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Allicin-Induced Cellular Destruction of Haemoflagellate Protozoa, *Trypanosoma evansi* in Mice

(Kemusnahan Sel Protozoa Hemoflagelat *Trypanosoma evansi* Aruhan Alisin dalam Mencit)

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ABSTRACT

*Allicin or diallyl thiosulfinate ($C_3H_5SS(O)C_3H_5$), an active compound of garlic (*Allium sativum*) is known for its pharmaceutical properties. In this study, the cellular destructive effects of allicin on a haemoflagellate protozoa parasite* Trypanosoma evansi *was investigated. Groups of male ICR mice were infected with a lethal dose of the parasite (1×10^5 parasites per mouse) and each group was either treated intraperitoneally with berenil (0.01 mL per mouse, a commercial anti-trypanosomal drug) on D+3 post-infection as the positive control group, treated orally with allicin (0.1 mL of 15 µg/mL allicin solution per mouse) for 30 days starting from D-7 pre-infection as the experimental group, or left untreated as the negative control group. Thin-stained blood smears were prepared from each mouse every alternate day, starting from D+3 post-infection and continued until the animal succumbed or until D+90 post-infection. Parasitaemias were determined using light microscope. Unstained blood smears were also prepared for direct observation under Phillips XL30 and Leo 1450VP scanning electron microscopes. All mice in the negative group succumbed to the infection with drastic increase of parasitaemias while all the positive control mice had minimal parasitaemias and cleared from the infection and survived for more than 100 days. On the other hand mice in the experimental group, experienced a prolonged suppressed parasitaemias which became patent later and caused death to all mice. Micrograph observations of parasites in the positive group showed that the parasites had adverse morphological changes due to berenil treatment which lead to cell destruction and death within 5 – 6 hours post-treatment. Likewise parasites in the experimental group too had undergone profound physical damages which caused cell death. This is the first report which shows that allicin actually induced cellular damage to haemoflagellate cells of* T. evansi in vivo.

Keywords: Allicin; cellular destruction; haemoflagellate protozoa; parasitaemia; Trypanosoma evansi

ABSTRAK

Alisin atau 'diallyl thiosulfinate' ($C_3H_5SS(O)C_3H_5$) adalah sebatian aktif bawang putih (Allium sativum) *yang mempunyai banyak khasiat farmaseutikal. Dalam kajian ini kesan kemusnahan alisin ke atas sel parasit protozoa hemoflagelat* Trypanosoma evansi *telah diselidiki. Kumpulan-kumpulan mencit jantan strain ICR telah dijangkitkan dengan takaran maut parasit (1×10^5 parasit per mencit) dan setiap kumpulan mencit sama ada dirawat dengan berenil iaitu sejenis dadah anti-tripanosom kemersial secara suntikan intraperitonem pada hari D+3 pasca-jangkitan (kumpulan kawalan positif), atau mencit dirawat dengan alisin secara oral (0.1 mL of 15 µg/mL larutan alisin per mencit) selama 30 hari bermula pada hari D-7 pasca-jangkitan (kumpulan kajian), atau mencit tidak mendapat sebarang rawatan (kumpulan kawalan negatif). Slaid darah calitan nipis terwarna disediakan daripada setiap ekor mencit berselang hari bermula pada hari D+3 sehingga mencit maut atau sehingga hari D+90 pos-jangkitan. Parasitemia telah ditentukan di bawah mikroskop cahaya. Calitan darah tak berwarna turut disediakan untuk cerapan langsung menggunakan mikroskop elektron pengimbasan Phillips XL30 dan Leo 1450VP. Didapati semua mencit kumpulan kawalan negatif mati akibat peningktan parasitemia yang cepat manakala mencit dalam kumpulan kawalan positif menunjukkan parasitemia yang minima dan bebas daripada jangkitan parasit dan bermandiri melebihi 100 hari. Sebaliknya mencit dalam kumpulan kajian mengalami perkembangan parasitemia tertindas yang kemudiannya meningkat semula dan menyebabkan kematian mencit. Cerapan ke atas mikrograf darah daripada kumpulan kawalan positif menunjukkan morfologi parasit mengalami kerosakan teruk kesan tindakan berenil yang menyebabkan kemusnahan dan kematian sel parasit 5-6 jam pasca-rawatan. Sebagai perbandingan parasit daripada kumpulan kajian juga mengalami kerosakan fizikal yang parah dan membawa kepada kematian. Hasil kajian ini merupakan laporan pertama yang menunjukkan alisin mengaruh kemusnahan sel-sel parasit* T. evansi *secara* in vivo.

