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Antifungal Activities of MeOH Extracts from Three Korean Mistletoes against *Tyromyces palustris*, *Endothia nitschkei* and *Trichophyton rubrum*[1*]

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3종의 한국산 겨우살이 메탄올 추출물의 *Tyromyces palustris*, *Endothia nitschkei* 그리고 *Trichophyton rubrum*에 대한 항균활성[1*]

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ABSTRCT

The traditional Korean medical book already recorded various biological activities of the Korean mistletoes. The objective of this study was exmine antifungal activities of MeOH extract from the Korean mistletoe through column chromatography on three fungi, such as *Tyromyces palustris*, *Endothia nitschkei* and *Trichophyton rubrum*. No mistletoes had anti-fungal activity against *T. palustris*. Extracts of *V. album* var. *coloratum* showed the highest hyphal growth-inhibitory activity against *E. nitschkei*, and leaf extract of this species had higher activity than twig extract. Further fractionation of most active fraction and following antifungal assay showed that its anti-fungal activity might be caused by synergism of its components. It was suggested that *Viscum album* var. *coloratum* shows significantly antifungal activities against *E. nitschkei* and *T. rubrum*. Further examination is needed to find out more exact active compounds.

요 약

본 실험에서는 3종의 한국산 겨우살이 메탄올 추출물을 유기용매 분획과 컬럼크로마토그래피를 이용한 분획을 실시한 후, *Tyromyces palustris*, *Endothia nitschkei* 그리고 *Trichophyton rubrum*에 대한 항균활성을 평가하여 겨우살이 추출물의 항균활성 자료로 활용하고자 하였다 그 결과 모든 겨우살이 추출물은 갈색 부후균에 대한 활성은 매우 낮게 나타났다. 그러나 *Viscum album* var. *coloratum* 추출물중 부탄올 분획은 황색줄기 마름 병원균의 성장을 억제하는 것으로 나타났으며, 이 겨우살이의 EtOAC 분획은 족부백선균에 대하여 높은 포자 발아 억제효과를 나타내었다. 시험된 3종의 한국산 겨우살이류 중 *V. album* var. *coloratum*은 병원균에 대하여 항균활성 성분을 함유할 가능성이 가장 높을 것으로 제시되었다.

Key words : MeOH extract, Korean mistletoes, antifungal activities, *Viscum album* var. *coloratum*

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INTRODUCTION

The term mistletoe is a general name for a parasitic plant that occurs on branches or trunk of a woody host plant, and is a common name for several families in the Sandalwood, Order Santalales[15]. Many people in the British Isles, France and Germany have used mistletoe medicinally for convulsions, delirium, hysteria, neuralgia, and heart conditions[12]. An European mistletoe, *Viscum album* L., is widely distributing throughout the Europe, and it was first used for the treatment of epilepsy dermatitis in Europe. Later, it was believed to have biological activities such as hypotensive, vasodilator, cardiac depressive, sedative, antispasmodic and anticancer[10]. Mistletoe extracts are sold under several trade names such as Iscador, Helixor, Eurixor, Isorel, and so on, and most of them are available in Europe[6]. Mistletoe also occurs in America and Korea, but only the European species has been used for cancer treatment[10]. Mistletoe preparations are used to stimulate the immune system and to kill cancer cells. They reduce tumor size and improve the quality of life and survival of some cancer patients[5].

Leaves and twigs of the Korean mistletoes dried in shade have been used to treat pain by paralysis, lumbago, uneasiness of guickening, heart attack, hypertension, convalescence after child birth, and agalactia[7] European mainly have used purified extract of fermented mistletoe. Additionally, they have used mistletoes extracts as vasodilator, cardiac, sedative, spasmolytic, and anticancer agents since thousands years ago[10].

