J. Protozool. Res. 17, 16-24 (2007)

Inhibitory effects of *Melia azedarach* L. extracts on the growth of *Trichomonas vaginalis* - Ultrastructural changes of *Trichomonas vaginalis* treated by *Melia azedarach* L.-

Yun Sik Lee[a], In Bai Chung[a], Won Hyung Choi[a], Yoo Jung Cho[a], Jong Phil Chu[a], Byung-Il Min[b], Eun-Hee Shin[c]**

[a] Department of Medical Zoology and [b] Department of Physiology, College of Medicine, KyungHee University, Seoul, South Korea, [c] Department of Parasitology and Tropical Medicine, College of Medicine, Seoul National University, Seoul, South Korea

****** Address correspondence and reprint requests to Jong Phil Chu, Department of Medical Zoology, College of Medicine, KyungHee University, #1 Hoegi-dong, Dongdaemoon-gu, Seoul, 130-701, South Korea.
Tel:+82-2-961-0919; Fax:+82-2-967-8401; E-mail:depara@khu.ac.kr

ABSTRACT

Meliae cortex (*Melia azedarach* Linne. *var*. *japonica* Makino, *M. toosendan* Siebold & Zucc) is one of the herbs used in oriental medicine for the treatment of various types of vaginitis. The present study was performed to examine the effects of *Meliae cortex* extracts on the growth and fine structure of *Trichomonas vaginalis* (*T. vaginalis*).

Meliae cortex dose-dependently reduced the viability of *T. vaginalis*. In the electron microscopy study, we also observed the ultrastructural changes of *T. vaginalis* following the treatment with *Meliae cortex*. One to two hours after the addition of the extract of *Meliae cortex*, the movement of flagella and axostyle decreased, but the cell death did not occur. The fine structure of the cytoplasm was changed 30 min to 2 hour after the treatment of the extract of *Meliae cortex*. The number of polyribosome decreased, whereas the number of single ribosomes in the cytoplasm increased.

These results indicate that *Meliae cortex* has the antiprotozoal effects on *T. vaginalis* cells through the inhibition of cell multiplication as well as the impairment of protein synthesis.

Key words: *Trichomonas vaginalis*; *Meliae cortex*; *Meliae azedarach*; inhibition of cell multiplication; protozoa; ultra structure

INTRODUCTION

Trichomonas vaginalis (*T. vaginalis*) is a widely prevalent parasitic flagellate causing sexually transmitted diseases, such as trichomoniasis. The number of people suffering from this form of vaginitis has explosively increased worldwide, with over 3 million new cases *per* year only in the United States (Lee *et al*., 1996). Symptoms of vaginitis include profuse leukorrhea, redness and a burning painful sensation. Endometritis is one of the complications associated with this disease and may result in infertility and reproductive failure (Chu *et al*., 1974). In the last decade, numerous articles regarding the prevalence, immunodiagnostic methods and therapeutic agents against *T. vaginalis* have been reported (Landers, 1988; Skirrow and Bondurant, 1988; Muller *et al*., 1988; Sears and O'Hare, 1988; Ahmed *et al*., 1988; McCutchan *et al*., 1992; Rivera *et al*., 1996). Metronidazole is the drug of choice used to treat trichomoniasis and is a relatively well-tolerated drug that is available in an oral or parenteral form. However, annoying adverse effects of this drug are common, including nausea, vomiting, diarrhea, numbness, and tingling of extremity (Finegold, 1980). In addition, animal and bacterial studies suggest that metronidazole may be mutagenic and

carcinogenic (Roe, 1983). It is thus of value to search for additional therapeutic options with less side effects (Weihe *et al*., 1988; Robertson *et al*., 1988).

Meliae cortex, {*Melia azedarach* Linne. *var*. *japonica* Makino, *M. toosendan* Siebold & Zucc (*M. azedarach*)}, a natural plant has long been used in oriental society, especially in Korea, for the treatment of bacterial, fungal or protozoal vaginitis (Kim *et al*., 1998). *M. azedarach* contains triterpenes, merosin, kulinone, toosendanin, nimbolin A, nimbolin B and fraxinellone gedunin, but little is known about the pharmacological actions or mechanisms of these agents (Kim *et al*, 1998; MacKinnon *et al*, 1997; Shi and Wang, 2004). Therefore, we attempted to evaluate the antiprotozoal activity of *Meliae cortex* in the present study. With this aim, the effects of *Meliae cortex* extracts on the growth and fine structure of *T. vaginalis* were examined using electron microscopy.

