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Effect of Korean arbor vitae (*Thuja koraiensis*) extract on antimicrobial and antiviral activity

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The present study was carried out to develop a new natural product reagent which has antimicrobial and antiviral effect, so we assayed the extract from Korean Arbor vitae (*Thuja koraiensis*) on antimicrobial and antiviral *in vitro*. The antimicrobial activity was assayed at two gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*); two gram-negative bacteria (*Escherichia coli, Salmonella typhimurium*) and the results were measured by the paper disc diffusion assay and minimum inhibitory concentration (MIC). The antiviral activity of *T. koraiensis* extract was assayed at the Bovine viral diarrhoea (BVD) virus which is a RNA virus replication in Madin-Darby bovine kidney (MDBK) cells and the results were measured by maximum non cytotoxic concentration (MNCC) and maximum non-toxic dose (MNTD). The result of paper disk diffusion assay showed that extract had the high antimicrobial effect at *S. aureus* strain. The MNCC of extract on MDBK cells was 0.031% and the MNTD of extract was 0.0195% on BVD virus. These results suggested that *T. koraiensis* extract had antimicrobial and antiviral effect, especially at low concentrations which had a strong antiviral effect at BVD virus. The *T. koraiensis* extract could also be useful as disinfectant for bacterial. The study of *T. koraiensis* function perhaps would be the first and more research is needed in the future.

Key words: *Thuja koraiensis* extract, antimicrobial activity, minimum inhibitory concentration, maximum non cytotoxic concentration, maximum non-toxic dose, antiviral activity.

INTRODUCTION

Arbor vitae is the common name for any of the coniferous evergreen trees or shrubs comprising the genus *Thuja* in the cypress family (William and Jackson, 1967). The foliage of *Thuja* is rich in Vitamin C, and was used by Native Americans and early European explorers as a cure for scurvy. The leaves have been used as a treatment for rheumatism. The oil of arbor vitae is often quoted as a herbal remedy topically used to aid in the treatment of human papillomavirus (HPV), genital or common warts (Aljos, 2005). Korean Arbor vitae (*Thuja koraiensis*) is a species of *Thuja*, an evergreen shrub or small tree growing up to 3 to 10 m tall. The foliage forms flat sprays with scale-like leaves of about 2 to 4 mm long (up to 15 mm long on strong-growing shoots), matt dark green above and with broad and vivid white stomata wax bands below. The cones are oval, yellow-green ripening

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to red-brown, 7 to 11 mm long and 4 to 5 mm broad (opening to 6 to 9 mm broad), with 8 to 12 overlapping scales ("Flora of China" editorial board, 1978).

Thuja is very resistant to most pests (Aljos, 2005). A lot of research has shown that Arbor vitae exhibits anti-inflammatory (Kim et al., 2013), antidiuretic (Dubey and Batra, 2008), antimicrobial (Jain and Garg, 1997), fungi toxic (Guleria et al., 2008) and antiviral (Loizzo et al., 2008) activities. However, there are no studies for *T. koraiensis*. In this study, we investigate the antimicrobial and antiviral activities of the extract of *T. koraiensis* in order to develop a new natural product reagent. For the antimicrobial activity, two strains each of Gram-positive (*Staphylococcus aureus, Bacillus subtilis*) and Gram-negative bacteria (*Salmonella typhimurium, Escherichia coli*) were selected and the experiment conducted. For antiviral activity, the Bovine viral diarrhoea (BVD) virus which resulted in large losses of domestic animals was selected for the experiment. BVD virus is an enveloped positive-strand RNA virus that holds generic status in the family Flaviviridae (Collett et al., 1988). BVD virus causes major economic losses in domestic ruminants worldwide (Houe, 1999). It can lead to a variety of clinical outcomes that range from subclinical infections to the more severe presentations including abortion, infertility and the fatal mucosal disease (Baker, 1995).

MATERIALS AND METHODS

Plant

T. koraiensis was purchased from the Natural Space and dissolved in 100% dimethyl sulfoxide (DMSO).

Solvent extraction

T. koraiensis was extracted by steam distillation method (Cassel et al., 2009).

Micro-organisms

The bacteria used in this study were two kinds of Gram-positive bacteria *B. subtilis* (ATCC11774), *S. aureus* (KCCM1335) and two kinds of Gram-negative bacteria *E. coli* (KCCM12181), *S. typhimurium* (KCCM11862). The strains were purchased at the Korean Culture Center of Microorganisms (KCCM). The medium used was Muller Hinton Broth (Difco, USA).