Ishidoya[18] reported that *Viscum coloratum* was used as a remedy for lumbago, rheumatism, and weak muscles in Korea. Monachino[26] reported that *Loranthus yadoriki* Sieb. and Zucc. had the bark being effective in treatment of hypertension, and grows in China, Japan, and Korea. Chung[8] reported that leaves and twigs of *Viscum album* were used as a tonic to treat the common cold in Korea. Ohta and Yagishita[27] studied Japanese mistletoe, *Viscum album* Linnaeus var. *coloratum* Ohwi in *Viscaceae*, which grows wild in Saghalien, Kurles, Honshu, Shikoku, Kyushu, Formosa, Korea and China. They identified flavonoid constituents in the leaves of *Viscum album* Linnaeus var. *coloratum* Ohwi (*Viscaceae*) epiphyting to *Pyrus communis* Linnaeus, and three kinds of flavonoids named flavoyadorinin-A, flavoyadorinin-B, and homo-flavoyadorinin-B from the leaves of this plant. Smolenski *et al.*[28] suggested that stems and leaves of *Viscum coloratum* Nakai var. *lutescens* Miyabe in Korea contain no alkaloids. However, Choi[7] appointed that the Korean mistletoe *Viscum album* var. *coloratum* had several alkaloids, flavonoids, triterpenes, and esters. Khwaja *et al.*[19] also reported the existence of alkaloids in the Korean mistletoe. In their previous work[20], the acetic acid extract of the Korean mistletoe showed activity against L1210 leukemia in vitro and against P388 leukemia in mice. They suggested that biologically activity of the preparation include cytotoxic proteins and glycosidic alkaloids. Khwaja *et al.*[19] ascribed that semi-purified extracts of *Viscum album*, the Korean mistletoe, exhibited anticancer activity against a variety of experimental tumors *in vitro* and *in vivo*. With similar results, Kim[23] also suggested that the alkaloids in *Pseudixus japonicus* might have anticancer activities. Many researchers have tried to find out new biological activities of alcohol extracts from mistletoes and to use them as a novel medicine sources. Dichloromethane, MeOH and water extracts from *Viscum sapense* of

the *Loranthaceae* family, were tested for antimicrobial activities against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*[2]. MeOH extract was also tested for activity against seizures in albino mice. MeOH extract of *V. capense* inhibited the growth of *S. aureus* and protected the mice against tonic seizure. The data indicate that the extract of *V. capense* has antibacterial activity against *S. aureus* and anticonvulsant activity.

Although mistletoe has been used prevalently for cancer treatment, a large number of researches have tried to find out new biological effects of mistletoes in the world. The traditional Korean medical book already recorded various biological activities of the Korean mistletoes. Therefore, new biological activities should be found in the Korean mistletoe extracts. The objective of this study was to examine antifungal activities of MeOH extract from the Korean mistletoe against three fungi.

MATERIALS AND METHODS

2.1 Materials

Mistletoe species and parts used in this experiment are represented in Table 1. This experiment did not consider variable characteristics of the mistletoe sample depending on sampling season and host species. Fresh tissue was stored in frozen state, and dried in shade to air-dried tissue. Leaves and twigs were collected separately from each species, but whole part of *P. japonicus* was used as a sample.

2.2 Extraction and fractionation

Mixture of plant sample and 80% MeOH solution was homogenized for 30 minutes[14]. MeOH extracts dissolved in small volume of 10% MeOH solution were partitioned between aqueous and organic solvent layer in a separatory funnel. Organic solvents were $CHCl_3$(C), EtOAc(E), n-BuOH(B) and water(W) layers to higher solvent polarity. Each layer was concentrated to a syrupy or sticky residue at less than 40℃. Filterate cake was re-extracted with 2% AcOH solution to get crude alkaline extracts[14]. Homogenized mixture of AcOH and residual solid was stored for 24 hours at room temperature. Supernatant was neutral- ized and concentrated to 1/5 of initial volume. Turbid basified solution was centrifugated and precipitate gave brown powder. UV spectra of brown powders showed maximum absorbent peaks at 250−303 nm in 0.1 M H_2SO_4. Further fractionation of n-BuOH partition gave three fractions of X1, X2 and X3 on chromatography by elution with water, MeOH-water(1:1) and MeOH on Amberlite XAD-7[9]. Eluate was separately charged in a refrigerator, freeze-dried and weighed. However LTB, the n-BuOH fraction of *L. yadoriki* twig, could not be eluted out from Amberlite XAD-7.

Table 1. Characteristics of the Korean mistletoe samples

Mistletoe Scientific Name*	Part	Sampling Date	Host Scientific Name	Symbol	Tissue
Pseudixus japonicus H_{AYATA}	Leaf & Twig	July '95	*Camellia japonica* Linnaeus	KJ	Fresh
Viscum album var. *coloratum* O_{HWI}	Leaf Twig	October '95	*Quercus acutissima* Carruthers	VL VT	Fresh Air-dried
Loranthus yadoriki $S_{IEB.}$	Leaf Twig	October '94	*Camellia japonica* Linnaeus	LL LT	Air-dried Air-dried

* named according to Lee[25].

