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Tinospora crispa extract inhibits MMP-13 and migration of head and neck squamous cell carcinoma cell lines

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ABSTRACT

Objective: To investigate the effect of *Tinospora crispa* (*T. crispa*) extract on matrix metalloproteinase-13 (MMP-13) expression and cell migration.
Methods: The cytotoxicity of *T. crispa* extract was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on head and neck squamous cell carcinoma (HNSCC) cell lines. The effect on expression of MMP-13 was analysed by RT-PCR and ELISA. The migration was assessed by wound healing assay.
Results: MMP-13 mRNA was highly expressed in the metastatic human HNSCC cell lines, HN22 and HSC-3. *T. crispa* extract at a concentration of 100.0 μg/mL caused about 50% reduction of cell survival. *T. crispa* extract at a non-toxic concentration of 12.5, 25.0 and 50.0 μg/mL significantly suppressed MMP-13 mRNA expression and secreted MMP-13 in both HN22 and HSC-3. The expression of tissue inhibitors of metalloproteinase-2 (TIMP-2) by HSC-3 cells was attenuated by 25.0 and 50.0 μg/mL of *T. crispa* extract. Addition of the extract to cells in a wound healing assay showed inhibition of cell migration by HN22 cells.
Conclusions: These data suggest that *T. crispa* could be considered as a potential therapeutic drug to prevent metastasis of HNSCC.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies, resulting in morbidity and mortality worldwide [1]. Local invasion, lymphatic dissemination and subsequent distant metastasis contribute to the poor prognosis and decrease in survival rate [2]. Tumor metastasis is a complex and multistage process. Degradation of the extracellular matrix, *e.g.*, the basement membrane, by proteinases such as matrix metalloproteinases (MMPs) is a key event of tumor invasion and metastasis.

MMPs are a family of zinc-dependent endopeptidases, which contain more than 25 members. MMPs are grouped as collagenases, gelatinases, stromelysins and membrane-type MMPs based on their structure and substrate specificity. MMP-13 or collagenase 3 plays a key role in the modulation of extracellular matrix degradation and cell–matrix interactions associated with inflammatory processes and metastasis [3,4]. In addition to type I collagen as its main substrate, MMP-13 can cleave collagen types II, III, IV, IX, X and XIV, laminin and fibronectin [5]. MMP expression and activity are regulated at different levels such as at the transcriptional level, mRNA stabilization and at the translational level. In addition, the activity of MMPs is modulated by tissue inhibitors of metalloproteinases (TIMPs). Thus far, there are four different TIMPs (TIMP-1, -2, -3, -4), whereas TIMP-1 and TIMP-2 are commonly identified in HNSCC [6].

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There is evidence of an increase of MMP-13 expression by many types of human cancer cells including breast, larynx, stomach and intestine [7,8]. MMP-13 is highly expressed in HNSCC compared to normal skin or oral mucosa [9,10]. It is well established that the level of MMP-13 expression is correlated with the invasive and metastatic phenotype of the HNSCC [11]. Targeted inhibition of this enzyme in both hepatocellular carcinoma and mouse models can decrease tumor invasion and growth [8]. Moreover, treatment with recombinant MMP-13 protein promoted angiogenesis *in vitro* and *in vivo*, leading to aggressive tumor progression and metastasis [12]. These data encourage the choice of MMP-13 as a therapeutic target in cancer treatment.

Phytochemicals, bio-active components from plants, are recently used as an alternative treatment for cancer due to their extensive availability, but more importantly, because of their potential of anti-cancer activity with minimal adverse effects compared with chemotherapy. Epidemiological studies revealed that the daily consume of certain phytochemicals effectively decline the incidence of several types of cancers [13,14]. Bishayee *et al.* proposed the synergistic effects of phytochemicals in fruits and vegetables on cancer prevention [15].

