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In Vitro Activities of 2,2'-Dipyridyl Against *Trichomonas vaginalis*, *Candida albicans*, and *Gardnerella vaginalis*

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Abstract The *in vitro* activity of 2,2'-dipyridyl, an iron-chelator, against clinical isolates of *Trichomonas vaginalis*, *Candida albicans*, and *Gardnerella vaginalis* was evaluated and compared with those of four other vaginal suppositories, ornidazole, clotrimazole, povidone-iodine, and Cenacert® (Methylbenzethonium Chloride mixed with 9-aminoacrydine undecylenate and hydrochloric acid N-myristyl-3-hydroxy butyl amine). The 2,2'-dipyridyl killed *T. vaginalis* and *G. vaginalis* at concentrations of 410 μg/ml and 205 μg/ml, respectively, however, this agent was less active against *C. albicans* (80% of which was inhibited at 410 μg/ml). The inhibition of these three pathogens by 2,2'-dipyridyl was similar to clotrimazole. In addition, the effect of 2,2'-dipyridyl on the ultrastructure of *T. vaginalis*, *C. albicans*, and *G. vaginalis* was examined. Transmission electron microscopy indicated that 2,2'-dipyridyl induced modifications of the cellular contents and cell envelope concomitant with the degradation of the three pathogens. These results suggest that 2,2'-dipyridyl has an inhibitory effect on *C. albicans* and *G. vaginalis*, as well as *T. vaginalis*.

Key words: 2,2'-Dipyridyl, *Trichomonas vaginalis*, *Candida albicans*, *Gardnerella vaginalis*

Candida albicans, *Trichomonas vaginalis*, and bacterial vaginosis (*Gardnerella vaginalis* and/or other species) represent the three major causes of vulvovaginitis [6]. Ferris *et al.* [8] reported that vulvovaginal candidiasis was diagnosed in 20.0%, vaginal trichomoniasis in 7.4%, and bacterial vaginosis in 52.1% of 501 symptomatic women using a DNA hybridization test. Fourteen percent of the subjects had multiple vaginal infections.

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Iron is vital for living organisms because it is an essential component in many metabolic processes, including oxygen and electron transport, and DNA synthesis [3]. Iron limitation caused by ferric iron chelators such as desferrioxamine and deferoxamine methanesulfonate (Desferal) have been reported to inhibit the growth of viruses such as cytomegalovirus [10], bacteria including *Neisseria gonorrhea* and *Proteus mirabilis* [18], fungi like *Trichophyton mentagrophytes* [13], and protozoan like *Pneumocystis carinii* [29]. In addition, desferrioxamine B and deferiprone have been demonstrated to be effective adjunct therapeutic agents for malaria [9, 19].

2,2'-Dipyridyl, a ferrous iron chelator, is known to inhibit enzyme functions, such as those of cyclo-oxygenase inhibitors of platelet functions and bovine lens aldehyde dehydrogenase, and to prevent cerebral vasospasm by continuous intravenous administration in a primate model of a subarachnoid hemmorrhage [11, 12, 23]. In addition, 2,2'-dipyridyl has been reported to suppress the growth of *T. vaginalis*, decrease the synthesis of adhesin proteins, and reduce the adherence of *T. vaginalis* to epithelial cells [16, 17].

However, it is not known whether 2,2'-dipyridyl also affects the viability of *Candida albicans* or *Gardnerella vaginalis*. Therefore, the aim of this study was to examine the *in vitro* inhibitory activity of 2,2'-dipyridyl on *Gardnerella vaginalis*, *Candida albicans*, and *Trichomonas vaginalis*, all of which are responsible for vulvovaginitis. Also, the ultrastructural changes caused by 2,2'-dipyridyl on the three pathogens were examined.

Materials and Methods

Agents

2,2'-Dipyridyl and vaginal suppositories were obtained from the following sources: 2,2'-dipyridyl (Sigma, St. Louis,

U.S.A.), clotrimazole (Chung-Gei Pharma. Co., Korea), ornidazole (Chong-Kun dang Pharmaceutical Co., Korea), povidone-iodine (Hyun-dai Pharm. ind,. Co., Korea), and methylbenzethonium chloride mixed with 9-aminoacrydine undecylenate and hydrochloric acid N-myristyl-3-hydroxy butyl amine (Cenacert®, 2 mg/tablet, H-pharm. Co., Korea). All of these drugs were dissolved in PBS.