2.3 Antifungal activity

Three kinds of fungi screened were *Trichophyton rubrum* ATCC 44766, an human pathogenic fungus, which causes *Tinea* spp. diseases in foot or hand, *Endothia nitschkei* of plant pathogenic fungus, and *Tyromyces palustris*, a wood decaying, brown rot fungus. Antifungal activities were measured against *E. nitschkei* and *T. palustris* by agar dilution method[11]. All of the strains were maintained on PDA(Bacto® Potato Dextrose Agar Medium) slants at 5℃. Appropriate amounts of test substances were mixed with PDA at 40~50 ℃, and agar plates were prepared in Petri dishes containing 20 ㎖ media. Then 5mm diameter agar disks of the pre-incubated fungi were inoculated as on the opposite edges of the plate with (sample) or without (control) samples. Inoculated dishes were incubated at 25±1℃. The radial growths of the fungal colonies were measured daily for 1-8 days after innoculation. The antifungal activity (AFA) was determined by evaluating the linear regression slope from plotting Gt with Gc, where Gc and Gt are radii of the hyphal or mycelial growth on the control media and on the media containing the test substances, respectively. The Gc and Gt were calculated from the average values of three replicates. To investigate the antifungal activity against *T. rubrum*, an automated turbidometer, Bioscreen (Labsystems Oy), was used according to the method of Laine *et al.*[24], because *T. rubrum* has spore reproduction character. Sixty micro liters of the sample solution and 30 ㎕ of the indicator organism in saline were dispensed to microtitre plate wells (96 wells) with 210㎕ of the growth medium, PDA. In the control wells the sample solutions were replaced by an equal volume of sample-dissolving solvent. The indicator was prepared by collecting spores with sterile swabs immersed in saline and adjusting 10^{-4} spores mL^{-1} with a light microscope. The microtitre plates prepared were incubated at 25℃ for 60 hours with gentle shaking. The turbidity was measured in wide range of wavelengths 420-580㎚. The concentrations of sample solutions were 50, 500 and 5,000 ppm.

2.4 Apparatus and chemical analysis

Column packing materials for separating systems were Sephadex® LH-20 (Lot No. 227621, Pharmacia Biotech, Sweden) and Amberlite XAD-7 (Nonionic polymeric adsorbent, Lot 45H1153, Sigma Co., USA) Instruments are follows : homogenizer (ULTRA-TURRAX T50, JANKE & KUNKEL, IKA®-LABORTECHNIK, Germany), rotary evaporator (N-N Series, EYELA, Japan), centrifuge (UNION 5KR, HANIL, Korea), freeze dryer (BONDIRO FD8512, ILSHIN, Korea), incubator (CAN & AM Deluxe, Great Britain), UV-VIS spectrophotometer (UV-1601PC, Shidmazu, Japan) and automated Bioreactor (Bioscreen; Labsystems Oy).

RESULTS AND DISCUSSION

3.1 Effect of mistletoe extracts on the growth of *Tyromyces palustris*

T. palustris is a brown-rot fungus of genus *Tyromyces*, beloning to family Polyporaceae, subdivision *Aphyllophorales*, and division Basidiomycotina[31]. This fungus reduces degree of polymerization of hemicelluose and cellulose in the early period of degradation of wood and cause following decrease of wood strength[29-30]. Therefore the inhibition of this fungus would give maintenance of wood strength, especially bending strength and important wood pre-

servation[21-22]. Diameter of hyphal growth of *T. palustris* in the medium was measured daily for 5 days, after inoculated Petri dishes were in incubator at 25℃ for two days. Concentration of samples applied to 20 ㎖ PDA medium a Petri dish was 500 ppm.

Numerated diameter values, a distance from inoculation center to hyphal growth edge of *T. palustris* grown in testing medium, were plotted with those of control medium. There was yellow pigmentation around inoculation center in the medium. Only VTBX2 showed significant AFA (antifungal activity) against *T. palustris* (Fig. 1). There was not other sample being significantly active in inhibition, and rather LTE promoted the growth of *T. palustris* (Fig. 2). Judging from the result that t-test and slope value of each curve, VTBX2 with t-value of 5.05 (95 % confidence level) and slope constant of 0.79±0.03 (R^2=0.997), in mistletoe samples, was the most active against *T. palustris*. In addition, AFA of VTBX2 was 21±3% at 500 ppm dose when analogized from the slope in Fig. 1. However MeOH extracts from the Korean mistletoes showed no apparent antifungal activity against *T. palustris* in this experiment.

