

# 1 **Evaluation of the inhibitory effects of drugs on the growth of** 2 ***Babesia* Parasites using real-time PCR method**

3 *Xiao-hu Zhai<sup>1</sup>, Xiao-xiao Feng<sup>2</sup>, Xian Wu<sup>2</sup>, Wei-hua He<sup>1</sup>, Yan-yan Li<sup>1</sup>, Da-wei Yao<sup>2\*</sup>¶, Bin Zhang<sup>1\*</sup>¶*

4 *1 Jiangsu Agri-Animal Husbandry Vocational College, Jiangsu Taizhou, 225300, China*

5 *2 College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu, 210095,*  
6 *China*

7 *\*Corresponding Author Da-wei Yao, Bin Zhang*

8 *Email: yaodawei@njau.edu.cn*

9 *¶These authors contributed equally to this work*

## 11 Abstract

12 In order to evaluate the inhibitory effects of drug on the growth of *babesia* parasite, relative  
 13 quantification real-time PCR method was developed in this study. The *18S rRNA* gene was used as  
 14 target gene for the  $2^{-\Delta\Delta C_t}$  method analysis. Meanwhile, Chicken RNA was added into the parasitized  
 15 blood for total RNA extraction. The  $\beta$ -actin gene of chicken was selected as internal control gene  
 16 for the  $2^{-\Delta\Delta C_t}$  method analysis. Parasitized blood 100  $\mu$ L, 50  $\mu$ L, 25 $\mu$ L, 12.5  $\mu$ L, 6.25  $\mu$ L was  
 17 prepared for *B. gibsoni* relative quantification. Regression analysis results revealed that significant  
 18 linear relationships between the relative quantification value and parasitemia. The *18S rRNA* gene  
 19 expression was significantly decreased after the treatment of Diminazene aceturate and Artesunate  
 20 in vitro drug sensitivity test. It suggested that this relative quantification real-time PCR method can  
 21 be used in evaluating the effects of inhibitory of drug.

22 **Keywords:** relative quantification real-time PCR; *babesia*; drug sensitivity

## 24 Introduction

25 *Babesia* is transmitted by ticks to vertebrates and results in serious economic losses in the  
26 livestock industry world-wide. Human babesiosis is also worldwide problem and presents a  
27 significant health burden in areas where it is endemic [1]. Several antibabesial drugs that have been  
28 in use for years such as diminazene aceturate, imidocarb dipropionate, artesunate, atovaquone and  
29 atovaquone with azithromycin [2-4]. However, most drugs have proven to be ineffective owing to  
30 problems related to toxicity and the development of resistant parasites [4, 5]. Therefore, developing  
31 more effective drugs against *babesia* with low toxicity to the hosts has been desired.

32 At present, there are no recognized standard methods for drug screening or activity evaluation.  
33 More recently, in vitro assays have been developed for evaluation of the susceptibilities of *Babesia*  
34 to drugs [6-9]. Parasite-infected red blood was diluted with uninfected blood to obtain the blood  
35 stock with parasitemia. Then blood with parasitemia were cultured in culture medium containing  
36 the indicated concentration of drugs and incubated at 37°C in a humidified multigas water-jacketed  
37 incubator. The sensitivity against drugs was evaluated by measuring the rate of parasite growth  
38 inhibition. The rate of parasite was calculated by counting the parasitized red blood cells to  
39 approximately 1000 red blood cells in Giemsa-stained thin blood smears [10]. However,  
40 microscopic examination of Giemsa-stained thin blood smears that requires good-quality smears is  
41 a time-consuming technique and significant differences in parasitemia estimated by different  
42 personnel may be found [11].