Trichomonas vaginalis and Tube Dilution Test

Five Korean isolates (KT4, KT9, KT11, KT12, and KT-Kim isolates) and Metronidazole resistant CDC85 were used in this study [25]. The trophozoites were axenically cultured in a TYM medium [4].

In order to determine the minimum inhibitory concentrations (MIC), culture tubes were inoculated with 1×10^6 trophozoites/ml at the early log phase, followed by the additions of the agents. The concentration ranges of the agents tested are indicated in Table 1. Tubes were incubated for 24 h at 37°C. Each isolate was evaluated in triplicate. The MIC was defined as the lowest concentration at which a live trichomonad could not be detected by the trypan blue staining method.

Candida albicans and Tube Dilution Test

Two isolates of *Candida albicans* (KC-1, KC-2) were isolated from Korean vaginal candidiasis for this study, and identified by the Department of Clinical Pathology, Hanyang University Hospital. The yeast phase organisms were extracted with a Hank's Balanced Salt Solution (HBSS) from a culture grown on a Sabouraud's agar (Sabouraud dextrose agar, BBL, Cockeysville, U.S.A.) plate for 24 h at 37°C [14]. The suspension was then centrifuged at 150 ×g for 10 min. The cell pellet was washed with HBSS, resuspended in a RPMI medium to a concentration of 1×10^6 organisms per ml, and incubated for 24 h at 37°C. The viability of the yeast was assessed by examining trypan blue stained smears. The lowest concentration of drug (MIC in micrograms per mililiter) that inhibited visible growth of the organism in a pure RPMI medium culture was determined using the tube dilution susceptibility test.

Gardnerella vaginalis and Agar Dilution Test

G. vaginalis ATCC 14018 and 14019 were grown in HBT (human blood bilayer agar with Tween 80) [28]. The MIC testing of *G. vaginalis* was performed by the agar dilution method. Two-fold serial concentrations of the agents were incorporated into the medium. The inocula were prepared from a 24 h culture on an agar medium and diluted to obtain 10^4 CFU per spot in the *G. vaginalis* test. After the incubation of the inocula for 48 h in 10% CO_2 at 37°C, the MIC was determined as the lowest concentration of each drug in which bacterial growth was completely inhibited.

Transmission Electron Microscopy

Two days' incubation of *T. vaginalis*, *C. albicans* and *G. vaginalis* with various concentrations of 2,2'-dipyridyl (31.2 μg/ml, 102 μg/ml, and 102 μg/ml, respectively) showed survival rates of about 50%. Harvested cells were washed three times with a PBS solution. In the case of *C. albicans*, the yeast was embedded in melted agar at 45°C before the fixation. The fixation procedures were different depending on the pathogen. *T. vaginalis* was fixed for 2 h in 3% glutaraldehyde in 0.1 M Millonig's phosphate buffer, at pH 7.3: *C. albicans* for 3 h in 3% paraformaldehyde, 1% glutaraldehyde, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% DMSO in a 0.1 M sodium cacodylate buffer [5]; and *G. vaginalis* in 0.15% ruthenium red and 5% glutaraldehyde in a 0.1 M cacodylate buffer, pH 7.2, for 2 h at 22°C. This buffer was used for all subsequent washing, yet with the inclusion of ruthenium red (0.05% w/v) [26]. The cells were then post-fixed with 1% osmium tetroxide in a veronal buffer, dehydrated, and embedded in Araldite for electron microscopy [31].

RESULTS

Inhibition of 2,2'-Dipyridyl on *T. vaginalis*, *C. albicans*, and *G. vaginalis*

When *T. vaginalis* was exposed to 2,2'-dipyridyl at a concentration of 6.4 μg/ml, it showed a survival rate of 81.4%. Its inhibitory effect was dose-dependent and almost all the trophozoites were killed at a concentration of 410 μg/ml. The inhibitory activity of 2,2'-dipyridyl against the 6 isolates of *T. vaginalis* was similar to that of clotrimazole. 2,2'-Dipyridyl proved to be more active than povidone-iodine and less active than ornidazole and Cenacert® in terms of its trichomonacidal activity (Fig. 1, Table 1).