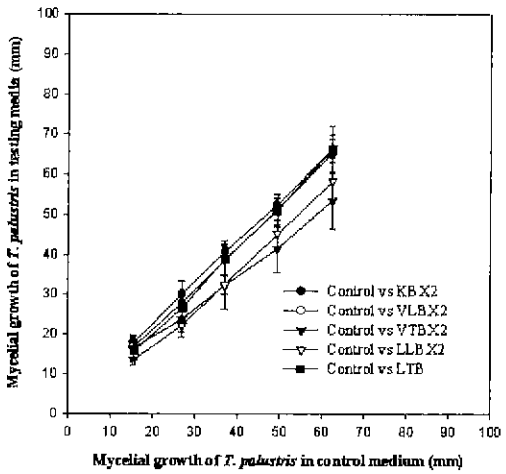

Fig. 1. Effect of *n*-butanol X2 fractions of MeOH extracts from mistletoe on mycelial growth of *Tyromyces palustris*.

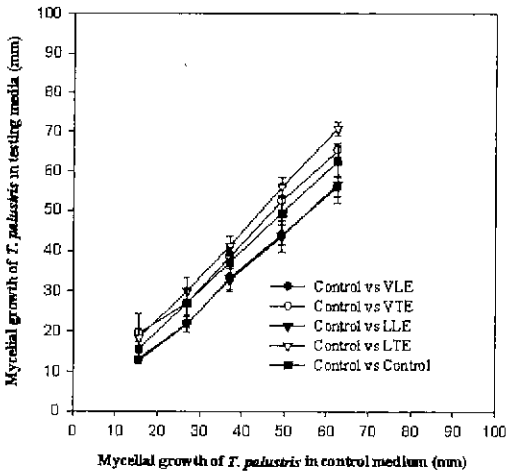

Fig. 2. Effect of ethyl acetate partitions of MeOH extracts from mistletoe on mycelial growth of *Tyromyces palustris*.

3.2 Effect of mistletoe extracts on the growth of *Endothia nitschkei*

E. nitschkei is a plant pathogen fungus belonging to genus *Endothia*, phylum Ascomycota, which causes withering of small branch or even main stem with 3-4cm diameter of *Quercus* species and thus inhibits the growth of living trees by causing wood defects such as cave-in where this occurs[1]. The inhibition of the fungus would give normal physiological growth of trees. The growth-inhibitory response to *E. nitschkei* was examined by the same method in the case of *T. palustris*. Diameter of hyphal growth in testing medium was measured daily after incubation for two days at 25 ℃.

VLE was most active in EtOAc partitions. VTE and LLE also had significant activities (Fig. 3). All n-BuOH X1 fractions had no significant activities and rather showed promotion activity, and did the other n-BuOH X2 fractions except VLBX2 as shown in Fig. 4. The most prominently ac-

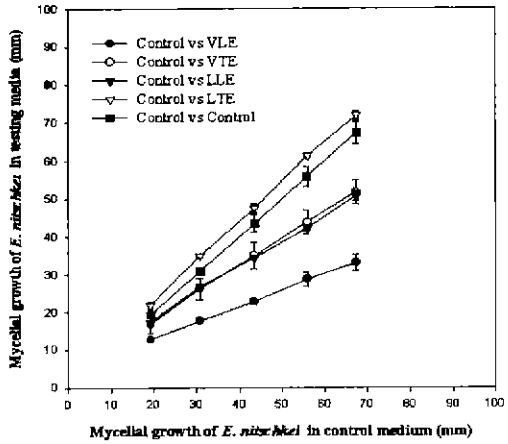

Fig. 3. Effect of ethyl acetate partitions of mistletoe MeOH extracts on mycelial growth of *Endothia nitschkei*.