Kata kunci: Alisin; kemusnahan sel; parasitemia; protozoa hemoflagelat; Trypanosoma evansi

Introduction

Garlic from *Allium sativum* has long been recognized for treating many ailments in various societies in the world and its most biologically active compound, the allicin ($C_3H_5SS(O)C_3H_5$) (Ali et al. 2000) is known to possess antibacterial, antiviral, anticancer as well as antiparasitic properties (Oommen et al. 2004). However, as far as antiparasitic properties of garlic are concerned, only limited information are available. Garlic extracts are effective against some important parasitic protozoa such as *Entamoeba histolytica, Trypanosoma brucei* and *Leishmania* (Ramos et al. 2006) and for the treatments against giardiasis and the infections by *E. histolytica* and *Trichomonas vaginalis* (Saleehen et al. 2004). Its effective mechanism against these parasites are largely unknown but in other studies, allicin which is readily permeable through phospholipid membranes (Oommen et al. 2004) is proposed to exert its activities either through anti-proliferative action or by causing oxidative damage to the cells (An et al. 2009). It is also considered as a modulating agent which can regulate enzymatic activity of SH containing enzymes by a thiol-disulphide exchange reaction (Rabinkov et al. 1998).

The surra disease caused by the etiological agent haemoflagellate protozoa *Trypanosoma evansi* is still a great concern for some tropical countries where cattle industry is practiced and that drug-resistant become almost unavoidable. The disease is endemic in Malaysia (Zainal-Abidin 1992) but sporadic epidemic may occur. Since its isolation from naturally infected cattle in late 1980's, the maintenance of *T. evansi* in laboratory animals has become established and very useful for the study of trypanosomiasis in general. In the present study, the possible action of allicin on this parasite was explored by providing possible cellular evidence to it.

Materials and Methods

Experimental animal

Male eight-week old ICR mice (20-25 g) obtained from the Animal House, Universiti Kebangsaan Malaysia (UKM) were used in this study. The care and handling of the mice for research were in accordance to the UKM Animal Ethical Committee Guidelines (UKMAEC) 2003 (Anon. 2003). The mice were maintained on standard commercial mouse pellets plus drinking water *ad libitum*.

Parasite inoculum

Trypanosoma evansi was maintained in ICR mice by intraperitoneal (i.p) injection of infected blood from donor mice every 4-5 days. The inoculum consists of 1×10^5 parasites per 0.1 mL was prepared by serial dilutions of infected blood in Alsever's solution. Each mouse was inoculated i.p. 0.1 mL of the prepared inoculum.

Allicin and berenil

Garlic tablets from Natural Factors Garlic 500 (Canada) were used in this study. Each tablet (500 mg) contains a minimum weight of 750 μg allicin ($(C_3H_5SS(O)C_3H_5)$). Two tablets were dissolved in sterile distilled water at room temperature and shake in vortex until completely dissolved. The solution was kept at 10°C-20°C in dark bottle until used. A 0.1 mL of the solution (containing about 0.3 μg allicin) was force-fed to each mouse for treatment.

Anti-trypanosomal drug berenil (Hoechst Ag from Sigma-Aldrich) was used to treat mice infected with the parasite. The dose of 3.5 mg/kg (Verma et al. 1976) contained in 0.01 mL solution was prepared as instructed and injected i.p. into each mouse.

Treatments

Three groups of mice (6 mice per group) infected with the lethal dose of *T. evansi* (1×10^5 parasites per mouse) were used. The first group was treated with berenil once on D+3 post-infection as the positive control group. The second group was treated with allicin given orally for 30 days starting from D-7 post-infection as the experimental group and the third group was left untreated as the negative control group. Thin- and -Giemsa's stained blood smears were prepared from each mouse every alternate day, starting from D+3 post-infection and continued until the animal succumbed or until D+90 post-infection. Unstained thin blood smears were also prepared at the same time.

Parameters

General microscopy: observation and parasitaemias In this study thorough observations on the parasite's morphology were carried out under normal light microscope using the stained blood smears. Using the same blood smears, parasitaemias (rate of infection in the mice) were calculated as described elsewhere (Zainal-Abidin 1992). The prepatent period and mortality/survival rate of each group of the mice were also recorded.

SEM microscopy Unstained thin blood smears were also prepared and used for close observation of the morphology of the parasites at higher magnifications under scanning electron microscopes. The smears were first coated with gold at 20A° for 90-120sec before observation under Phillips XL30 and Leo 1450VP scanning microscopes. Micrographs were prepared for close scrutiny of the cellular damages due to treatments employed in this study.