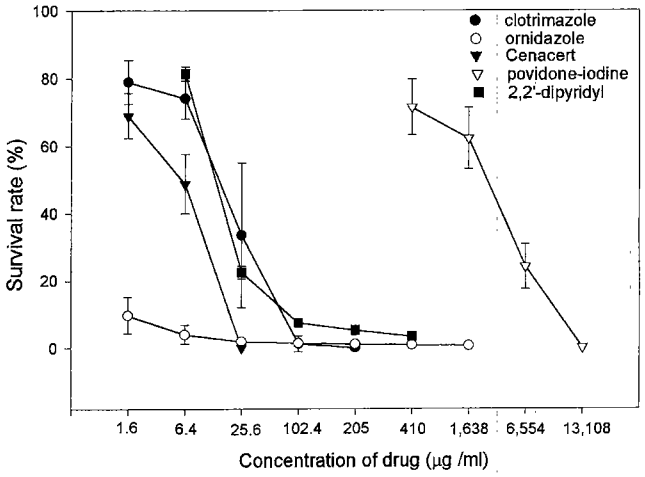

Fig. 1. Survival rate (%) of *Trichomonas vaginalis* treated with 2,2'-dipyridyl and four vaginal suppositories.

Table 1. Minimum inhibitory concentrations (μg/ml) of *Trichomonas vaginalis*, *Candida albicans*, and *Gardnerella vaginalis* treated with 2,2'-dipyridyl and four vaginal suppositories.

Organisms (No. of Isolates)	Drugs	Test ranges (μg/ml)	Minimal Inhibitory Conc. (μg/ml)		
			50%	90%	100%
T. vaginalis (6)	Clotrimazole	1.6-205		102	205
	Ornidazole	0.4-410		1.6	410
	Cenacert®	0.4-25.6	6.4	12.8	25.6
	Povidone-Iodine	410-13,107	6,554		13,107
	2,2'-Dipyridyl	6.4-410		102	410
C. albicans (2)	Clotrimazole	0.4-3,277	1.6	410[a]	
	Ornidazole	0.4-3,277	1,638		
	Cenacert®	0.4-410	6.4	25.6	410
	Povidone-Iodine	1,638-13,107	3,277		
	2,2'-Dipyridyl	25.6-410	102	410[a]	
G. vaginalis (2)	Clotrimazole	0.5-8.5			4.3
	Ornidazole	8.5-4,369			2,185
	Cenacert®	0.53-8.5			4.3
	Povidone-Iodine	546-17,476			13,107
	2,2'-Dipyridyl	12.8-410			205

[a]The inhibitory rates of *C. albicans* treated with 410 μg/ml of clotrimazole and 2,2'-dipyridyl were about 80%.

The suppressive effect of 2,2'-dipyridyl on the growth of the two vaginal isolates of *Candida albicans* was similar to that of clotrimazole. The survival rate of *C. albicans* treated with 2,2'-dipyridyl at a concentration of 410 μg/ml was 19.1%. Its inhibitory effect was stronger than ornidazole and povidone-iodine. Of the vaginal suppositories, Cenacert® had the strongest anti-candidal effect and killed all the tested *C. albicans* at a concentration of 410 μg/ml (Fig. 2, Table 1).

All the chemicals tested killed the 2 isolates of *Gardnerella vaginalis*, although the killing concentrations of each drug were varied. 2,2'-Dipyridyl completely prevented the growth of *G. vaginalis* at 205 μg/ml. Its inhibitory concentration was lower than that of ornidazole and povidone-iodine, yet higher than that of clotrimazole and Cenacert® (Table 1).

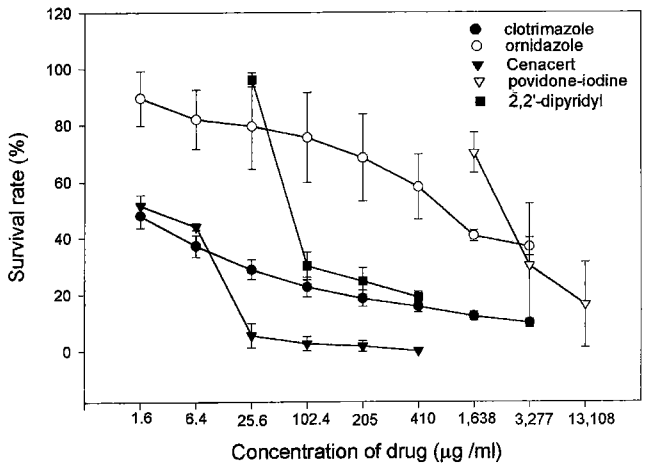

Fig. 2. Survival rate (%) of *Candida albicans* treated with 2,2'-dipyridyl and four vaginal suppositories.