MATERIALS AND METHODS

Culture of *T. vaginalis*

T. vaginalis (KT4) isolate was provided by the Dept. of parasitology, college of medicine, Hanyang university. Trophozoites were axenically cultured in TYM medium.

Extraction of *Meliae cortex*

Used *Meliae cortex* {*Melia azedarach* Linne. *var*. *japonica* Makino, *M. toosendan* Siebold & Zucc (*M. azedarach*)}, provides and vouches by KyungHee Medical Center Herb Pharmacy. 1,500 ml of distilled water in 5L flask was pressurized and sterilized under the condition of 120^{o}**C**, 3 kg/cm^{3}of pressure, for 1 hour in an autoclave. 97.5 g of *Meliae cortex*, herb medicine, was washed and concussed in a vacuum container (Misung Scientific Co. Seoul, Korea) for 3 hours. The concussed solution was concentrated with reduced pressure using a rotary evaporator and then centrifuged at 3,000 rpm, 4^{o}**C**, for 15 min. The supernatant was sterilized by filtration with a 0.22 μm filter and kept at 4^{o}**C** as a stock solution and diluted with PBS (Phosphate buffer saline: pH 7.4) just before the experiments and use.

Determination of viability and ultrastructural change of *T. vaginalis*

1) Survival rate

Each 5, 10, 15, 20, and 25 mg/ml sample of concentrated solution were diluted with dried *M. azedarach* extracts in PBS was added to the TYM media containing *T. vaginalis* 3.0 x 10^{6}/ml. Each sample was cultivated at 36^{o}**C** in a CO_2 incubator for 1, 1.5, 2, 2.5 and 3 hours, respectively. Cell death was evaluated by exclusion of trypan blue (GIBCO, Grand island, N.Y., USA) dye.

2) Ultra structural change of *T. vaginalis*

Trophozoites treated with each concentration of *M. azedarach* were fixed for 4 hrs in 2.5% glutaraldehyde, post fixed with 1% osmium tetraoxide (pH 7.4) for 2 hrs, then dehydrated with grade alcohol and experimented with a SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy).

For SEM observation, the samples were treated with isoamylacetone, dried with a critical point dryer, attached to the aluminum stab with colloidal silver, coated with a thickness of 200 Å of gold in JEC-1, 100 ion coater and observed with 15 kV of accelerated voltage under SEM (JSM-35C, Japan). For TEM observation, the samples were treated with propylene oxide, and embedded with epon 812. Tissues were cut into 50~70nm thickness of ultra thin segments with ultramicrotome (LKB III, U.S.A.). The ultra thin sections were stained with uranyl acetate and lead citrate, and observed with 60-80kV of accelerated voltage under TEM (Hitachi H 600A, Tokyo, Japan).

Statistics

The experiments were performed in triplicates and repeated twice to confirm similar results. Significance of the differences in mean value was determined using the Student's *t*-test.

Table 1. Viabilities of *T. vaginalis* incubated in PBS containing *M. azedarach* from 0.5 hour to 3 hours.

Concentration (mg/ml) / Time (hour)	5	10	15	20	25	Control
0.5	$1.95\pm0.5\times10^6$	$1.8\pm0.1\times10^6$	$7.4\pm0.2\times10^5$	$7\pm1\times10^5$	$1.7\pm0.1\times10^5$	$2.15\pm0.05\times10^6$
1.0	2.55 ± 0.5 x10^6	$2\pm0.1\times10^6$	$10.2\pm0.6\times10^5$	$7.1\pm0.9\times10^5$	$1\pm1\times10^4$	$2.2\pm0.3\times10^6$
1.5	$2.05\pm1.5\times10^6$	$7.4\pm0.8\times10^5$	$1\pm1\times10^4$	$0.5\pm0.5\times10^4$	0	$2.55\pm0.25\times10^6$
2.0	$2.25\pm1.5\times10^6$	$3.25\pm0.45\times10^5$	$0.5\pm0.5\times10^4$	$0.5\pm0.5\times10^4$	0	$2.1\pm0.1\times10^6$
2.5	$2.2\pm0.3\times10^6$	$5.5\pm0.5\times10^4$	$0.5\pm0.5\times10^4$	0	0	$2.55\pm0.05\times10^6$
3.0	$2.1\pm0.4\times10^6$	$3\pm1\times10^4$	$5\pm0.5\times10^4$	0	0	$2.7\pm0.1\times10^6$