Results

Parasitaemias

The pattern of parasitaemias in the three groups of mice were clearly defined. Parasitaemia in the negative group increased drastically and all mice succumbed to the

infection with a mean of peak parasitaemia of 38.35 ± 1.7%. The prepatent period of this group lasted for 04.56 ± 0.2 days. Although mice in the positive group (treated with berenil) had similar prepatent period (Table 1) their parasitaemias on the other hand, were short-lived and lasted for about 10 days post-infection with a moderate mean peak of parasitaemia (19.83 ± 1.2%). They survived from the infection for more than 100 days without signs of recurrence.

Mice in the experimental group (treated with allicin) experienced a prolonged and suppressed type of parasitaemias lasted for about 80 days before causing patent infection which caused death to all mice. The prepatent period of this group was delayed until about 20 days post-infection. The mean peak of parasitaemia was slightly higher than those in the other two groups of mice and the mice survived for more than 95 days (Table 1).

CHANGE IN MORPHOLOGY OF *T. EVANSI*

Observations on the micrographs prepared for this study revealed that the cells of the parasite undergone profound morphological changes due to treatment with berenil or allicin. Parasites in the positive group had adverse morphological changes due to berenil. Morphological changes were observed as soon as 2-4 h following treatment and the overall morphology of the cells quickly deteriorated. By 5-6 h post-treatment, total cell destruction was evidenced (Figure 1e) which led to cell death.

By comparison, the parasites in the experimental group (which had suppressed parasitaemias) also undergone profound cellular damages but occurred at much slower rate compared to action by berenil. Cellular changes started to appear soon after the termination of the prepatent period. During the patent infection, it was evidenced that there were actually mixed populations of normal and deteriorated parasite cells in the blood (Figure 1a, b, c, d). More interestingly it was noticed that granular or globular type of structures were formed and dotted underneath or close to the plasma membrane adjacent to undulating membrane (next to the flagellum) and later distributed within the cell. In the later stage, the cells became crescent with the undulating membrane and cytoplasm destroyed. Destruction of these cells lead to total disintegration of the cell and cell death (Figure 1d).

DISCUSSION

The time taken by the parasite to increase its numbers to detectable level in the peripheral blood may vary between species and influenced by some intrinsic factors. In light of the present study, the prepatent period of the negative control group and that of the berenil-treated or positive control group were similar and relatively shorter as compared to that of the allicin-treated or experimental group. This delayed prepatent period in the later group could be due to an early action of allicin which suppressed parasite cells growth and development. In turn this prolonged prepatent period may have caused a slow increase of and suppressed parasitaemias in the mice for 80 days before finally the infection became apparent and lethal. The higher mean peak parasitaemia in this group may also indicate the host's capacity to counter high parasitaemias with protective activities albeit immunity which was developed during the onset of prolonged and suppressed parasitaemias.

The detection of a mixed population of normal and deteriorated parasite cells in the peripheral blood need further explanation. It may indicate that the effect(s) of allicin on the cells was not total. Some cells were left undeterred by the action of this compound and thus continued to grow which later became predominant in the peripheral blood and causing patent and lethal infection to the mice. Due consideration should also be put forward here that the less effective action of this compound may be due to a lower dosage used in this study. Allicin at 30μg/mL was used to efficiently inhibit the growth of protozoan parasites such as *Entamoeba histolytica*, *Giardia lamblia* and *Leishmania major* and a much lower dose of between 0.15-1.9 μg/mL was used to inhibit some fungal growth including *Candida* spp. (Mirelman et al 1987; Yamada and Azuma 1997). Since the dose used in this study was only 15 μg/mL further study is required to determine whether a (i.e 30 μg/mL) or even higher dose should be applied to cause total inhibition of allicin on *T. evansi* cells *in vivo*. The mechanism of action of berenil on trypanosomes is well understood and related to the inhibition of DNA synthesis (Pilch et al. 1995) which led to cell death. Evidence from this study showed that damage to parasite cells by allicin may not be due to nuclear interference but more likely due to the indirect interference with cellular enzymes or cell membrane structures or interference with both.