Tinospora crispa (*T. crispa*) or Guduchi, a plant from the Menispermaceae family, is an indigenous climber plant widely distributed in Southeast Asia, particularly in Vietnam, Malaysia, Thailand, Indonesia and India [16,17]. This medicinal herb is traditionally used for treatment of inflammation, diabetes, contusion, septicemia, fever, scabies and other tropical ulcer-related disorders [18,19]. *T. crispa* has a potential to be a source of natural antioxidants and anti-cancer agents [20]. A previous study showed that *T. crispa* exhibited anti-proliferative and anti-angiogenesis effects and could induce apoptosis in certain human cancer cell lines such as MCF-7 (breast carcinoma), HeLa (cervical carcinoma), Caov-3 (ovarian carcinoma) and HepG2 (hepatocellular carcinoma) [20–22]. Methanol extract of *T. crispa* was found to have a more potent anti-proliferative effect on MDA-MB-231 and MCF-7 human breast squamous cell carcinoma than extracts obtained with water or chloroform [20].

Although many pharmacological activities of *T. crispa* have been reported, the effect of *T. crispa* as an anti-cancer drug in HNSCC is still unknown. The aim of this study is to investigate the effect of *T. crispa* on MMP-13 expression and HNSCC cell migration.

2. Materials and methods

2.1. Preparation of T. crispa extract

Whole plants of *T. crispa* (300 g) were blended in 95% ethanol and macerated for 5 days. The mixture was filtered and evaporated under reduced pressure to yield 7.32 g crude *T. crispa* extract. This extract was dissolved in dimethyl sulfoxide as a stock solution at 50 μg/μL.

2.2. Cell culture

Four human cell lines derived from HNSCCs were used. HN8 and HN22, primary and metastatic type of laryngeal carcinoma, were provided by Professor J. Silvio Gutkind (NIDCR, NIH, USA). HSC-7 and HSC-3, primary and metastatic type of tongue carcinoma, were provided by Professor Teruo Amagasa (Tokyo Medical and Dental University, Japan). All experimental protocols were approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University, Thailand (No. 071/2013). Cells were cultured at 37 °C in dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL amphotericin B (Gibco BRL, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cell viability of HNSCC cells was determined by MTT test (Sigma, St. Louis, MO, USA). Briefly, the cells were serum-starved for 6 h and incubated with various doses of *T. crispa* extract in serum-free DMEM for 48 h. The culture medium was then aspirated, and incubated in 0.5 mg/mL MTT solution for 15 min in a CO_2 incubator. Formazan crystals were eluted in a detergent solution containing 1:9 dimethyl sulfoxide and glycine buffer (0.1 mol/L glycine/0.1 mol/L sodium chloride at pH 10). Optical density of solubilized solution was measured at 570 nm in a microplate reader (Elx800; Biotek, Winooski, VT, USA). The viable cell number of experimental groups was calculated as relative to control group.

2.4. RNA extraction and RT-PCR

The total cellular RNA was extracted with Trizol reagent (Molecular Research Center, Cincinnati, OH, USA). One microgram of RNA sample was converted to cDNA by using reverse transcriptase (ImpromII, promega, UK). Taq polymerase (Invitrogen, Brazil) was used for PCR to detect MMP-13, TIMP-1, TIMP-2 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as a reference gene. Primers were designed by published sequences from GenBank. The amplification profile for MMP-13, TIMP-1 and TIMP-2 was denatured for 1 min at 94 °C, annealed for 1 min at 60 °C, and extented for 1.3 min at 72 °C (30 cycles). The same profile with 22 cycles was used for GAPDH. PCR product was then subjected to electrophoresis on 2% agarose gel and the band intensity was measured by BIO-1D software (Scion, Frederick, MA, USA).

2.5. ELISA

Measurement of secreted MMP-13 in the conditioned medium was performed by using anti-human pro-MMP-13 ELISA kits (R&D Systems, Minneapolis, MN, USA) followed the manufacturer's protocol. The optical absorbance was measured at 450 nm in a microplate reader.

2.6. Wound-healing migration assay

A wound healing assay was performed as described previously [23]. Briefly, HN22 cells were seeded on 6-well plates and incubated overnight yielding a confluent monolayer. A “wound” was made by dragging a sterile blue pipette tip along the center

of the plate. Detached cells were washed out and the remaining cells were incubated with 50.0 μg/mL *T. crispa* extract in serum-free DMEM. Images of cell monolayer were captured at 0 and 24 h time points under the phase-contrast microscope with a digital camera. The distance between the margins of the wound in randomly selected fields was measured for the wound wideness. Calculating method was:

Relative migration ratio = (distance at 0 h−distance at 24 h)/ distance at 0 h.