43 The mRNA had been used as a marker of viability, because mRNA is a highly labile molecule  
44 with a very short half-life (seconds) [12]. Therefore, mRNA could provide a more closely correlated  
45 indication of viability status. Ribosomal RNA (rRNA) has also been selected as an indicator of  
46 viability [13] and has been found to be positively correlated with viability under some bacterial-  
47 killing regimes [14]. In addition, detection of *16S rRNA* from *Chlamydia pneumoniae* was  
48 demonstrated to provide a more suitable indication of active infection than immunocytochemical  
49 detection of specific antigens [15]. In this research, we used *18S rRNA* gene of *B. gibsoni* parasites

as molecular target for relative quantification using real-time PCR method.

## Materials and methods

### *Chemical reagents*

Diminazene aceturate and Artesunate were purchased from Macklin Ltd. (Shanghai, China).

Stock solutions of 20 mM in physiological saline were prepared and stored at -20 °C until use.

### *Parasite*

*B. gibsoni* parasites were isolated from a naturally infected dog in Nanjing, China. This parasite was identified to be *B. gibsoni* according to the 18S rDNA sequences analysis. The blood from sick dog naturally infected with *B. gibsoni* was kept in liquid nitrogen. Then this blood was sub-inoculated into a Beagle. Parasites were first detected in the blood 12 days after inoculation. The blood used in this research was collected from Beagle infected with *B. gibsoni*. Samples were collected in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Jiangsu province. The protocol was approved by the Committee on the Ethics of Animal Experiments of JiangSu Agri-Animal Husbandry Vocational College.

### *B. gibsoni* relative quantification assay

Real-time PCR was used for *B. gibsoni* relative quantification. The 100 µL, 50 µL, 25µL, 12.5 µL, 6.25 µL parasitized blood were prepared. Each blood samples were added 3 µg chicken RNA isolated from spleen tissue and mixed together for RNA extraction. The total RNA of mixture was extracted using RNAiso Plus reagent (Takara, Japan) according to the manufacturer's instructions. The RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.). Reverse transcription-PCR was conducted using a PrimeScript One-Step RT-PCR kit (Takara, Japan) according to the manufacturer's instructions. One primer pair ( $\beta$ -actin F 5'-GAGAATTGTG CGTGACATCA-3' and  $\beta$ -actin R 5'-CCTGAACCTCTCATTGCC A-3') was used to amplify 157 bp fragment of  $\beta$ -actin gene of chicken [16]. Another primer pair (B.com 339 F 5'-GTCTT-GTAATTGGAATGATGGTGAC-3' and B.com 339 R 5'-ATGCCCCCAA CCGTTCCTATTA-3') was used to amplify 339 bp fragment of the 18S rRNA gene of *Babesia*

76 *gibsoni* [17]. Real-time PCR was performed with an Applied Biosystems 7500 Real-time PCR  
77 System (Applied Biosystems). The cDNA was diluted continuously to make the calibration curves.  
78 PCR amplification efficiencies of *B. gibsoni* 18S rRNA gene and Chicken  $\beta$ -actin gene were  
79 established by means of calibration curves. The expression levels of 18S rRNA gene, which  
80 indicated the quantity of *B. gibsoni*, were analyzed with the  $2^{-\Delta\Delta C_t}$  method. Each test was performed  
81 in triplicate. The group of 100  $\mu$ L blood was identified as control.

82 The quantity of *B. gibsoni* was calculated according to the Eq.1.

$$83 \quad -\Delta\Delta C_T = -[(C_{T \text{ 18SrRNA of } B.g} - C_{T \text{ } \beta\text{-actin of chicken}}) \text{ test group} - (C_{T \text{ 18SrRNA of } B.g} - C_{T \text{ } \beta\text{-actin of chicken}}) \text{ control} \\ 84 \quad \text{group}] \quad (1)$$

85 where  $C_{T \text{ 18SrRNA of } B.g}$  was the value of the *B. gibsoni* 18S rRNA gene.  $C_{T \text{ } \beta\text{-actin of chicken}}$  was the  
86 value of the Chicken  $\beta$ -actin gene.