TABLE 1. Prepatent period, peak parasitaemia and survival time of all groups used in the study

Treatment / group	Prepatent period (days)	Peak parasitaemia (%)	Survival time (days)
Negative control	04.56 ± 0.2	38.35 ± 1.7	09.36 ± 0.1
Positive (berenil-treated) control	04.56 ± 0.3	19.83 ±1.2	> 100
Experimental (allicin-treated)	20.42 ± 0.3	47.09 ± 0.2	96.58 ± 0.2

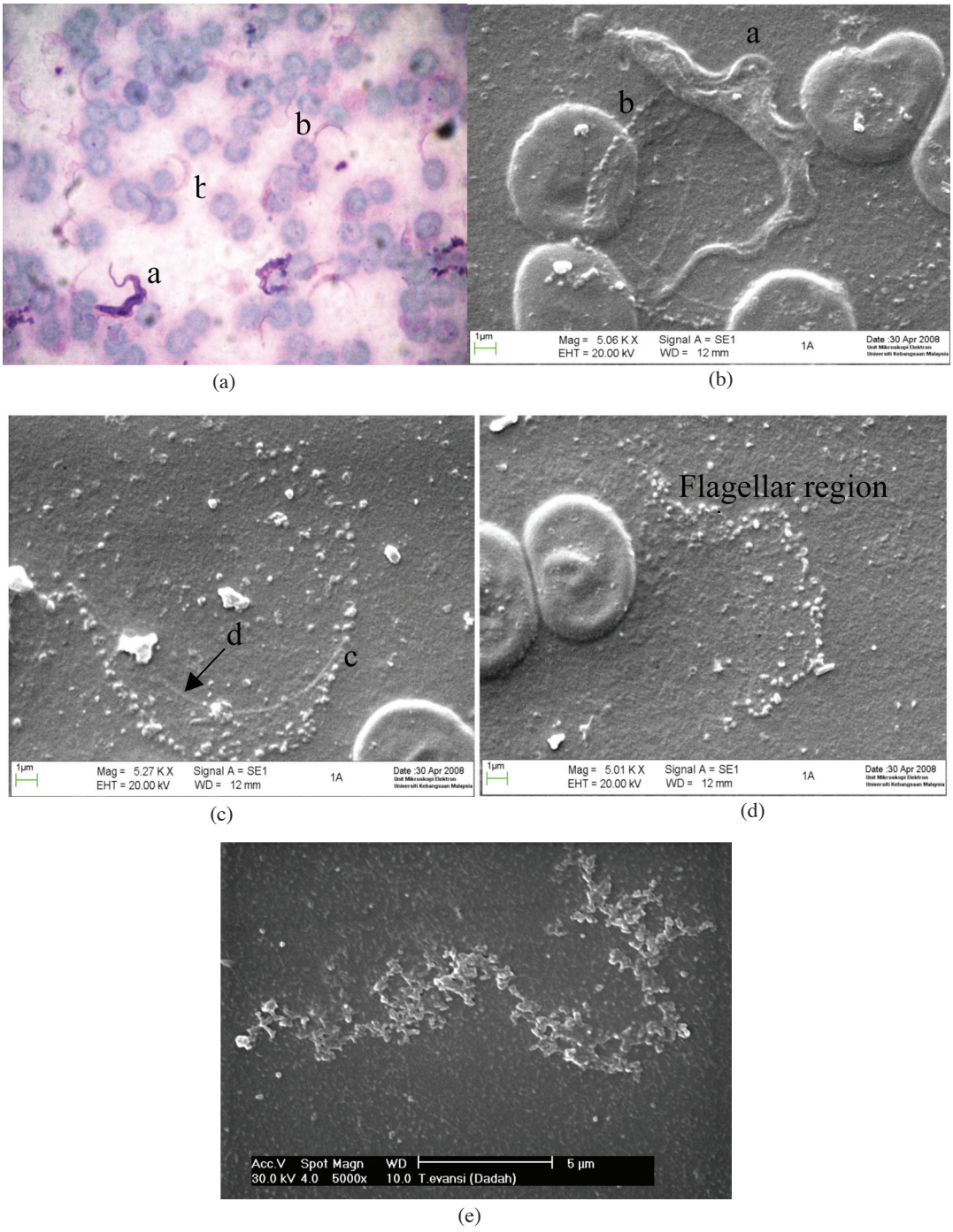

FIGURE 1. Normal and deteriorated parasite cells as seen under under light microscope at x100 magnification (a) and scanning electron micrographs of parasite cells after treatment with allicin (b, c and d) and treatment with berenil (e). (a = normal parasite cell; b = deteriorated parasite cell; c = ganules / globules on/in plasma membrane, d = intact plasma membrane)

