นิพนธ์ต้นฉบับ

Bioactivity Study of *Tadehagi triquetrum* Extracts Used in Thai Folk Medicine

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Abstract

Tadehagi triquetrum (L.) H.Ohashi (*tankhoma* in Thai) is a medicinal plant that has been traditionally used as an antimicrobial agent for extending the expiration date of products containing water as vehicle. Thai folk medicine practitioners have also used a decoction of *T. triquetrum* leaves for improving physical and sexual performances in men. The aim of our study was to determine the bioactivity of *T. triquetrum* extracts using different polar and nonpolar solvents. We evaluated the antioxidant activity of extracts using DPPH and ABTS assays, investigated phosphodiesterase-5 (PDE-5) inhibitory activity of the extracts, which is associated with enhancing sexual and physical performance, and measured antimicrobial activity of the extracts using disc diffusion and minimal inhibitory concentration (MIC). The results revealed that the ethanolic and water extracts showed potent antioxidant and antimicrobial activities. Water extract presented the highest antioxidant activity (IC50 = 12.45 ± 0.14 μg/mL). Polar extracts inhibited the growth of microorganisms *S. aureus, E. coli* and *P. aeruginosa*. The non-polar extract effectively suppressed fungal growth. Interestingly, the Thai folk medicine formulation of *T. triquetrum* water extract at 50 μg/mL inhibited PDE-5 activity by 60.45% (SD = 0.14). This level of inhibitory bioactivity is considered good. As these findings are promising, future *in vitro, in vivo*, and clinical studies should be conducted to examine properties of this plant in greater detail.

Key words: antimicrobial activity, PDE-5 inhibition, antioxidant activity, *Tadehagi triquetrum*

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การศึกษาฤทธิ์ทางชีวภาพของสารสกัดหญ้าตานคอม้าตามการนำมาใช้ประโยชน์ทางการแพทย์พื้นบ้าน

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บทคัดย่อ

หญ้าตานคอม้า [*Tadehagi triquetrum* (L.) H. Ohashi] เป็นพืชสมุนไพรที่ใช้กันมาแต่โบราณเพื่อเป็นสารฆ่าเชื้อจุลชีพซึ่งทำให้สามารถยืดอายุของผลิตภัณฑ์ที่มีน้ำเป็นองค์ประกอบหลักได้ นอกจากนี้การแพทย์พื้นบ้านแนะนำให้ใช้ยาต้มจากใบหญ้าตานคอม้าเพื่อเพิ่มสมรรถภาพทางกายและสมรรถภาพทางเพศสำหรับเพศชาย และตามการใช้ประโยชน์ที่แพทย์พื้นบ้านได้แนะนำไว้ การศึกษานี้มุ่งที่จะตรวจวัดฤทธิ์ทางชีวภาพของสารสกัดหญ้าตานคอม้าที่ได้จากการสกัดด้วยตัวทำละลายที่มีขั้วแตกต่างกัน สารสกัดกลุ่มที่มีขั้วและไม่มีขั้วที่ได้ถูกนำไปทดสอบฤทธิ์ทางชีวภาพ ซึ่งได้แก่ ฤทธิ์ต้านอนุมูลอิสระโดยการประเมินด้วยวิธี DPPH และ ABTS รวมถึงการตรวจวัดฤทธิ์ในการต้านการทำงานของเอ็นไซม์ฟอสโฟไดเอสเทอเรส-5 (PDE-5) ซึ่งมีความเกี่ยวข้องกับสมรรถภาพทางเพศและทางกายของผู้ชาย สำหรับฤทธิ์ในการต้านเชื้อจุลชีพของสารสกัดจะถูกประเมินโดยวิธีดิสค์ดิฟฟิวชั่น และค่าความเข้มข้นที่น้อยที่สุดในการยับยั้งเชื้อจุลชีพ (MIC) จากผลการศึกษาแสดงให้เห็นว่าสารสกัดที่เตรียมจากแอลกอฮอล์และน้ำแสดงฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านจุลชีพที่สูง โดยสารสกัดน้ำแสดงฤทธิ์ในการต้านอนุมูลอิสระสูงที่สุด (IC50 = 12.45 ± 0.14 μg/mL) ส่วนการเจริญเติบโตของเชื้อ *S. aureus*, *E. coli* และ *Ps. Aeruginosa* สามารถถูกยับยั้งด้วยสารสกัดกลุ่มมีขั้ว ในขณะที่สารสกัดกลุ่มไม่มีขั้วสามารถยับยั้งการเจริญเติบโตของเชื้อราได้ดี ฤทธิ์ในการยับยั้งการทำงานของเอ็นไซม์ PDE-5 แสดงผลการยับยั้งที่ 60.45% ± 0.14 ที่ความเข้มข้น 50 μg/mL ของสารสกัดน้ำ จากการศึกษาแสดงให้เห็นว่าหญ้าตานคอม้ามีฤทธิ์ทางชีวภาพเบื้องต้นตามคำแนะนำของแพทย์พื้นบ้านที่ได้ระบุไว้ แต่อย่างไรก็ตามควรมีการศึกษาในห้องปฏิบัติการรวมถึงการศึกษาทางคลินิกอื่น ๆ เพิ่มเติม