### 87 **Drug sensitivity test**

88 For the in vitro drug sensitivity test, fifty microliters parasitized blood and two hundred  
89 microliters of Artesunate (0, 0.005, 0.085 and 0.340 ng/mL, respectively) were distributed per well  
90 in 24-well plates in three replicates. These plates were incubated at 37 °C for 24 h. The RNAiso  
91 Plus reagent and 3  $\mu$ g chicken RNA was added into the well for the total RNA extraction. Real-time  
92 PCR was used for *B. gibsoni* relative quantification. The group of 0 ng/mL was identified as control.  
93 The different concentrations of Diminazene aceturate solution (2500 ng/mL, 250 ng /mL, 25 ng/mL  
94 and 2.5 ng/mL, respectively) were used for the in vitro drug sensitivity test as well.

### 95 **Statistical analysis**

96 The data in the figure are presented as the arithmetic mean  $\pm$  standard deviation (SD). The  
97 statistical analysis was performed by one-way analysis of variance (ANOVA) using Predictive  
98 Analytics Software 18.0. Duncan's multiple-range test was used, with differences considered to be  
99 significant at  $P < 0.05$ .

## 100 **Results and discussion**

### 101 ***B. gibsoni* relative quantification assay**

At present, due to the toxicity and resistance of drugs, the development of new drugs that has a chemotherapeutic effect against babesiosis with high specificity to the parasites and low toxicity to host is urgently needed. Development of a simple in vitro system to evaluate susceptibility of *Babesia* to drugs is very important. Reverse transcription and polymerase chain reaction has proven to be a powerful method to quantify mRNA gene expression. Relative quantification real-time PCR describes the change in expression of the target gene compared to reference group. The method of presenting quantitative real-time PCR data is the comparative CT method, also known as the  $2^{-\Delta\Delta C_t}$  method. Using this method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control [18]. For the untreated control sample,  $\Delta\Delta C_t$  equals zero and  $2^0$  equals 1, so that the fold change in gene expression relative to the untreated control equals 1, by definition. Housekeeping genes usually sufficed as internal control genes and it is not affected by experimental treatment. Suitable internal controls for real time quantitative PCR include *GAPDH*,  *$\beta$ -actin*, and *rRNA*.

In this research, *18S rRNA* gene was used quantified the parasites as target gene. It had been reported that the mRNA transcripts of the *B. bovis tubulin* beta chain and small subunit rRNA genes were not affected by the treatment with lower concentration of apicoplast-targeting antibacterials [19]. Due to instability of mRNA, it was sequenced degradation when the parasites were died. Therefore, mRNA could provide a more closely correlated indication of viability status of the parasites.

In this research, *18S rRNA* gene of *B. gibsoni* parasites was selected as target gene rather than internal control genes. Because of degradation of mRNA, there was no gene can be used as internal control genes. In this study, chicken RNA was added into the parasite RNA and the  *$\beta$ -actin* gene of chicken was selected as internal control genes. The same quantification of chicken RNA was added into both treated group and untreated control group to confirm the same quantification expression of  *$\beta$ -actin* gene. The quantity of *B. gibsoni* were calculated according to the equation where  $-\Delta\Delta C_T = -[(C_{T \text{ 18SrRNA of } B.g} - C_{T \text{ } \beta\text{-actin of chicken}}) \text{ test group} - (C_{T \text{ 18SrRNA of } B.g} - C_{T \text{ } \beta\text{-actin of chicken}}) \text{ control group}]$ .

128 The melting curve of *B. gibsoni* *18S rRNA* gene and Chicken  $\beta$ -*actin* gene indicated that the primer  
129 used in this study were specific for real-time PCR amplification. PCR amplification efficiencies of  
130 *B. gibsoni* *18S rRNA* gene and Chicken  $\beta$ -*actin* gene were 97.7% and 96.7%. These results  
131 suggested real-time PCR used in this study was reliable. *B. gibsoni* relative quantification was  
132 shown in the Fig 1. The magnitude of linearity ranged from 12.5 $\mu$ L to 100  $\mu$ L. The equation of the  
133 parasitized blood volume versus the *B. gibsoni* relative quantification obtained was  $y=0.01x$  with a  
134  $R^2$  of 0.987. Significant linear relationships between the relative quantification and parasitemia  
135 were observed.