Paper disc diffusion assay

Antimicrobial activity of *T. koraiensis* extract was determined by the disc diffusion method (Bauer et al., 1966). The bacteria were cultured in Mueller–Hinton broth. The concentrations of the cultures were 10^6 colony forming units (CFU) per ml. The extract to be tested was dissolved in 100% DMSO. For the purpose of screening, 10 ul of each extract solution was loaded on paper disc (Whatman no. 6 mm). The disc was placed on the surface of the Mueller–Hinton agar plate previously inoculated with bacteria. The agar plates were then inverted and incubated for 24 h at 37°C. The antimicrobial activity was recorded by measuring the zone of inhibition (in mm) around each disc and the diameter zone of inhibition was measured using a transparent plastic ruler. Each test was carried out in triplicate. Amoxicillin (10 ug/ml) and collistin sulfate (10 ug/ml) were used as a positive control and 100% DMSO as a negative control in the assays.

Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration (MIC) assay was determined by following the micro broth dilution method (Shin and Kim, 2005) performed with the 96-well plate. A range of two-fold dilution (50 to 0.3%) of the *T. koraiensis* extract in Muller Hinton Broth (MHB) containing 100% DMSO was prepared. The extract suspensions (100 ul) were added to each well. The test bacterial strains were adjusted with MHB to match the 10^6 colony forming units (CFU) per ml. Subsequently, 100 ul volume of the strain medium was added to each well and the plate were incubated at 37°C for 24 h under micro plate spectrophotometer (Biotech, Eon, USA) using the absorbance of 600 nm measured once every 4 h. MHB was used as a negative control and each strain medium were used as a positive control. The MIC was defined as the lowest concentration of a test sample that completely inhibited visible bacterial growth.

Cell and virus cultures

The Madin-Darby bovine kidney (MDBK) cell and Bovine viral diarrhoea (BVD) virus were purchased at the American Type Culture Collection (ATCC). MDBK cells were grown in 75T-Flask by using minimum essential medium (MEMA, Thermo, USA) supplemented with 5% (v/v) fetal bovine serum (FBS, GIBCO/ BRL, USA) and 1% (v/v) anti-anti (Antibiotic-Anti mycotic, GIBCO/ USA). The BVD virus was propagated using MDBK cells and cytopathic effect (CPE) was observed. Repeated freezing and thawing was done twice and then centrifuged for 5 min at 2000 rpm. The aqueous layer was removed and put into cryogenic vials, stored at -70°C until use. Titer of virus infection was done using the Spearman-Karrer method (Jung, 2001), 50% tissue culture infective dose ($TCID_{50}$) was calculated and the result of 1.5×10^6/ml was obtained.

Cell cytotoxicity test

The cytotoxicity assay for dimethyl sulfoxide (DMSO) was used to obtain the cell attachment test. MDBK cells were seeded into 96-well plate at a density of 3.5×10^3 cell/ml. DMSO was diluted with MDBK cell growth medium by two fold serial dilutions in 96-well plate and incubated for 72 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation, cytopathic effect (CPE) was observed. The plate was examined under binocular microscope and the dead cells in each well were counted. Monolayers of MDBK cells incubated only with growth medium were used as a control. The result for DMSO concentration is 0.7% than that of non-cytotoxic at MDBK cells. For extract cytotoxicity assay, 1% *T. koraiensis* extract was diluted in 0.7% DMSO and in the same way as DMSO cytotoxicity assay to be experimented. CPE was observed and the maximum non-cytotoxic concentration of extract was determined.

Antiviral activity test

The antiviral activity of *T. koraiensis* extract was also assayed in 96-well plates, since the initial dilution of the extract was done in 0.7% DMSO and the MNCC of extract in MDBK cells were used. The

Table 1. Antimicrobial activity of the Korean Arbor vitae (Thuja koraiensis) extract on several micro-organisms.

Strain		Zone of inhibitory (mm)	
		*Control	Extract 100%
Gram(+)	*Bacillus subtilis* (ATCC11774)	10	13
	Staphylococcus aureus (KCCM1335)	30	17
Gram(-)	*Escherichia coli* (KCCM12181)	15	15
	Salmonella typhimurium (KCCM11862)	15	12

*Control: Gram (+), Amoxicillin; Gram (-), Collistin Sulfate.