RESULTS

Viability of *T. vaginalis*

Viability of *T. vaginalis* incubated in PBS containing a given concentration of *M. azedarach* extracts are shown on table 1. The number of cells with the control group, cultivated in 5 mg/ml of *M. azedarach* extract for 3 hours were reduced slightly from 2.6~2.8 x 10^6 to 1.7~2.5 x 10^6. Even through there was no remarkable decrease of trophozoites with the treatment of 5 mg/ml of *M. azedarach* extract, the cellular viability of *T. vaginalis* was reduced remarkably from 6.6~8.2 x 10^5 under 1.5 hours of cultivation to 2~4 x 10^4 under 3 hours of cultivation with more than 10 mg/ml. At 15 mg/ml, cellular viability began to reduce after 1 hour of cultivation, showing nearly 0% viability after 1.5 hours of cultivation. At 20 mg/ml, 0% cellular viability was observed after 2 hours cultivation. In 25 mg/ml, living cells were not observed after 1 hour of cultivation.

As the time passed by at a given concentration, there were statistically significant results shown by a reduction of viable cells of *T. vaginalis,* shown on table 1. Values are means (SEM is some cases).

Electron Microscopic observation

There were significant differences in morphology of *T. vaginalis* with each concentration, when observed using SEM. In control groups, the body appeared slightly swollen, in general, and undulating membrane was absent. The axostyle and 4 flagella were observed clearly (Fig. 1). There was not remarkable difference between the control group and the cultivated groups after 2 hours at 5 mg/ml.

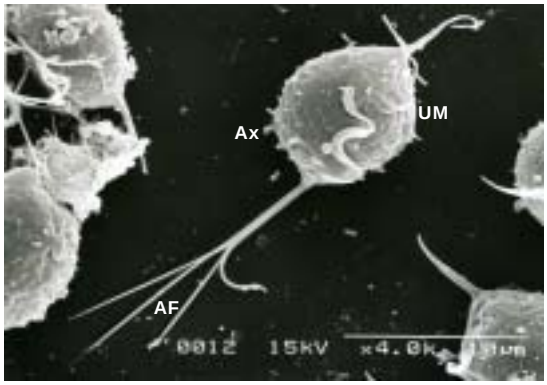

Fig 1. Scanning electron micrograph of *T. vaginalis* cultivated in TYM medium. Normal trophozoite of *T. vaginalis* shows 4 anterior flagella (AF), undulatin membrane (UM) and an Axostyle (Ax) (×4,000).

In the group cultivated for 3 hours at 5 mg/ml and the group cultivated for 1 hour at 10 mg/ml, almost all the cells external appearance had an irregular shape, and the axostyle gradually disappeared. Some of the *T. vaginalis* showed the axostyle, but their length was shortened. There was also a tendency of shortening of the flagella (Fig. 2, 3).

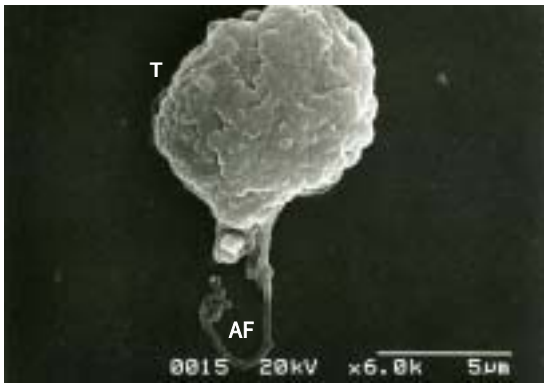

Fig 2. Scanning electron micrograph of *T. vaginalis* (T) cultivated in TYM medium containing 10 mg/ml of *M. azedarach* for 2 hours. An axostyle is disappear and anterior flagella (AF) are not clear and short. The body of the trophozoite has an irregular shape, is wrinkled and shows small tubercle like structures on the surface (×6,000).