136

137 **Fig 1. Linearity assessment between *B. gibsoni* relative quantification and parasitized blood**  
138 **volume**

139

140

141 ***Drug sensitivity test of Artesunate and Diminazene aceturate***

142 Significant linear relationships between the relative quantification value and parasitemia were  
143 observed using the real-time PCR method. In order to evaluate the inhibitory effects of drug,  
144 Diminazene aceturate and Artesunate were added into the parasitized blood. It was found that the  
145 *18S rRNA* gene expression was decreased relatively to the untreated control. Both Diminazene  
146 aceturate and Artesunate can dose-dependently inhibit the growth of *B. gibsoni* in vivo.

147 The expression levels of *18S rRNA* gene in Artesunate treated group were down-regulated  
148 compared to control group ( $P<0.05$ ) (Fig 2). The levels of *18S rRNA* gene were 27% lower at 0.340  
149 ng/mL Artesunate. It was suggested that *B. gibsoni* was significantly suppressed in the presence of  
150 0.005 ng/mL, 0.085 ng/mL and 0.340 ng/mL Artesunate. The higher Artesunate was used, the better  
151 the inhibitory effects achieved. Artesunate can dose-dependently inhibit the *B. gibsoni* in vivo.

152

153

## Fig 2. Drug sensitivity test of Artesunate

The expression levels of *18S rRNA* gene in Diminazene aceturate treated group (250 ng/mL, 2500 ng/mL) were down-regulated compared to control group and 25 ng/mL Diminazene aceturate treated group ( $P < 0.05$ ) (Fig 3). The levels of expression were found to be significantly reduce (28%) in the presence of Artesunate 2500 ng/mL ( $P < 0.05$ ). The lower concentration of Diminazene aceturate (25 ng/mL) had no inhibitory effects.

## Fig 3. Drug sensitivity test of Diminazene aceturate

In summary, relative quantification real-time PCR based RNA was developed in this study. This method can determinate live parasites and be used for the evaluation of inhibitory effects of on the growth of *Babesia* Parasites.

## Acknowledgements

We would like to thank Yun Liao and Ze-nan Su for assistance in animal housing.

## References

1. Tonnetti L, Townsend RL, Deisting BM, Haynes JM, Dodd RY, Stramer SL. The impact of *Babesia microti* blood donation screening. *Transfusion*. 2019;59(2):593-600. pmid: 000459614500022.
2. Tuvshintulga B, Sivakumar T, Yokoyama N, Igarashi I. Development of unstable resistance to diminazene aceturate in *Babesia bovis*. *Int J Parasitol Drugs Drug Resist*. 2019;9:87-92. pmid: 000462490500012.
3. Goo Y-K, Terkawi MA, Jia H, Aboge GO, Ooka H, Nelson B, et al. Artesunate, a potential drug for treatment of *Babesia* infection. *Parasitol Int*. 2010;59(3):481-6. pmid: 000281461500028.
4. Checa R, Montoya A, Ortega N, Luis Gonzalez-Fraga J, Bartolome A, Galvez R, et al. Efficacy, safety and tolerance of imidocarb dipropionate versus atovaquone or buparvaquone plus azithromycin used to treat sick dogs naturally infected with the *Babesia microti*-like piroplasm. *Parasit Vectors*. 2017;10. pmid: 000396899800001.
5. Mosqueda J, Olvera-Ramirez A, Aguilar-Tipacamu G, Canto GJ. Current Advances in