2.7. Statistical analysis

The data were reported as mean ± SD relative to the control from three independent experiments. Statistical differences were assessed by using student's *t*-tests for two-group comparisons or One-way ANOVA followed by Dunnett's test for three or more groups. A significance level of 0.05 was considered in all statistical comparisons.

3. Results

3.1. MMP-13 expression in HNSCC cell lines

Figure 1 demonstrates the endogenous MMP-13 mRNA expression in metastatic HNSCC cell lines compared with that in primary HNSCC cell lines. A striking lower MMP-13 expression was observed in both HN8 and HSC-7. The quantitate data showed that MMP-13 expression of HN22 was 2.5 fold of HN8 and HSC-3 was 5 fold of HSC-7.

3.2. Cytotoxic effect of T. crispa extract on HNSCC cell lines

To evaluate possible cytotoxic effects of the *T. crispa* extract, HN22 and HSC-3 were treated with a concentration of 12.5, 25.0, 50.0 and 100.0 μg/mL or dimethyl sulfoxide (control) for 48 h and analyzed by MTT assay. *T. crispa* extract at a concentration of 100.0 μg/mL significantly reduced cell viability to about 50% in HN22 cells and 60% in HSC-3 cells, while the lower concentrations had no significant effect on cell viability (Figure 2A). By phase contrast microscope, the morphology of HN22 cells incubated with 50.0 μg/mL of *T. crispa* extract was not seen to change compared to the control (Figure 2B).

3.3. Inhibitory effect of T. crispa extract on MMP-13 expression and protein released

The non-toxic concentrations (12.5, 25.0 and 50.0 μg/mL) of *T. crispa* extract were added to HN22 and HSC-3 cells and after 24-h-culture, the level of MMP-13, TIMP-1 and TIMP-2 mRNA expression was analyzed. *T. crispa* extract significantly decreased MMP-13 gene expression in a dose-dependent manner in both cell lines (Figure 3). The expression of TIMP-1 and TIMP-2 by HN22 cells was not changed in the presence of *T. crispa* extract, but *T. crispa* extract at the dose of 25.0 and 50.0 μg/mL attenuated the TIMP-2 expression by HSC-3 cells.

The analysis by ELISA showed that the release of MMP-13 in both cell lines was inhibited after treatment with *T. crispa* extract. However, the inhibitory effect of *T. crispa* extract was stronger for the HN22 than for the HSC-3 (Figure 4).

3.4. Migration of HN22 cell inhibited by T. crispa extract

The influence of *T. crispa* extract on the migratory potential of HNSCC cells was determined by using the scratch-wound healing assay. After 24-h-culture, non-treated HN22 cell almost completely filled up the space created between cells, but *T. crispa* extract significantly inhibited the cell migration to 65% compared to the control as shown in Figure 5.

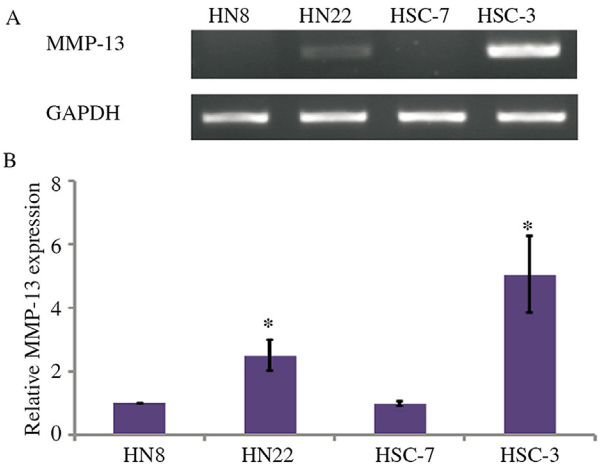

Figure 1. MMP-13 gene expression in metastatic HNSCC compared with that in primary HNSCC.
A: The level of MMP-13 mRNA expression in HN8, HN22, HSC-7 and HSC-3 analyzed by RT-PCR; B: Quantitation of MMP-13 expression in HN22 and HSC-3 was normalized to GAPDH and represents as the mean ± SD relative to HN8 and HSC-7, respectively; *: $P < 0.05$.