คำสำคัญ: ฤทธิ์ต้านเชื้อจุลชีพ, ฤทธิ์ต้านอนุมูลอิสระ, ฤทธิ์ต้านเอ็นไซม์ PDE-5, หญ้าตานคอม้า

Introduction & Objectives

Tadehagi triquetrum (L.) H. Ohashi has been used as a medicinal plant in several countries in Asia.[1-4] Chinese medicine has used this plant as an anti-viral and anti-inflammatory agent.[5-6] It is often incorporated into drug recipes for treating sore throat and common cold.[5,7] Ayurveda uses the leaf of *T. triquetrum* for patients who have intestinal tract disorders such as dysentery, bloated stomach, and parasites. In Thailand, *T. triquetrum* is called *Ya-tarn-kor-ma* or *Kaow-moa-nok*. Folk medicine practitioners in the northern region of Thailand use *T. triquetrum* to support men's physical

ability. It is often incorporated into a traditional drug recipe for improving sexual and physical performance.[1,8] In addition, *T. triquetrum* is traditionally used as an anti-microbial agent in aqueous herbal products. The herb prevents the growth of microorganisms and extends the products'expiration date, Moreover, topical applications of the herb have been used to treat infectious wounds.[1,4,7]

Researchers have also found this herb useful in food preservation and has effects on different types of cells. Researchers found that maggot growth in pickled fish was limited when *T. triquetrum* extract was incorporated as an ingredient. Moreover, bacterial growth was suppressed, so the food expiration date was extended.[9] Recently, researchers revealed evidence that *T. triquetrum* contained ligand and phenylated isoflavones that prevented cell activity. They inhibited effects of chemical toxicity on hepatocyte cells by decreasing the expression of NFκB.[10] Tadehaginosin and 3,4-dihydro-4-(40-hydroxyphenyl)-5,7-dihydroxycoumarin were isolated from the aerial part of *T. triquetrum*. These two compounds displayed hypoglycemic activity by increasing glucose consumption and reducing blood glucose levels.[11] Phenylpropanoid glucosides and tadehaginosides increased glucose uptake by inducing peroxisome proliferator-activated receptor γ (PPARγ) activity and glucose transporter-4 (GLUT-4) expression.[12] The alternative products developed from *T. triquetrum* for diabetic patient health promotion are promising. Although recent *in vitro* studies revealed the beneficial effects of *T. triquetrum*, the bioactivity of folk medicine formulations has been not identified.

This study will determine the bioactivity of *T. triquetrum* extract in dosages and formulations found in Thai folk medicine practice. Specifically, we aim to investigate the herb's antioxidant activity, anti-microbial activity, and the inhibition activity of the PDE-5 enzyme.

Methodology

Materials

Tadehagi triquetrum (L.) H. Ohashi leaves were collected and identified by a botanist of Tungsleangluang National Park, Petchaboon Province, Thailand. DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid), MTT ([3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-] tetrazolium bromide) and 95% ethanol were AR grade and purchased from Sigma (Sigma-Aldrich, Singapore)

Instruments

- UV-Visible spectrophotometer (Evolution 201, Thermo Scientific)

- Microplate reader (Eon, Biotek)

Methods

1. Plant extraction

Leaves of *T. triquetrum* were chopped into tiny pieces and then macerated with one of the following solvents: 50% ethanol, 95% ethanol, or hexane. Each of extract solutions was filtered. Then the organic solvent was evaporated by rotary evaporator. To create the water extract, the leaves of *T. triquetrum* were boiled with hot water for 15 minutes. The water extract was filtered. The water was removed from the extract by freeze drying. All extracts were kept at -20°C and protected from light.

2. Cytotoxicity testing

Fibroblast cells were isolated from the foreskin of male infants who underwent neonatal circumcision in the hospital. The protocol of sample collection was approved by Ethics Committee for Research in Human Subject Naresuan University with approval number 140/62. The fibroblast cells were isolated from the dermis layer and cultured with 10% fetal bovine serum in Dulbecco's Modified Eagle Medium (DMEM). The culture was incubated in a humidified atmosphere of 5% of CO_2 at 37 °C to allow cell attachment. Cell culture medium was changed every 3 days.