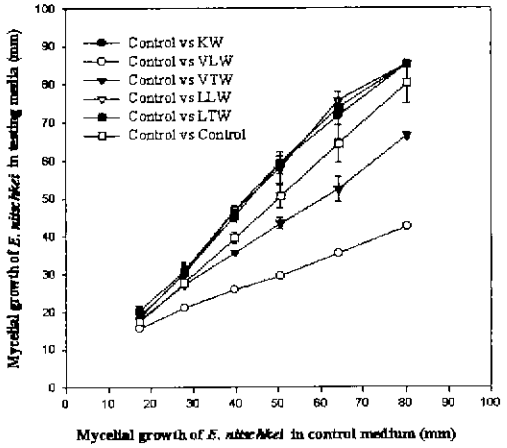

Fig. 5. Effect of water partitions of mistletoe MeOH extracts on mycelial growth of *Endothia nitschkei*.

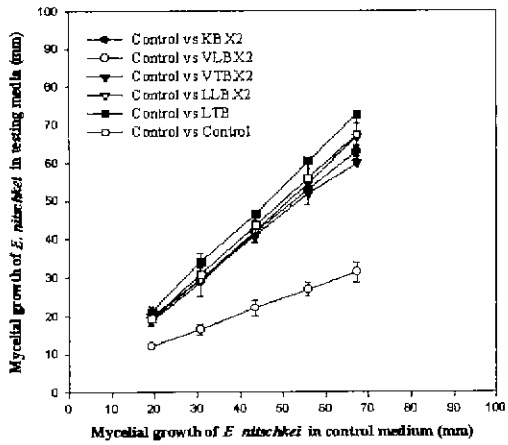

Fig. 4. Effect of n-butanol X2 fractions of mistletoe MeOH extracts on mycelial growth of *Endothia nitschkei*.

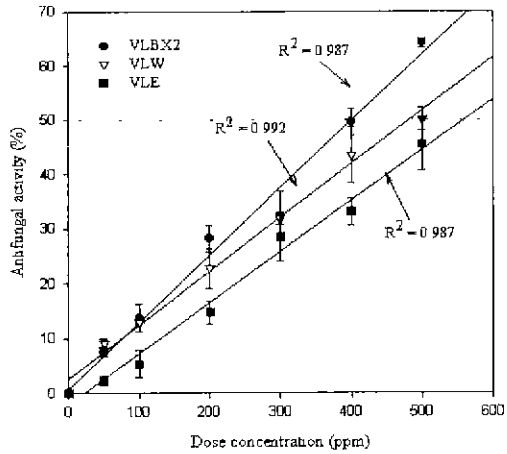

Fig. 6. Antifungal activities of significant partitions and fraction against *Endothia nitschkei* depending upon dose concentration.

Table 2. Korean mistletoe extracts which are significantly active against *Endothia nitschkei*

Sample	Regression coefficient*	R square	t-value**	P-value***	AFA(%)****
KBX3	0.73±0.01	0.999	3.48	0.03	+
VLE	0.42±0.01	0.998	4.16	0.01	+++
VLBX2	0.40±0.00	0.999	4.22	0.01	+++
VLBX3	0.71±0.05	0.993	3.35	0.03	+
VLW	0.42±0.01	0.998	2.54	0.05	+++
VTE	0.72±0.01	0.999	3.11	0.04	+
VTW	0.75±0.02	0.997	3.31	0.02	+
LLE	0.68±0.02	0.998	3.30	0.03	++

* the coefficient of linear regression in plotting growth diameter of samples with that of control
** the t-value at the P=0.05 level
*** the probability that estimation is incorrect in stating that the two means are different.
**** the value calculated with equation, AFA = (1-a)×100, +++ : 50 - 100 %, ++ : 30 - 50%, + : 0 - 30%

tive fraction was VLBX2, and VLBX3 and VTBX3 showed significant activities although they were lower than that of VLBX2. Two partitions, VTW and VLW, had significant inhibition activity, and the other water partitions had no inhibition activity but rather promotion activity or no change (Fig. 5). Generally extracts of *V. album* var. *coloratum* showed higher inhibition activity against *E. nitschkei* than those of other species.

Active partitions or fractions against *E. nitschkei* showed their linear inhibition to incubation time significantly at the 5% confidence level (Table 2). Simple statistic values about the AFA of samples estimated to act positively, which means growth inhibition against *E. nitschkei*. AFAs showed (+) at VTW, KBX3, VLBX3, and VTE, (++) at LLE, and (+++) at VLW, VLBX2, and VLE. The order of AFA was in VLBX2 > VLW ≒ VLE >> LLE > VLBX3 > VTE > KBX3 > VTW.