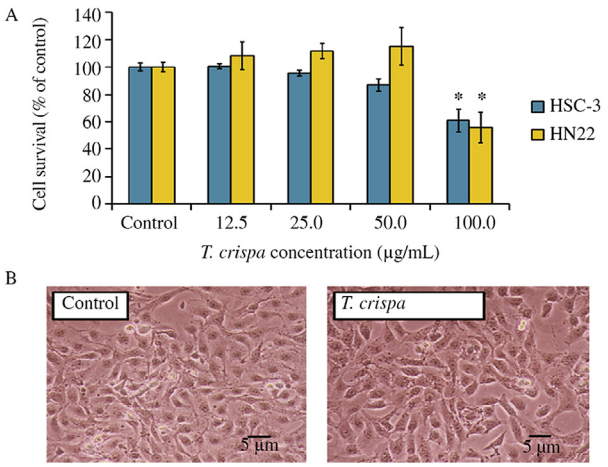

Figure 2. Cytotoxicity of *T. crispa* extract measured by MTT assay.
A: HN22 and HSC-3 cell viability using MTT assay after treated with *T. crispa* extract at concentrations of 12.5, 25.0, 50.0 and 100.0 μg/mL or DMSO (control) for 48 h; B: The cell morphology observed by phase contrast microscope; *: Significant difference ($P < 0.05$) compared to control group.

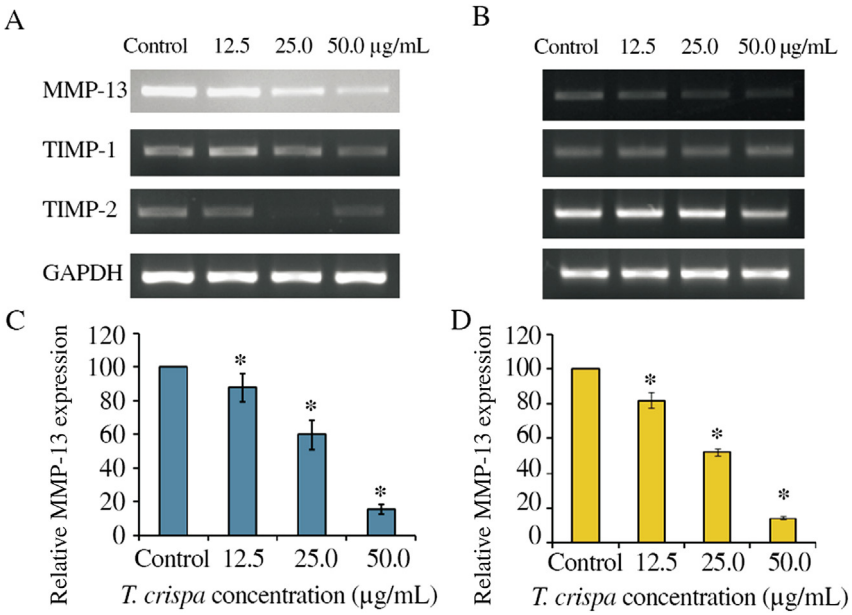

Figure 3. Effect of *T. crispa* extract on MMP-13 gene expression.
RT-PCR analysis of HSC-3 (A) and HN22 (B) treated with *T. crispa* extract at different concentrations for 24 h; MMP-13 expression in HSC-3 (C) and HN22 (D) were normalized to GAPDH; The results represent the mean ± SD relative to the control from three independent experiments; *: $P < 0.05$.

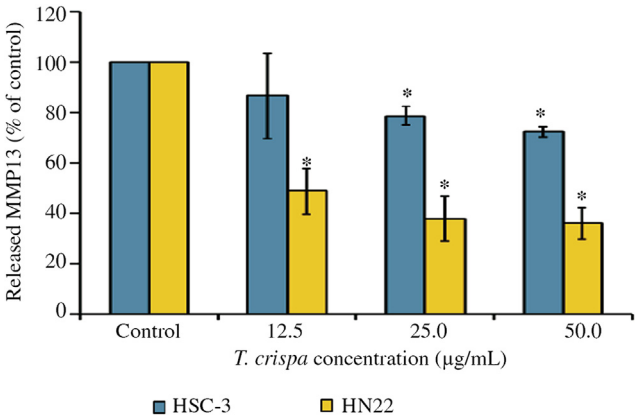

Figure 4. Inhibition of secreted MMP-13 by *T. crispa* extract.
Release of MMP-13 in HN22 and HSC-3, treated with various concentrations of *T. crispa* extract, was analyzed by ELISA; The data was represented as a relative amount of secreted MMP-13 compared to control. *: $P < 0.05$.

