jasco DIP-370 digital polarimeter. Mass spectra were recorded on JEOL JMS-SX102A and JMS-AX500 spectrometers. NMR spectra were recorded on a JEOL JNM-EX 270 FT-NMR spectrometer and on a Bruker AM-500 FT-NMR spectrometer. Column chromatography was conducted with silica gel 60 (Kanto Chemical). Analytical thin-layer chromatography was performed with silica gel 60 F_{254} (Merck).

Plant materials: Nine medicinal plants [*Berberis vulgaris* bark (Berberidaceae), *Marrubium vulgare* leaves (Lamiaceaeae), *Vinca minor* leaves (Apocynaceae), *Taraxacum officinale* roots, *Helianthus annus* leaves, *Inula viscosa* leaves (Asteraceae), *Balanites aegyptica* fruits (Balanitaceae), *Rosa damascene* flowers (Rosaceae) and *Tamarindus indica* fruits (Leguminosae)] were purchased from a local shop in Cairo, Egypt, in October 2005 and identified by Dr Salwa Kawashty (Division of Chemistry and Plant Systematics, National Research Center, Egypt).

Preparation of plant extracts: Air-dried samples (5 g) were boiled in 100 mL of water for 30 min. The extract was concentrated under reduced pressure, from which four different concentrations were prepared (1000, 100, 10, and 1 µg/mL), and evaluated for their anti-babesial activity using *Babesia gibsoni* as a test parasite.

In vitro test for anti-babesial activity: The anti-babesial assay was performed against *Babesia gibsoni in vitro* according to the reported methods [15].

Extraction and isolation: Air-dried bark of *Berberis vulgaris* (200 g) was extracted with boiling water (2 x 4 L) for 30 min. The combined extract was filtered and concentrated to 500 mL under reduced pressure and then partitioned with EtOAc (500 mL x 4). The EtOAc layer was dried over Na_2SO_4 and then concentrated under reduced pressure. The residue (1.17 g) was chromatographed on a silica gel column (100 g) eluting with $CHCl_3$

(1 L), MeOH-$CHCl_3$ (3:97, 1 L), MeOH-$CHCl_3$ (20:80, 1 L), MeOH-$CHCl_3$ (50:50, 1 L), and MeOH (1 L), successively. The MeOH-$CHCl_3$ (3:97) extract was re-chromatographed on a silica gel column (30 g) by elution with MeOH-$CHCl_3$ (5:95, 500 mL) to give two fractions, Frs I and II. Fr. I was further purified by PTLC on silica gel by development with MeOH-$CHCl_3$-AcOH (8:92:1). The bands were collected and eluted from the silica gel to derive compounds **1** (5.2 mg, Rf = 0.60) and **2** (1.9 mg, Rf = 0.44). Fr. II was further purified by HPLC (Inertsil ODS, 10 μm, 20 mm × 250 mm) using CH_3OH-H_2O-AcOH (3.0:7.0:0.01, *v/v/v*) at a flow rate of 5 mL/min, to derive **3** (1 mg, t_R: 35.47 min). The MeOH-$CHCl_3$ (20:80) extract was re-chromatographed on a silica gel column (70 g), eluting with MeOH-$CHCl_3$ (20:80, 500 mL) to give two fractions, Fr. III and IV. Fr. III was further purified by HPLC (Inertsil ODS, 10 μm, 20 mm × 250 mm) using CH_3OH-H_2O-AcOH (5.0:5.0:0.01, *v/v/v*) at a flow rate of 5 mL/min, to derive Fr. III-1 and III-2. Fr. III-1 was further purified by PTLC on silica gel by development with MeOH-$CHCl_3$-AcOH (5:95:1). The bands were collected and eluted from the silica gel to give **4** (5.0 mg, Rf = 0.51). Fr. III-2 was re-chromatographed on a silica gel column (30 g) eluting with MeOH-$CHCl_3$-AcOH (5:95:1) to yield **5** (3.5 mg). Fr. IV was re-chromatographed on a silica gel column (50 g) by elution with MeOH-$CHCl_3$-AcOH (5:95:2) to give two fractions, Fr. IV-1 and IV-2. Fr. IV-1 was further purified on Sephadex LH-20 by eluting with MeOH-$CHCl_3$ (30:70) to give two fractions, IV-1-1 and IV-1-2. Fr. IV-1-1 was further purified by HPLC (Capcell Pak C_{18}, 5 μm, 15 mm × 250 mm, Shiseido) using CH_3OH-H_2O-AcOH (5.0:5.0:0.01, *v/v/v*) at a flow rate of 5 mL/min, to yield **6** (1 mg, t_R: 13.70 min). Fr. IV-1-2 was purified by HPLC in the same way as Fr. IV-1-1 to give **7** (2 mg, t_R: 13.39 min) and a mixture of compounds **5** and **8**, which were separated by PTLC on silica gel. The plates were developed with MeOH-$CHCl_3$-AcOH (8:92:1). The bands were collected and the compounds eluted from the silica gel to give **5** (2.5 mg, Rf = 0.67) and **8** (2.0 mg, Rf = 0.56). Fr. IV-2 was further purified using Sephadex LH-20 by eluting with MeOH-$CHCl_3$ (30:70) to yield **9** (7.0 mg).

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特集

呼吸器と寄生虫

肺吸虫

丸山治彦* 名和行文*

要 旨

日本に分布するウエステルマン肺吸虫および宮崎肺吸虫には，感染型の幼虫が寄生しているモクズガニやサワガニ，イノシシ肉を生食して感染する。虫体はおもに肺に寄生し，胸膜および肺実質の炎症を引き起こす。自覚症状は咳嗽・胸痛・呼吸困難等で，胸水貯留を中心に多彩な胸部画像所見を示す。通常，末梢血好酸球数が高値である。虫卵の検出率は高くなく，診断はおもに血清抗体検査により行われる。治療はビルトリシドの内服が有効である。

Key words：肺吸虫，モクズガニ，サワガニ，胸水，酵素抗体法/lung fluke, mitten crab, freshwater crab, pleural effusion, enzyme-linked immunosorbent assay

1 はじめに

肺吸虫症はかつて日本全国に広くみられ，とくに西日本に濃厚な流行地があった。その後，感染源に関する知識の広まりや食生活の変化などによって，その発生は激減した。ところがここ十数年ほどで症例数の増加がみられ，現在も年間30〜40例の新規患者が発生している。肺吸虫症の存在を知らないでいると，肺癌や肺結核，好酸球性肉芽腫症などを疑って診断の遅れや誤診につながることがある。数は少ないといえども注意が必要である。

2 病原体とその生活環

肺吸虫類はおもにイヌ科やネコ科の野生動物に寄生する比較的大型の吸虫で，日本のほか東アジアから東南アジア，インドなどに広く分布している。肺吸虫類の生活環は図1に示す通りで，第1中間宿主はカワニナなどの淡水産の巻貝，第2中間宿主はモクズガニやサワガニなどの淡水産甲殻類である。第2中間宿主のカニ類にはメタセルカリアと呼ばれる感染型の虫体が寄生しており，ヒトへの感染はカニ類を生または十分に加熱されないまま食べて起きる。また，待機宿主であるイノシシの肉を生食して感染することもある。代表的な人獣共通寄生虫疾患である。終宿主内

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