Among the Korean mistletoes, *V. album* var. *coloratum* had the most highest hyphal growth inhibitory activity to *E. nitschkei*, and leaf part 이d higher activity than twig part. All fractions showing (+++) AFA occurred in leaf extracts of *V. album* var. *coloratum*, and especially VLBX2 showed 60% AFA at 500 ppm dose. In Fig. 6 which shows dose dependency on AFA, all fractions tested were statistically significant in linear regression and dose-dependent. MIC50 of tested samples was around 400, 500, and 550 ppm for VLBX2, VLW and VLE, respectively.

VLBX2 fraction was further fractionated into VLBX2A, VLBX2B, VLBX2C and VLBX2D on Sephadex LH-20 by elution with water, MeOH-water(50:50), MeOH-water(70: 30) and MeOH, respectively. There was no promotion in AFA by fractionating VLBX2, and only VLBX2C showed steady

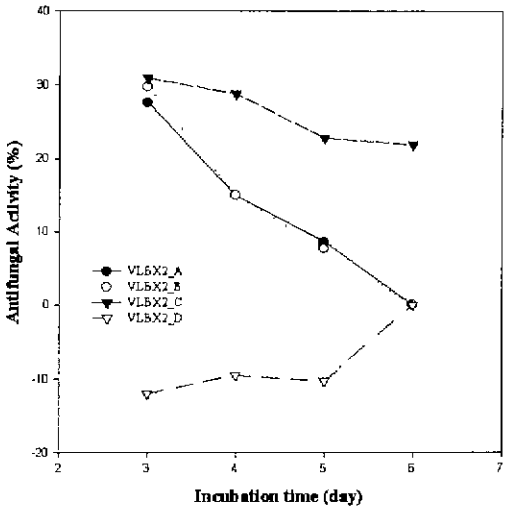

Fig. 7. Antifungal activities of VLBX2 subfractions from Sephadex LH-20 column chromatography against the mycelial growth of *Endothia nitschkei*.

(+) AFA against *E. nitschkei* according to incubation period and its AFA was lower than that of VLBX2 (Fig. 7). Therefore, AFA of VLBX2 against *E. nitschkei* is thoght to be caused by synergism of its chemical components.

Hull and Leonard[17] suggested that if host tree has allelopathic compounds against some pathogen, parasitic plant will have same compounds and it is easier to trap antipathogenic compounds which can be produced by host plants, will be easily collected continuously by gathering parasitic plant. Considering that the host was *Quercus* species (Table 1), there is also possibility to apply this suggestion to collect antipathogenic compounds against *E. nitschkei* from *V. album* var. *coloratum*.

3.3 Effect of mistletoe extracts on the growth of *Trichophyton rubrum*

Dermatophytes are a unique group of fungi that can invade the hair, skin, and nails of a living host. The etiologic agents of the dermatophytoses are classified in three anamorphic (asexual or imperfect)

genera, *Epidermophyton*, *Microsporum*, and *Trichophyton*[4]. Species capable of reproducing sexually belong in the teleomorphic genus, *Arthroderma*, of the Ascomycota. On the basis of primary habitat association, they may be grouped as geophilic, zoophilic, and anthropophilic. Adaptation to growth on humans by most geophilic species resulted in diminished loss of sporulation, sexuality, and other soil-associated characteristics[32]. The dermatophytes have the ability to invade keratinized tissue (skin, hair, and nails) but are usually restricted to the nonliving cornified layer of the epidermis because of their inability to penetrate viable tissue of an immunocompetent host. Acid proteinases, elastase, keratinases, and other proteinases reportedly act as virulence factors[3]. Especially *T. rubrum* is major pathogenous fungus of *Tinea barbae*, *Tinea capitis*, *Tinea corporis*, *Tinea cruris*, *Tinea pedis*, *Tinea unguium* and *T. manus*, and it causes dry squamous type dermatomycosis which is diffuse scaled, relative lack of inflammative, and extremely chronic[13,32].

The control curve showed that spore germination continued to 22 hours, mycelial growth from 22 to 35 hours, and the stagnation period began at around 35 hours.