Ultrastructure of *T. vaginalis*, *C. albicans*, and *G. vaginalis* Treated with 2,2'-Dipyridyl

Normal *T. vaginalis* consists of a nucleus, cytoplasmic organelles, e.g. hydrogenosomes, glycogen granules, polysomes, vacuoles, and organelles for support and movement, of axostyle, an undulating membrane, and flagella (Fig. 3A). When *T. vaginalis* was incubated with 2,2'-dipyridyl for 48 h, its cytoplasm displayed a moderate rarefaction of the glycogenic component. This was indicated by the appearance of an electron-translucent zone (ETZ). Occasionally, the cytoplasm was filled with ETZ without cytoplasmic organelles. Membrane lining interstices (I) appeared in the cytoplasm and perinuclear area of *T. vaginalis*. The number of hydrogenosomes was decreased, whereas the number of vacuoles was increased (Figs. 3B, 3C, 3D).

C. albicans normally has a thick three-layered wall and its cytoplasm is filled with densely packed ribosomes (Fig. 4A). When treated with 2,2'-dipyridyl, it exhibited changed plasma membranes and cell walls, and large vesicles and vacuoles were also observed (Figs. 4B, 4C). The plasmalemma were indented and separated from the cell wall, and finger-

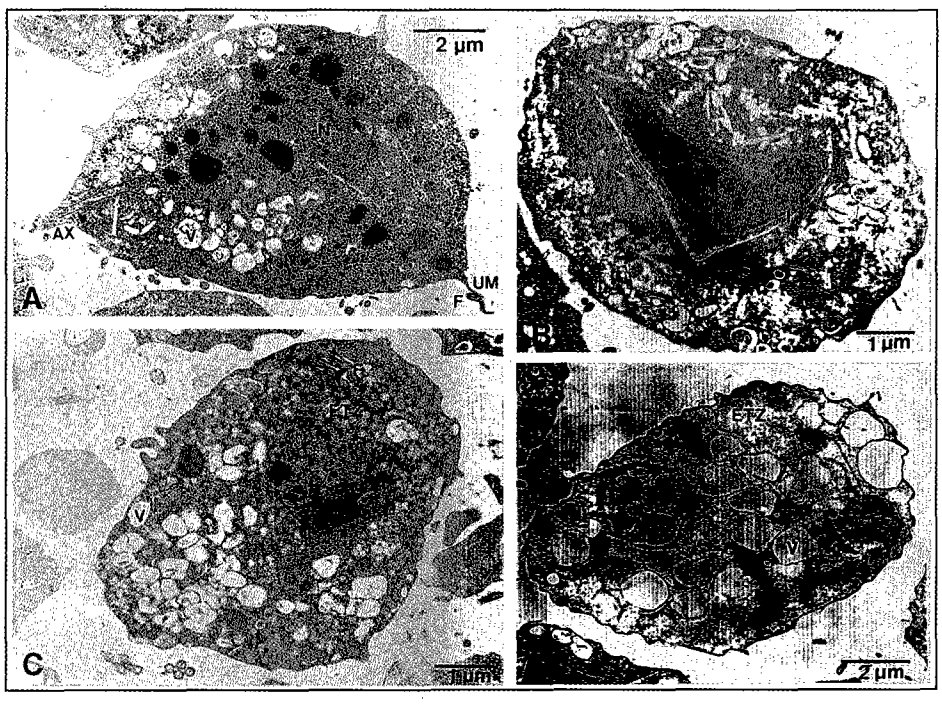

Fig. 3. Transmission electron micrographs of *Trichomonas vaginalis*.
Normal *T. vaginalis* (A), 2,2'-dipyridyl-treated trophozoites (B, C, and D) showing nucleus (N), hydrogenosomes (H), glycogen granules, vacuoles (V), axostyle (AX), undulating membrane (UM), and flagella (F). The 2,2'-dipyridyl-treated *T. vaginalis* shows interstices (I) and an electron translucent zone (ETZ) in the cytoplasm.