- 183       Detection and Treatment of Babesiosis. *Curr Med Chem*. 2012;19(10):1504-18. pmid:  
184       000302118600006.
- 185   6. AbouLaila M, Nakamura K, Govind Y, Yokoyama N, Igarashi I. Evaluation of the in vitro  
186       growth-inhibitory effect of epoxomicin on Babesia parasites. *Vet Parasitol*. 2010;167(1):19-27.  
187       PubMed pmid: 000273929300003.
- 188   7. Matsuu A, Yamasaki M, Xuan X, Ikadai H, Hikasa Y. In vitro evaluation of the growth  
189       inhibitory activities of 15 drugs against Babesia gibsoni (Aomori strain). *Vet Parasitol*.  
190       2008;157(1-2):1-8. pmid: 000260136100001.
- 191   8. Rizk MA, El-Sayed SAE-S, AbouLaila M, Yokoyama N, Igarashi I. Evaluation of the inhibitory  
192       effect of N-acetyl-L-cysteine on Babesia and Theileria parasites. *Exp Parasitol*. 2017;179:43-8.  
193       pmid: 000406081500006.
- 194   9. Silva MG, Domingos A, Alexandra Esteves M, Cruz MEM, Suarez CE. Evaluation of the  
195       growth-inhibitory effect of trifluralin analogues on in vitro cultured Babesia bovis parasites. *Int*  
196       *J Parasitol Drugs Drug Resist*. 2013;3:59-68. pmid: 000327535700008.
- 197   10. Salama AA, AbouLaila M, Moussa AA, Nayel MA, Ei-Sify A, Terkawi MA, et al. Evaluation  
198       of in vitro and in vivo inhibitory effects of fusidic acid on Babesia and Theileria parasites. *Vet*  
199       *Parasitol*. 2013;191(1-2):1-10. pmid: 000312355000001.
- 200   11. Rizk MA, El-Sayed SAE-S, Terkawi MA, Youssef MA, El Said ESES, Elsayed G, et al.  
201       Optimization of a Fluorescence-Based Assay for Large-Scale Drug Screening against Babesia  
202       and Theileria Parasites. *PloS ONE*. 2015;10(4). PubMed pmid: 000353659100114.
- 203   12. 12. Keer JT, Birch L. Molecular methods for the assessment of bacterial viability. *J Microbiol*  
204       *Methods*. 2003;53(2):175-83. pmid: 000182299100006.
- 205   13. Villarino A, Bouvet OMM, Regnault B, Martin-Delautre S, Grimont PAD. Exploring the  
206       frontier between life and death in Escherichia coli: evaluation of different viability markers in  
207       live and heat- or UV-killed cells. *Res Microbiol*. 2000;151(9):755-68. pmid: 000165784300006.
- 208   14. McKillip JL, Jaykus LA, Drake M. rRNA stability in heat-killed and UV-irradiated  
209       enterotoxigenic Staphylococcus aureus and Escherichia coli O157 : H7. *Appl. Environ.*  
210       *Microbiol*. 1998;64(11):4264-8. pmid: 000076694200021.
- 211   15. Meijer A, Roholl PJM, Gielis-Proper SK, Meulenbergh YF, Ossewaarde JM. Chlamydia  
212       pneumoniae in vitro and in vivo: a critical evaluation of in situ detection methods. *J Clin Pathol*.  
213       2000;53(12):904-10. pmid: 000165766400006.
- 214   16. Yao D-w, Zhan L, Hong Y-f, Liu J-x, Xu J-r, Yang D-j. Altered expression of the mismatch  
215       repair genes in DF-1 cells infected with the avian leukosis virus subgroup A. *Springerplus*.

216 2016;5. pmid: 000391805500018.

217 17. Yao D-w, Jiang J-y, Yu Z-z, Yao D-q, Yang D-j, Zhao Y-b. Canine Babesiosis in China Caused  
 218 by Babesia gibsoni: A Molecular Approach. Iran J Parasitol. 2014;9(2):163-8. pmid:  
 219 000337343400003.

220 18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative  
 221 PCR and the  $2^{-\Delta\Delta C_T}$  method. Methods. 2001;25(4):402-8. pmid:  
 222 000173949500003.