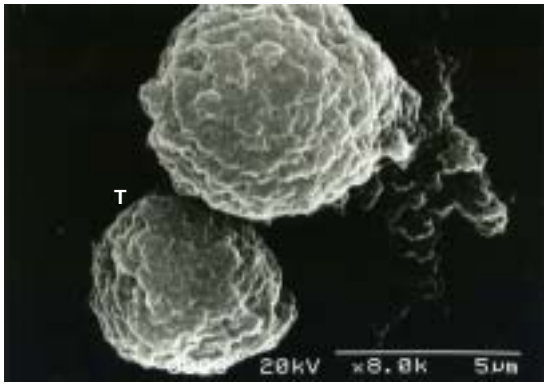

Fig 3. Scanning electron micrograph of *T. vaginalis* cultivated in TYM medium containing 15 mg/ml of *M. azedarach* for 2 hours. An axostyle and flagella are not seen. The body of the trophozoite (T) has an irregular shape and changes to small. The cell surface shows irregular and tubercular shape (×8,000).

In the group incubated for 2 hours at 10 mg/ml, the external appearance was irregular. The axostyle and flagella had disappeared. The cellular surface was wrinkled and small papillae-like shape were observed (Fig. 4).

Fig 4. Scanning electron micrograph of *T. vaginalis* cultivated in TYM medium containing 20 mg/ml of *M. azedarach* for 2 hours. The trophozoite (T) looks like a ghost cell. Various sizes of hollows (arrow) are broadly distributed on the cell surface. (×12,500)

In the group cultivated for 2 hours at 15 mg/ml, the axon and flagella quickly disappeared and granite-like, small pores resurfaced to the cellular surface, showing evidence of destroyed *T. vaginalis.*

Most cells were destroyed. Degenerative change was progressive and the surface appeared to be swollen like, round bumps (Fig. 5). From the groups cultivated for 1 hour at 25 mg/ml, more small pores were observed on the cell surface than Fig. 5. All cells degenerative changes became more severe (Fig. 6).

There were also significant morphologic changes of *T. vaginalis* with the change in concentration of *M. azedarach* and incubation time.

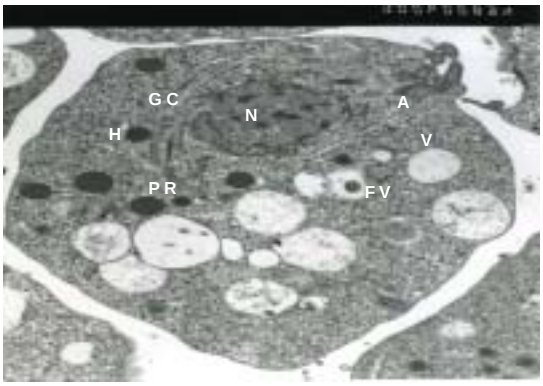

Fig 5. Transmission electron micrograph of *T. vaginalis* cultivated in TYM medium. Normal *T. vaginalis* consists of an irregular shaped nucleus (N), hydrogenosomes (H), Golgi complex (GC), polysomes (PR), vacuoles (V) and food vacuole (FV) in the cytoplasm. Nucleus (N) encircled by RER contains clusters of chromatinic materials in the nucleoplasm. Hydrogenosomes (H) are located around the axostyle (A) and surrounded by polysomes and glycogens. As the organelles for supporting and movement, an axostyle, costa are observed.

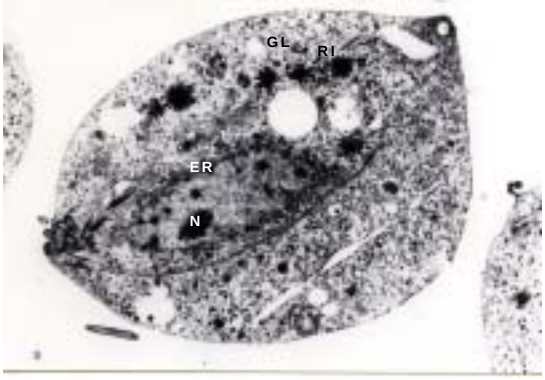

Fig 6. Transmission electron micrograph of *T. vaginals* cultivated in TYM medium containing 10 mg/ml of *M. azedarach* for 2 hours. The free ribosomes in the cytoplasm are mostly present as polyribosomes (RI). A nucleus (N), some of the perinuclear endoplasmic reticulum (ER), are also damaged. The electron-translucent zones (GL) are possibly extracted glycogen granules.