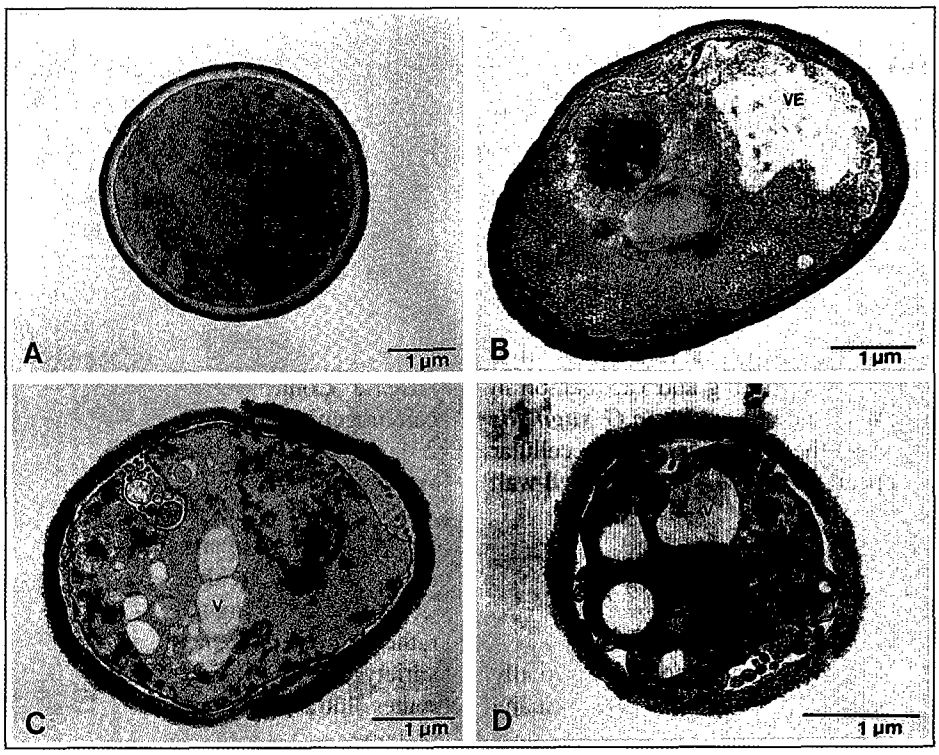

Fig. 4. Transmission electron micrographs of *Candida albicans*.
Many ribosomes (R) and a triple-layered smooth cell wall are observed in the normal *C. albicans* (A). A loosening of the cell wall, electron-lucent vesicles (VE), vacuoles (V), and polysaccharide materials (P) are seen in the 2,2'-dipyridyl-treated *C. albicans* (B, C, and D).

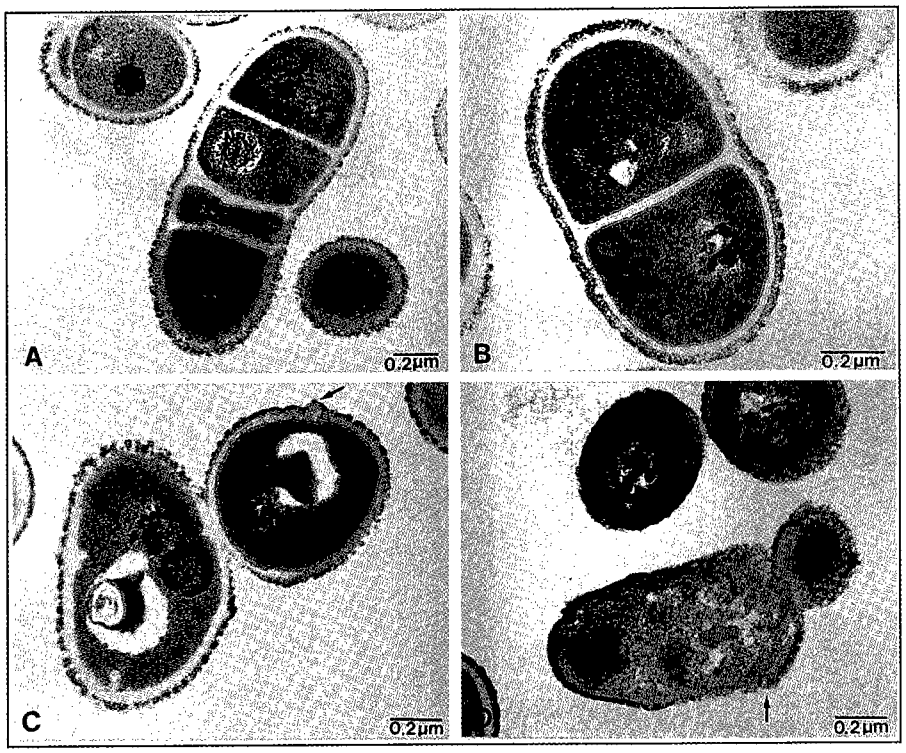

Fig. 5. Transmission electron micrographs of *Gardnerella vaginalis*.
Normal *G. vaginalis* has a nuclear region (NR), mesosome (MS), many ribosomes (R), and is surrounded by a triple-layered cytoplasmic membrane, cell wall, and fibrillar cell surface (A, B). The cell wall of the 2,2'-dipyridyl-treated *G. vaginalis* is damaged or ruptured (arrows). Vacuolation in the general area of the nucleoid material is also evident (C, D).