223 19. AbouLaila M, Munkhjargal T, Sivakumar T, Ueno A, Nakano Y, Yokoyama M, et al.  
 224 Apicoplast-Targeting Antibacterials Inhibit the Growth of Babesia Parasites. Antimicrob Agents  
 225 Chemother. 2012;56(6):3196-206. pmid: 000304432800054.  
 226  
 227

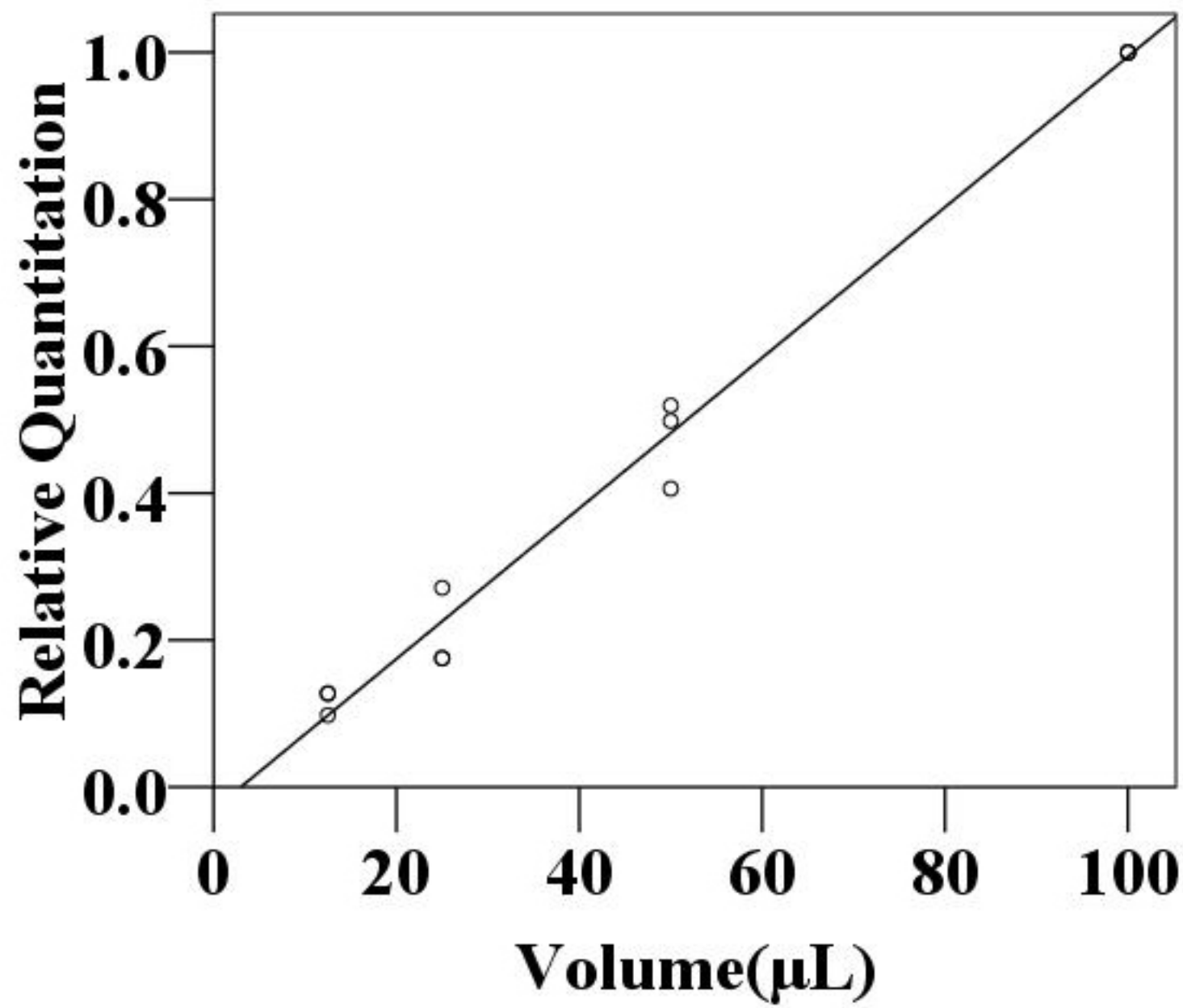


Figure 1

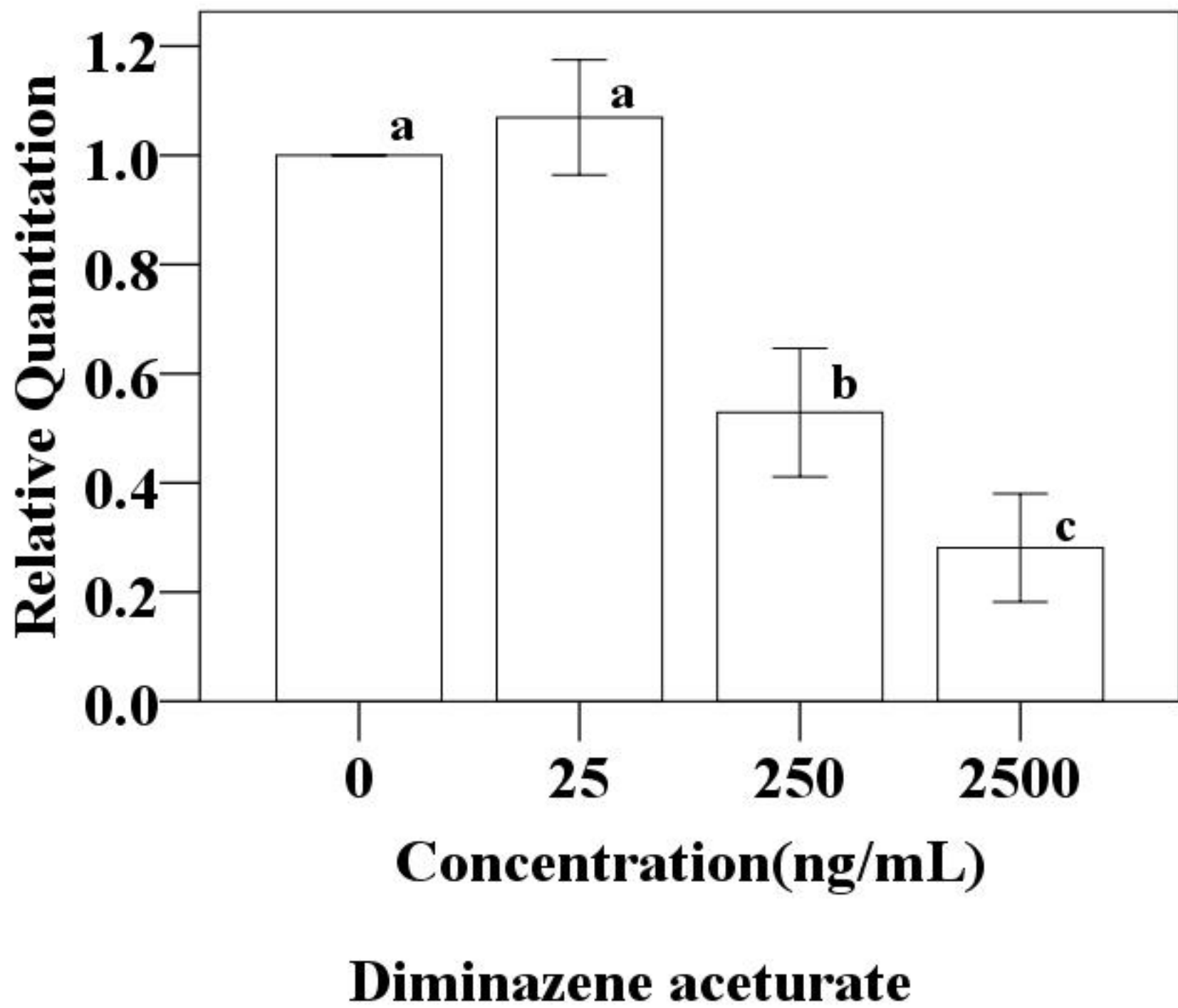


Figure 2

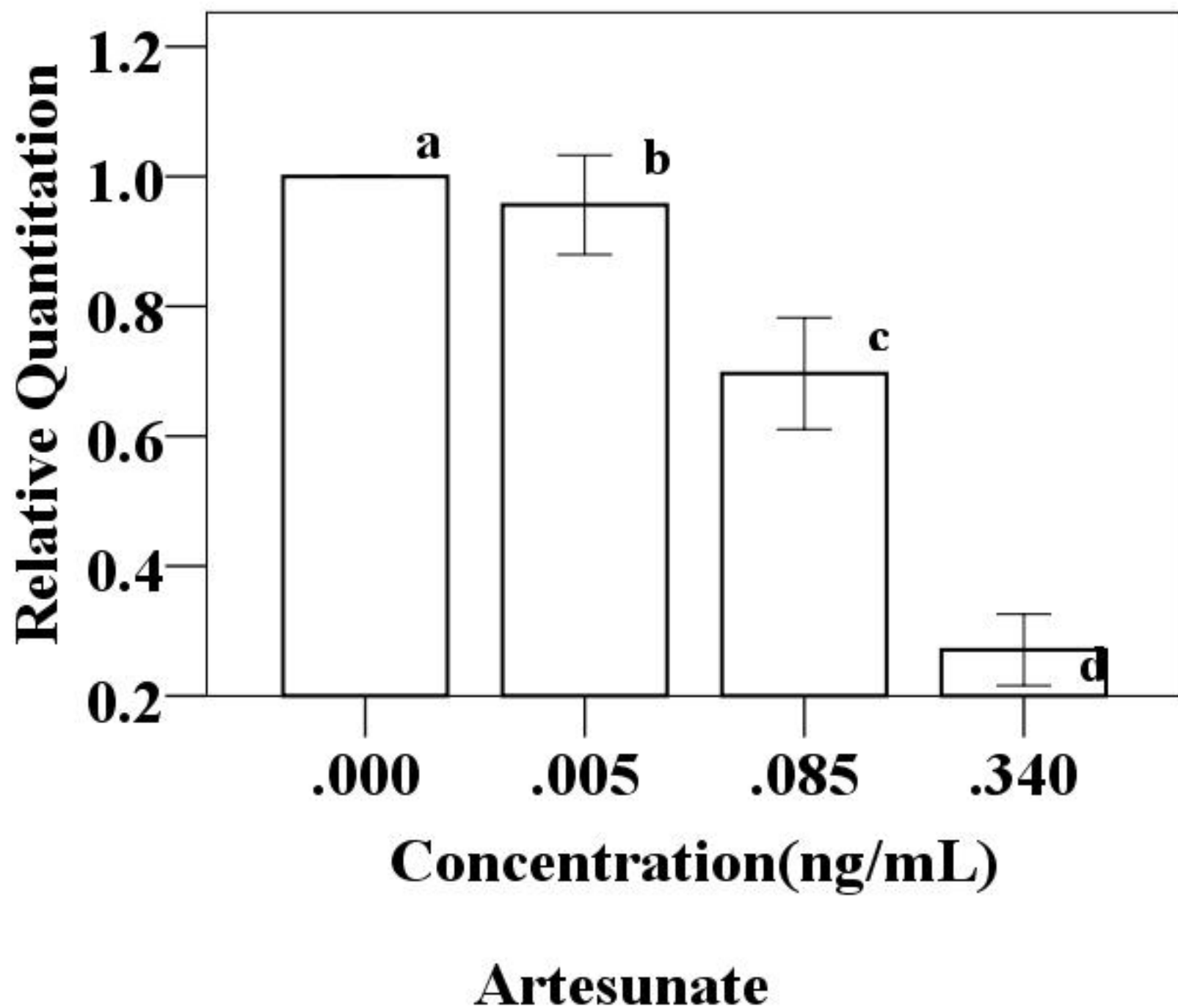


Figure 3