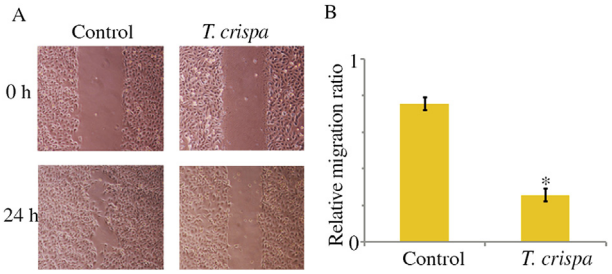

Figure 5. Effect of *T. crispa* on migration of HN22 cells.
A: Photographs of the wound in HN22 cells treated with DMSO (control) or 50 μg/mL extract at time 0 h and 24 h; B: Quantitation of the relative migration ratio between 0 h and 24 h; *: $P < 0.05$.

4. Discussion

The data presented in this study demonstrate that an extract obtained from *T. crispa* inhibits the expression of MMP-13 by HNSCC cell lines. This inhibitory effect was found both at the gene and at the protein level. Moreover, the extract also inhibited migration of these cells. Finally, the results confirmed a higher expression of MMP-13 by metastatic cell lines compared to the primary cell line. The latter finding is consistent with studies which showed that MMP-13 expression was significantly increased in tumors from lymph node metastases in head and neck squamous cell carcinomas and papillary thyroid carcinomas patients [11,24].

It has been shown that MMP-13 plays a role not only in matrix degradation but also in the activation of other MMPs and the enzyme appears to be critical in bone metabolism, tumor invasion and metastasis [5]. We hypothesize that the inhibitory effect of *T. crispa* on HNSCC cell migration might be related to the aberration of MMP-13 expression. Numerous findings presented in the literature demonstrated a role for MMP-13 in cell migration. MMP-13 silencing was shown to decrease tumor cell migration, whereas over-expression of MMP-13 increased cell migration and promoted metastasis [8,24]. Whether such a role of MMP-13 also counts for the cell types we have used, needs additional experiments.

The anti-proliferation, anti-angiogenic and apoptosis-inducing effect of *T. crispa*, were well-documented [21,25], but the effect on cell migration and MMP-13 suppression have not been reported before. There is one study demonstrating that octacosanol isolated from *Tinospora cordifolia*, another member of the Menispermaceae family, decreased gelatinolytic activity of both MMP-2 and MMP-9 in Ehrlich ascites tumor cells [26]. However, MMP-2 and MMP-9 expression was not altered by *T. crispa* in our cell lines (data not shown). Interestingly, the *T. crispa*-induced inhibition of MMP-13 release by HSC-3 cells proved to be less effective compared to its effect on HN22 cells. Similarly, the extract induced a decreased TIMP-2 expression by

HSC-3 cells but not by HN22 cells. These findings suggest considerable differences between cell lines in their response to the extract of *T. crispa*. The mechanisms underlying the difference in response are not yet known.

A series of chemical constituents such as terpenoids, alkaloids, flavones and phenolics have been isolated from *T. crispa* extracts. Some of these compounds were shown to exert biological activities. Terpenoids and alkaloids are major active ingredients and chemically investigated widely. The terpenoid glycosides are mainly composed of borapetosides A, B, C, D, E, F and H [25,27]. Hypoglycemic action of borapetoside has been shown. Borapetoside A, exerts a glucose-lowering effect, mainly through an enhanced glucose utilization by peripheral tissues. This effect was found in both streptozotocin-induced type 1 diabetes mellitus and diet-induced type 2 diabetes mellitus [18]. Alkaloids are well recognized as anti-cancer agents due to their anti-proliferative and apoptosis-inducing properties [25,28]. However, the active ingredients responsible for MMP-13 regulation as well as cell migration are still unknown and remain to be investigated.

In conclusion, *T. crispa* may exhibit anti-cancer properties through its ability to modulate MMP-13 expression and to inhibit cell migration by HNSCC. These data emphasize the importance of further development of phytochemical agent from medicinal herb as novel cancer therapeutic drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

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