In the control group, *T. vaginalis* showed an irregular shaped nucleus, hydrogenosome, flagella and axon (Fig. 7). The chromatin of the nucleus was less stained and heterochromatin was more abundant than euchromosome. The anterior motor organ was located in the anterior canal and each flagella were observed separately (Fig. 8).

In the group cultivated for 2 hours at 10 mg/ml, there was no remarkable difference compared with the control group in external appearance. This group had more electron dense portions observed, the golgi apparatus appeared reduced, and more food vacuoles were observed than in the control group. The hydrogenosomes were not observed, but the trophozoites surface was intact, and showed progressively degenerative changes of *T. vaginalis* (Fig. 6).

In the group cultivated for 2 hours at 15 mg/ml, free ribosomes and polyribosomes nearly disappeared in the cytoplasm. Damaged cell walls, less than stained nucleus, and abundant vacuoles in cytoplasm were observed. Vacuoles and food vacuoles were enlarged, and showed electron translucent. Expulsion of glycogen granules, were observed, and showed considerable progressive degenerative changes of *T. vaginalis* (Fig. 7).

In the group cultivated for 2 hours at 20 mg/ml, dead cells were observed, granules looked like hydrogenosomes and the cytoplasm was also degenerated. There was no nucleus, nearly scant polysomes and ribosomes were observed. The size of vacuoles was enlarged, so they couldn't be differentiated from food vacuoles. Trophozoites walls were nearly destructed and generalized degeneration changes were severe, so they could not be investigated by inner composition of *T. vaginalis* (Fig. 8).

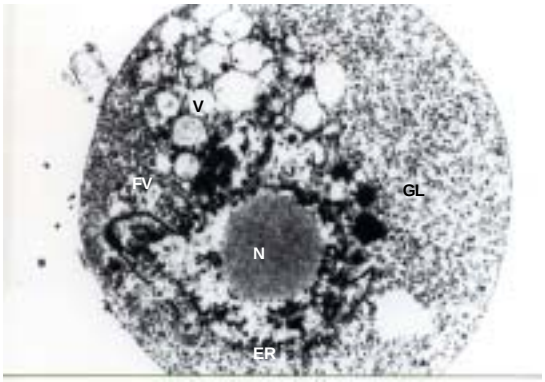

Fig 7. Transmission electron micrograph of *T. vaginalis* cultivated in TYM medium containing 15 mg/ml of *M. azedarach* for 2 hours. A nucleus (N), some of the perinuclear endoplasmic reticulum (ER), are damaged. The electron-translucent zones (GL) are possibly extracted glycogen granules. Some of the large food vacuole (FV), and vacuoles (V) show in the cytoplasm. A part of the cell membrane showed destruction.

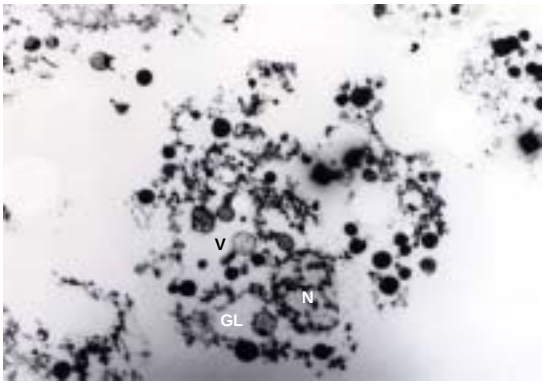

Fig 8. Transmission electron micrographs of *T. vaginalis* cultivated in TYM medium containing 20 mg/ml of *M. azedarach* for 2 hours. The electron translucent zone (GL) increased in the cytoplasm. The vacuoles (V) are destructed and the nucleus (N) has decreased compared with untreated trophozoites. Total destructed cell membranes are observed. Dead *T. vaginalis* are easily seen.

DISCUSSION

It has been reported that metronidazole inhibits the cellular division of *T. vaginalis* by blocking the synthesis of nucleic acid (Nielsen, 1976). Previous studies using electron microscopy showed an increased