Table 2. Minimum inhibitory concentration (MIC) of the Korean Arbor vitae (*Thuja koraiensis*) extracts several micro-organisms.

	Microorganism	MIC (mg/ml) extract (%)
Gram (+)	*Staphylococcus aureus* (KCCM1335)	12.5
	Bacillus subtilis (ATCC11774)	0.6
Gram (-)	*Salmonella typhimurium* (KCCM11862)	12.5
	Escherichia coli (KCCM12181)	12.5

virus were serially diluted in MEMA containing 1% (v/v) anti-anti and the virus titer was determined at 104 TCID50/ml. 50 ul of BVD virus growth medium was placed in each well and 50 ul of extract atserial dilutions corresponding to MNCC were added. BVD virus suspension (10^4 $TCID_{50}$/ml) of 50 ul was added in each well, incubated at 37°C and 5% CO_2 adsorbed in 90 min. MDBK cells (3.5×10^3 cells/well) of 100 ul were added in each well and incubated at 37°C in a humidified 5% CO_2 atmosphere, and viral plaques were counted after 72 h. The BVD virus growth medium containing the MDBK cells was used as a positive control. The BVD virus growth medium contained the MDBK cells and virus was used as a negative control.

RESULTS AND DISCUSSION

The antimicrobial activity of the *T. koraiensis* extract by paper disk diffusion assay against the test organisms are shown in Table 1. The extract inhibited Gram-positive bacteria *S. aureus* and *B. subtilis*, with diameters of inhibition zone 17 and 13 mm, Gram-negative bacteria *E. coli* and *S. typhimurium*, with diameters of inhibition zone 15 and 12 mm. Particularly, the antimicrobial effect of extract at the *B. subtilis* strain was higher than in the control. Table 2 shows the MIC values of the *T. koraiensis* extract against the test organisms. The MIC values ranged from 0.6 to 12.5% of the extract. The extract exhibited the highest activity against *B. subtilis*, with MIC of 0.6%.

The results showed that *T. koraiensis* extract has antimicrobial activity both at gram-positive and gram-negative bacteria. At other studies, the growth of gram-positive bacteria was inhibited very effectively than gram-negative bacteria in a study using quercetin and naringenin which are single compounds in a phenolic compound (Rauha et al., 2000), while it had similar analysis results for the *T. koraiensis* extract single compound. But accordingly, the results of the *T. koraiensis* extract had a good antimicrobial effect at gram-positive bacteria in this study which may contain phenolic compound in the *T. koraiensis* extract. Further study was done analysing *T. koraiensis* antimicrobial effective single compound using high performance liquid chromatography (HPLC) profiling.

The antiviral activity of 1% *T. koraiensis* extract against BVD virus is shown in Table 3. The result showed that the MNCC of *T. koraiensis* extract on MDBK cells was 0.031% and the MNTD of extract was 0.0195% on BVD virus replication in MDBK cells. Especially, the extract at low concentrations had a strong antiviral effect at BVD virus; BVD virus is a RNA virus. *T. koraiensis* extract had antiviral effect at RNA virus. *T. koraiensis* extract, which anti-virus affects, will appear at DNA virus and needs to be more experimental in the future.

In summary, *T. koraiensis* extract had good anti-bacterial and antiviral effect. And we had first identified that Korean Arbor vitae (Thujakoraiensis)extract has both effect about antibacterial and antiviral. We suggest that the Korean Arbor vitae (Thuja koraiensis) extract will be useful as disinfectant for gram positive bacterial. Future

Table 3. Antiviral activity of Korean Arbor vitae (*Thuja koraiensis*) extracts 1% against BVD virus.

Solvent	*MNCC (%)	†MNTD (%)
Korean Arbor vitae (*Thuja koraiensis*) extract	0.031	0.0195
Control (P.C)	nag	nag
Control (N.C)	-	-

*MNCC: maximum non cytotoxic concentration, †MNTD: maximum non-toxic dose, PC: Positive control, N.C: Negative control, 1% extract dissolved 0.7% DMSO

study, Korean Arbor vitae idendtifie effective single compound about antibacterial and antiviral, also another single compound will confirm pharmacology function.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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