VLE exhibited prominent inhibition against both spore germination and mycelial growth at 5,000 ppm dose (Fig. 8). There was no prominent activities in X2 fractions. All water partitions and most of X1 fractions showed promotion activities or no inhibitory activity. However LLBX1 showed significant inhibitory activity against *T. rubrum*, where it lengthens the spore germination period and decreases the mycelial growth slightly (Fig. 9). All alkaline fractions, except KA, had conspicuous inhibitory activities against the spore germination of *T. rubrum*, but they did not against mycelial growth (Fig. 10). Only LTA showed transitional growth

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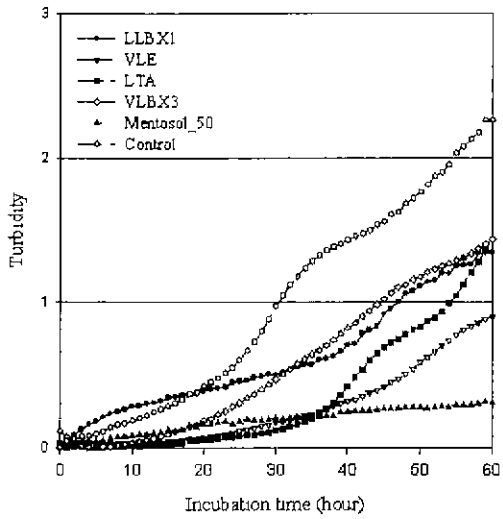

Fig. 8. Effects of significant partitions and fractions of the Korean mistletoe extracts on the growth of *Trichophyton rubrum*.

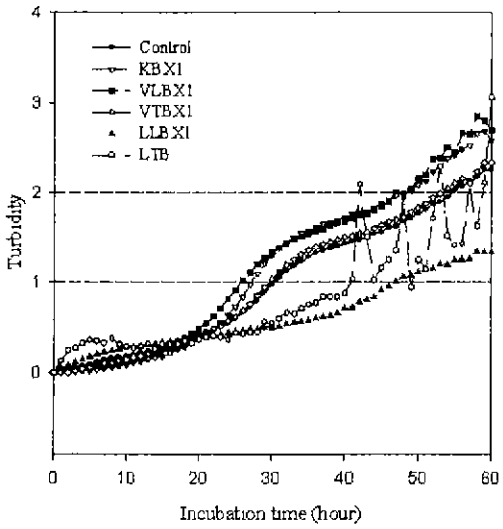

Fig. 9. Effect of n-butanol X1 fractions of mistletoe MeOH extracts on the growth of *Trichophyton rubrum*.

ration period within testing incubatin period, which means the inhibitory activity of LTA is dependent on incubation time. Conclusively LTA and VLE showed significant inhibition to spore germination during incubation period of 35 hours at 5,000 ppm. One can observe

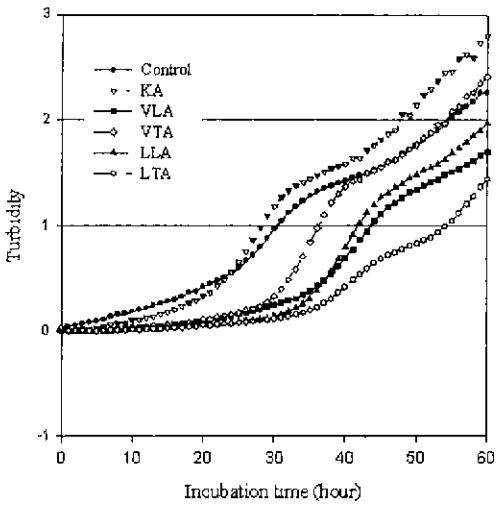

Fig. 10. Effect of 2% acetic acid partitions of mistletoe extracts on the growth of *Trichophyton rubrum*.

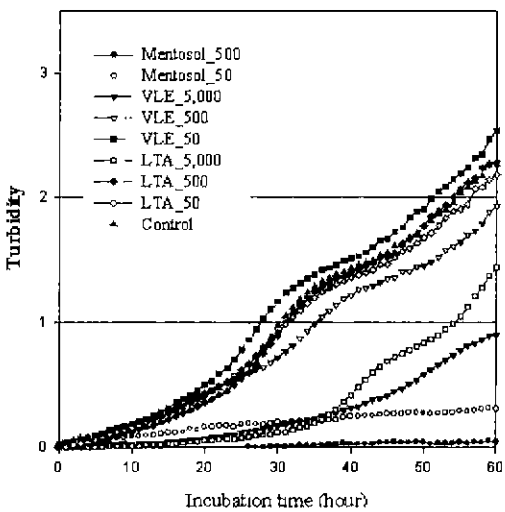

Fig. 11. Dependence of antifungal activities of LTA and VLE on dose concentration and their comparison with those of a reference compound, Mentosol™, against *Trichophyton rubrum*.