number of free ribosomes and fewer polyribosomes in *T. vaginalis* after the treatment with metronidazole (Fried and Fried, 1968; Edwards and Mathison, 1970). A high concentration of metronidazole has been reported to inhibit H^+ ion transfer (Fried and Fried, 1968; Edwards and Mathison, 1970). These findings suggest that metronidazole inhibits the cellular division of *T. vaginalis* as a result of blocking the interphase of proliferation (Fried and Fried, 1968; Edwards and Mathison, 1970). However, we could not observe the increase in number of intracellular free ribosomes and the decrease in polyribosomes following the treatment with *M. azedarach* in the present study. Therefore, there may be a difference in the mechanisms of the therapeutic effects between metronidazole and *M. azedarach*. It would be of high interest to clarify this difference in a future study. According to the result of the survival rate test and the changes on electron microscope, it can be proved that the therapeutic effect of *M. azedarach* may be more potent than that of metronidazole. A low concentration of metronidazole did not show therapeutic effects up to the 8-18 hours after the administration(Asami, 1963), whereas 15 mg/ml of *M. azedarach* extracts produced a therapeutic effect just 2 hours after the treatment.

Although the SEM results of the present study are similar to those of the study by Warton and Honigberg (1979), *M. azedarach* evoked the primary change of the axostyle even at the lower concentrations tested. Eventually, both the axostyle and flagella disappeared. These results are consistent with Kim *et al*. (2003) who showed the effects of Kalopanaxsaponin A on *T. vaginalis*. At higher concentrations, these findings were observed even earlier. The time- and dose-dependent findings of the TEM were also similar to the SEM observations.

To examine whether *M. azedarach* has a therapeutic effect against *T. vaginalis*, we observed the time- and dose-dependent ultra structural changes of *T. vaginalis* after treatment with *M. azedarach*. In the control group, *T. vaginalis* had an irregularly shaped nucleus, gydrogenosomes, a cut aspect of the flagella and part of the parabasal body. The pale nuclear and more abundant hetero-chromosomes than euchromosomes were also observed (Fig. 7, 8). Small holes in the cell surface were observed. It can be suspected that these holes were the openings of the pinocytosis path. The *T. vaginalis* group treated with 5 mg/ml of *M. azedarach* for 3 hours did not show any difference from the control group. Even though the *T. vaginalis* group treated with 10 mg/ml of *M. azedarach* for 2 hours had similar results to the control group, more intracytoplasmic electron dense lucent portions were observed. We confirm that this observation was due to the loss of glycogen granules. The nucleus was more pale and had more abundant abnormal chromosomes. The number of intracytoplasmic Golgi apparatuses and polysomes decreased, and more food vacuoles were observed compared with those of the control group. Hydrogenosomes were not observed. These observation indicate the regressive changes of *T. vaginalis* by *M. azedarach* (Fig. 6). A destructed cell wall, pale nucleus, and vacuolization were observed in the *T. vaginalis* group treated with 15 mg/ml of *M. azedarach* for 2 hours. The regressive changes were more aggravated to the extent that free ribosomes and polyribosomes disappeared within the scattered dead cells (Fig. 7). These results show similar effects in *Gleditsia sinesis*, a metronidazole resistant strain of *T. vaginalis* (Park et al., 2004). A vanished nucleus, destructed cell walls, and scattered intracellular debris from dead cells were noted in the *T. vaginalis* group treated with 20 mg/ml of *M. azedarach* for 2 hours. According to the above observations, a concentration of more than 15 mg/ml of *M. azedarach* has a parasiticidal effect against *T. vaginalis*.

In conclusion, we confirm the *in vitro* killing of *M. azedarach* by demonstrating survival rate and ultrastructural changes of *T. vaginalis*. Although triterpenes, merosin, kulinone, toosendanin, nimbolin A and fraxinellone gedunin have been identified as the chemical components of *M. azedarach*, it remains to be elucidated which of these components is responsible for the therapeutic effect against *T. vaginalis* (Chen,

1990). Further study is needed to clarify the action mechanism and the major active chemical component of *M. azedarach*.

ACKNOWLEDGEMENTS

We thank the Department of Parasitology, College of Hanyang University for giving us the strain of *T. vaginalis* and an advice for this research. This result was supported by Kyung Hee University foundation (KHU-20031118).

REFERENCES

Ahmed, J. I. H., Murray, A. E. and McKeown, J. 1988. Managing trichomonal vaginitis refractory to conventional treatment with metronidazole. Genitourin. Med. 64: 25-29.

Asami, K. 1963. Effects of metronidazole on *Trichomonas vaginalis* in culture and in an experimental host. Am. J. Trop. Med. 12: 535-538.

Chen, S. Y. 1990. Tu Shuo Han Fang Yi Yac Da Shi Dian. (Korean ed.) SongAk Pulishing Co., Seoul, Korea.