Since these cellular damages were seen beneath or close to plasma membrane, it may be possible that allicin induces or interferes with the thiol-enzymes activity or perhaps phospholipid peroxidation in the plasma membrane that lead to oxidative damage causing cell death. In trypanosomes and leishmanias trypanothione/trypanothione reductace and glutathione act as the antioxidant agents against the toxic effect of nitrogen (nitrogen oxide, NO)-derived reactive species (Ramão et al. 2006). Since trypanothione is one of the thiol-redox proteins (Ariyanayagam and Fairlamb 2001) therefore interference with its metabolism by allicin may lead to a highly oxidative intracellular environment causing cell damage. The results of the present study provide the first evidence that allicin can actually induce cell damage to *T. evansi* cells *in vivo*. Further studies are required not only for better understanding of the mechanism of action of allicin on

parasites and on *T. evansi* in particular and also looking into means of application by which allicin or garlic in general can be taken as a potential prophylactic agent or supplement to prevent parasitic disease such as trypanosomiasis in cattle or surra in particular in the near future.

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REFERENCES

Ali, M., Thomson, M. & Afzal, M. 2000. Garlic and onions: their effect on eicosanoid metabolism and its clinical relevance. *Prostaglandins Leukotrienes and Essential Fatty Acids* 62: 55-73.

An, MM., Shen, H., Cao, YB., Zhang, JD., Cai, Y., Wang, R. & Jiang, Y.Y. 2009. Allicin enhances the oxidative damage effect of amphotericin B against *Candida albicans*. *International Journal of Antimicrobial Agents* 33: 258-263.

Anonymous. 2003. *Universiti Kebangsaan Malaysia Animal Ethics Committee Guidelines (UKMAEC)*. UKMAEC Secretariat, Kuala Lumpur, 1-40.

Ariyanayagam, M.R. & Fairlamb, A.H. 2001. Ovothiol and trypanothione as antioxcidants in trypanosomatids. *Molecular and Biochemical Parasitology* 115: 189-198.

Mirelman, D., Monheit, D. & Varon, S. 1987. Inhibition of growth of *Entamoeba histolytica* by allicin, the active principle of garlic extract (*Allium sativum*). *Journal of Infectious Diseases* 156(1): 243-244.

Oommen, S., Anto, R.J., Srinivas, G. & Karunagaran, D. 2004. Allicin (from garlic) induces caspace-mediated apoptosis in cancer cells. *European Journal of Pharmacology* 485: 97-103.

Pilch, D.S. Kirolos, M.A. & Breslauer D.S. 1995. Berenil binding to higher ordered nucleic acid structures: complexion with a DNA and RNA triple helix. *Biochemistry* 34: 16107-16124.

Rabinkov, A., Miron, T., Konstaninovski, L., Wilchek, M., Mirelman, D. & Weiner, L. 1998. The mode of action of allicin: trapping of radicals and interaction with thiol containing proteins. *Biochemicaet Biophysica Acta* 1379: 233-244.

Ramos, F.A., Takaishi, Y., Shirotori, M., Kawaguchi, Y., Tsuchiya, K. & Shibata H. 2006. Antibacterial and antioxcidant activities of quercetin oxidation products from yellow anion (*Allium cepa*) skin. *Journal of Agricultural and Food Chemistry* 54: 3551-3557.

Romão, P.R.T., Tovar, J. Fonseca, S.G. Moraes, R.H., Cruz, A.K., Hothersall, J.S., -Noronha-Dutra, A.A., Ferreira, S.H. & Cunha, F.Q. 2006. Glutathione and the redox control system trypanothione/trypanothione reductase are involved in the protection of *Leishmania* spp. against nitrosothiol-induced cytotoxicity. *Brazilian Journal of Medical and Biological Research* 39(3): 355-363.

Saleehen, D., Ali, S.A. & Yasinzai M.M. 2004. Antileishmanial activity of aqueous onion extract *in vitro*. *Fitoterapia* 7(51): 9-13.

Verma, B.B., Gautam, O.P. & Malik P.D. 1976. *Trypanosoma evansi*: therapeutic efficacy of diaminazine aceturate in crossbred calves, *Bos Taurus and B. indicus*. *Experimental Parasitology* 40: 406-410.

Yamada, Y. & Azuma, K. 1997. Evaluation of the *in vitro* antifungal activity of allicin. *Antimicrob. Agents Chemother.* 11: 743-749.

Zainal-Abidin, B.A.H.1992. Infections of *Trypanosoma evansi* in Malaysia. *Malaysian Applied Biology* 2(10): 1-8.

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