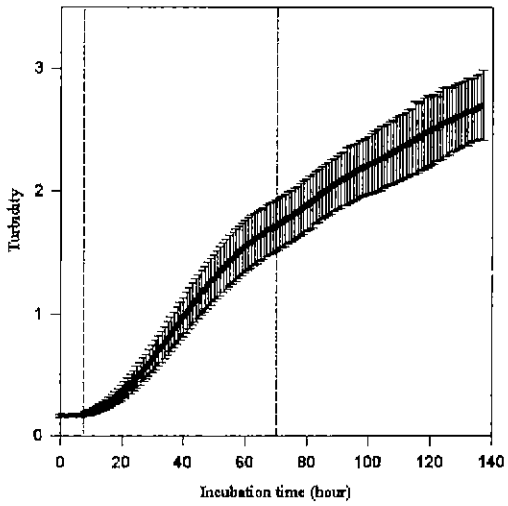

Fig. 12. Dependence of the growth of *Trichophyton rubrum* on incubation time in PDB medium.

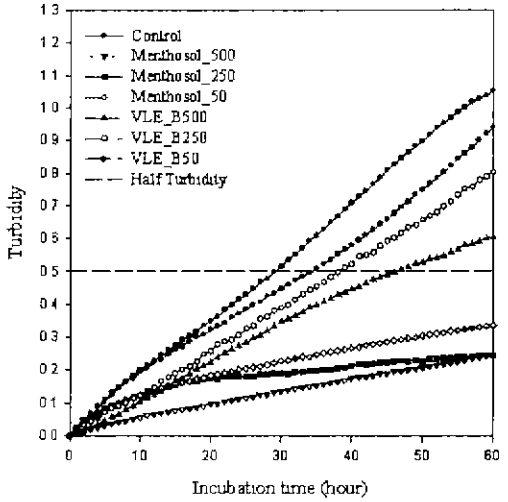

Fig. 13. Comparative effects of VLE_B fraction of mistletoe MeOH extract on the growth of *Trichophyton rubrum* at 500 and 250 ppm doses.

dose concentration (Fig. 11). Two samples applied with 5,000 ppm dose concentration showed more active against spore germination than Mentosol™ (based on chlorotrimazol concentration; solution containing 1.0g chlorotrimazol, 0.1g dlcamphor, 3.0g salicylic acid in 100 ㎖ EtOH; Uhan Co. Korea) at 50 ppm dose until about 35 hours. Inhibitory activities of VLE against both germination and mycelial growth were higher than those of LTA. Although VLE at 5,000 ppm dose had lower activity than Mentosol™ of even 500 ppm dose, VLE had comparable anti-spore germination activity with reference compound contain salicylic acid.

Therefore the VLE was confirmed as antifungal fraction that acts as anti-spore germination active agent.

Each fractions of VLE was examined at dose concentrations, 500, 250, 50, 10 and 2 ppm, and concentration was indicated at the position following the last word of sample name. The results were re-plotted to incubation time and reaction solution turbidity. Therefore growth-inhibitory activity of VLE_B was further investigated. In the lower inhibition activity than VLE_C and VLE_D apparently showed dose-dependent, dose levels of more 50 ppm were applied because 10 ppm and 2 ppm had no significant inhibition in Fig. 13.

VLE_B had growth-inhibitory activities of 34.6 %, 24.1% and 12.6 % at 500, 250 and 50 ppm dose concentrations, respectively(Fig. 13).

CONCLUSION

Generally, no mistletoes had anti-fungal activity against *T. palustris*. Extracts of *V. album* var. *coloratum* showed the highest hyphal growth-inhibitory activity against *E. nitschkei*, and leaf part had higher activity than twig part. Further fractionation of most active fraction and following antifungal assay showed that its anti-fungal activity might be caused by synergism of its components. VLE showed comparable anti-spore germination activity against *Trichophyton rubrum*, equivalent to a reference compound. As a result, it was suggested that *Viscum album* var. *coloratum* species of the Korean mistletoes has significantly antifungal activities against *E. nitschkei* and *T. rubrum*.

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