Chu, J. K., Chang, M. C., Chung, S. B. and Cho, M. J. 1974. Urinary tract infection with *Trichomomas vaginalis* in men. Korean Central Journal Medicine 26: 325-330.

Edwards, D. I. and Mathison, G. E. 1970. The mode of action of metronidazole against *Trichomonas vaginalis*. J. Gen. Microbiol. 63(3): 297-302.

Finegold, S. M. 1980. Metronidazole. Ann. Intern. Med., 93:585-587.

Fried, R. and Fried, L. W. 1968. Inhibition of oxidizing enzymes by metronidazole. Experientia 24(1): 56-57.

Kim, C. M., Shin, M. G., Ahn, D. G. and Lee, G.S. 1998. Wan Yi Zhang yao da shi dian. pp. 340-347. ChungDam Publishing Co., Seoul, Korea.

Kim, D. J., Cho, Y. J. and Chu, J. P. 2003. *In vitro* effect of Kalopanaxsaponin A on the ultrastructure of *Trichomonas vaginalis*. Infection and Chemotherapy 35(6): 446-453.

Landers, D. V. 1988. The treatment of vaginitis: Trichomonas, yeast, and bacterial vaginosis. Clin. Obstet. Gynecol. 31: 473-479.

Lee, S. H., Chai, J. I. and Hong, S. T. 1996. Clinical parasitology. pp. 336-338. Korea Medical Publishing Co., Seoul, Korea.

MacKinnon, S., Durst, T., Arnason, J. T., Angerhofer, C., Pezzuto, J., Sanchez-Vindas, P. E., Poveda, L. J. and Gbeassor, M. 1997. Antimalarial activity of tropical Meliaceae extracts and gedunin derivatives. J. Nat. Prod. 60: 336-341.

McCutchan, J. A., Ronald, A. R., Corey, L. and Handsfield, H. H. 1992. Evaluation of new anti-infective drugs for the treatment of vaginal infections. Infectious diseases society of America and the Food and Drug Administration. Clin. Infect. Dis. 15 Suppl. 1: S115-122.

Muller, M. Lossick, J. G. and Gorrell, T. E. 1988. *In vitro* susceptibility of *Trichomonas vaginalis* to metronidazole and treatment outcome in vaginal trichomoniasis. Sex. Transm. Dis. 15: 17-24.

Nielsen, M. H. 1976. *In vitro* effect of metronidazole on the ultrastructure of *Trichomonas vaginalis* Donné. Acta pathol. Microbiol. Scand. (B) 84: 93-100.

Park, W. S., Cho, Y. J. and Chu, J. P. 2004. Growth inhibitory effects of various herbal extracts on metronidazole resistant strain of *Trichomonas vaginalis*. Infection and Chemotherapy 36(2): 97-104.

Rivera, L. R., Trenado, M. Q., Valdez, A. C. and Gonzalez, C. J. 1996. Prevalence of bacterial vaginitis and

vaginosis : association with clinical and laboratory features, and treatment. Ginecol. Obstet. Mex. 64: 26-35.

Robertson, J. F., Azmy, A. F. and Cochran, W. 1988. Assent to ascent of the testis. Br. J. Urol. 61: 146-147.

Roe, F. J. 1983. Toxicologic evaluation of metronidazole with particular reference to carcinogenic, mutagenic, and teratogenic potential. Surgery 93: 158-164.

Sears, S. D. and O'Hare, J. 1988. *In vitro* susceptibility of *Trichomonas vaginalis* to 50 antimicrobial agents. Antimicrob. Agents. Chemother. 32: 144-146.

Shi, Y. L. and Wang, Z. F. 2004. Cure of experimental botulism and antibotulismic effect of toosendanin. Acta Pharmacol Sin. 25: 839-848.

Skirrow, S. Z. and Bondurant, R. H. 1988. Treatment of bovine trichomoniasis with ipronidazole. Aust. Vet. J. 65:156.

Warton, A. and Honigberg, B. M. 1979. Structure of Trichomonads as revealed by scanning electron microscopy. J. Protozool. 26:56-62.

Weihe, J., Metelmann, C., Borner, K., Meingassner, J. and Orfanos, C. E. 1988. Metro- nidazole-resistant trichomoniasis and sucessful therapy following high dosage. Hautarzt. 39: 237-239.