like protrusions projected into the space between the plasmalemma and the cell wall (Fig. 4D). Sometimes, the differentiation of the triple layer was difficult because of deterioration of the cell wall (Figs. 4C, 4D). The cytoplasm was relatively electron-lucent, and polysaccharide material (P) accumulated in the cytoplasm (Fig. 4C).

G. vaginalis has a fibrillar nucleoid, plus ribosomes dispersed throughout its cytoplasm. The triple-layered plasma membrane and outer fibrillar material are clearly visible (Figs. 5A, 5B). On the other hand, 2,2'-dipyridyl-treated *G. vaginalis* showed clumping and vacuolation in the general area of the nucleoid region. Some *G. vaginalis* exhibited complete lysis, the extrusion of the cellular contents, and the disappearance of the outer thick cell wall (Figs. 5C, 5D).

DISCUSSION

Trichomoniasis is the most common nonviral sexually transmitted diseases (STD) and associated with many perinatal complications, male and female genitourinary tract infections, and an increased incidence of HIV transmission [22]. Bacterial vaginosis is one of the most common infectious disorders affecting and associated with preterm deliveries and pelvic inflammatory disease in pregnant women and nonpregnant women [21].

The oral administration of metronidazole or other 5-nitroimidazoles is currently used for the treatment of bacterial and trichomonal vaginitis, and a topical formulation of a 100 mg clotrimazole vaginal tablet or 5 g of a 1% cream, applied intravaginally, provides effective treatment for vulvovaginal candidiasis [2]. Metronidazole is very effective and is approved by the WHO [30], yet is listed as the second drug of choice by the Centers for Disease Control in the US, because of its potential carcinogenicity in rats and mutagenicity in bacteria [15, 24]. In addition, reports on metronidazole-resistant *T. vaginalis* and *G. vaginalis* have recently increased [1, 27]. Also, metronidazole sometimes causes adverse effects: e.g, myopia, neuralgia, and allergic dermatitis [20]. For these reasons, alternative drugs for vaginitis are required.

Occasionally, vaginitis may be caused by two pathogens rather than a single pathogen. Drugs are administered in a variety of ways, e.g. oral, intravenous, and intravaginal routes. Intravaginal products may be expected to act to a certain extent on all pathogens in the vaginal environment. It has been reported that patients treated with intravaginal products, such as metronidazole vaginal gel or clindamycin vaginal cream, were more satisfied with the result,

although oral metronidazole and intravaginal products achieved nearly the same cure rates for the treatment of bacterial vaginosis [7]. Therefore, a new drug needs to be developed for the treatment of vaginitis, and ideally, this should be an intravaginal product.

In the present study, 2,2'-dipyridyl was applied to three vaginitis-causing pathogens and compared with four vaginal tablets frequently used for the treatment of vaginitis. The inhibitory activity of 2,2'-dipyridyl against *T. vaginalis*, *C. albicans*, and *G. vaginalis* was similar to that of clotrimazole, which is mainly used for *C. albicans*, and as expected, its activity in this respect was better than ornidazole and povidone-iodine. The ultrastructural changes of *T. vaginalis*, *C. albicans*, *and G. vaginalis* treated with 2,2'-dipyridyl showed a decrease in the number of cytoplasmic organelles related to the metabolism *of T. vaginalis* and *C. albicans*, and destruction of the cell wall of *C. albicans* and *G. vaginalis*. These activities of 2,2'-dipyridyl can cause damage or death to the three pathogens. In further study, the precise mechanism of antimicrobial action of 2,2'-dipyridyl should be elucidated.

In conclusion, these findings suggest that 2,2'-dipyridyl has an inhibitory effect on *C. albicans* and *G. vaginalis* as well as *T. vaginalis*, and may possibly be an antimicrobial agent against vulvovaginitis associated with *C. albicans*, *G. vaginalis*, and *T. vaginalis*.

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