The cytotoxicity of *T. triquetrum* extracts was evaluated using MTT assay. The cells were seeded into 96-well plates at $1x10^4$ cells per well. Cell attachment was allowed for 24 hours. Various concentrations of *T. triquetrum* extracts were added into the wells containing cells. The treated cells were re-incubated for 24 hours. Fifty microliters of MTT reagent ([3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-] tetrazolium bromide) in DMEM free serum (1 mg/mL) was then added into each well. The plates were incubated in a humidified atmosphere of 5% of CO_2 at 37 °C for 4 hours. After removing the medium, formazan crystals were dissolved by 100 μL of DMSO, and the absorbance was measured at 595 nm.

3. Antioxidant activity study by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay

One hundred and ninety micro-liters of 0.12 mM DPPH solution was mixed with 10 μL of *T. triquetrum* extract solution in 96-well plates. The extracts were dissolved in ethanol with 0.0001-5 mg/mL of concentration. The mixture was then mixed vigorously, and maintained at room temperature in the dark for 30 minutes. The decrease of absorbance was measured at 515 nm using a microplate reader. The positive control was trolox.

4. Antioxidant activity study by ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) assay

The stock solution was performed by dissolving 7.4 mM ABTS into 2.6 mM potassium persulfate. ABTS radical cation was prepared by mixing the two stock solutions in equal quantities. The mixture was allowed to stand for 12 hours in the dark place. ABTS

radical solution was adjusted with absolute ethanol to an absorbance of 0.7 ± 0.02 at 732 nm. Ten microliters of *T. triquetrum* extracts were mixed with 190 μL of ABTS radical solution in 96-well plates. The decrease of absorbance was measured at 732 nm using a microplate reader. The standard curve was linear between 0.025 and 0.9 mM Trolox. Results were expressed in the concentration of Trolox per g of *T. triquetrum* extract.

5. Phosphodiesterase-5 inhibitory activity

Erectile dysfunction (ED) is one of sexual dysfunction that correlates with phosphodiasterase-5 activity. Phosphodiesterase-5 (PDE-5) Inhibitors have been used as first-line therapy for ED. The mechanism of action of this drug class is inhibition of the enzyme PDE-5 which is an enzyme breaking down cGMP. Vasodilatory effect enhanced by cGMP improving penile erection. The basic screening of promising agent for enhancing sexual performance is often measured by phosphodiasterase-5 (PDE-5) inhibitory activity.[13] Current study, phosphodiasterase-5 (PDE-5) inhibitory activity of *T. triquetrum* extracts was investigated by Herb tech, Faculty of Pharmaceutical Sciences, Naresuan University, Thailand. The method was described in brief[14];

PDE-5 was obtained from mouse lung tissue. It was performed following as Temkifthawon and colleagues report.[5] The reaction mixture was composed of 25 μl of buffer A [100 mM Tris-HCl (pH 7.5), 100 mM imidazole, 15 mM $MgCl_2$ and 1.0 mg/mL BSA], 25 μl of 10 mM EGTA, 25 μl of PDE solution and 25 μl of test sample or only solvent (5% DMSO) as a control. The reaction mixture was mixed with a substrate, 25 μl of 1 μM [3H] cGMP and incubated at 30 °C for 10 min. After that, the reaction was stopped by placing the tube in boiling water for 1 min and cooled for 5 min. For the second enzymatic reaction, 25 μl of 2.5 mg/mL snake venom containing 5′-nucleotidase enzyme was added to the reaction mixture, incubated at 30 °C for 5 min. Then, 250 μl of 20 mM Tris-HCl, pH 6.8 (buffer 1) was added. The reaction mixture was transferred to a diethylaminoethyl (DEAE) ion exchange resin column and eluted 4 times with 500 μl of buffer 1 to obtain the hydrolysis product, uncharged [3H] guanosine. The eluant was mixed with a scintillant cocktail and the radioactivity was measured using a β-counter. The PDE in the study was standardized to have a hydrolysis activity of 15-20% of the total substrate counts. The PDE inhibitory activity is calculated from Eq. (1). The calculation of hydrolysis is shown in Eq. (2).

$$\%\ \text{PDE-5 inhibition} = \left[1 - \left(\frac{\%\text{hydrolysis}_{\text{sample}}}{\%\text{hydrolysis}_{\text{control}}}\right)\right] \times 100 \quad (1)$$

where % hydrolysis sample and % hydrolysis control were the enzyme activities of the sample and solvent (1% DMSO) used in the

assay, respectively.

$$\%\,\text{Hydrolysis} = \left[\frac{\left(\text{CPM}_{\text{control}} - \text{CPM}_{\text{background}}\right)}{\left(\text{CPM}_{\text{total count}} - \text{CPM}_{\text{background}}\right)}\right] \times 100 \ldots. \quad (2)$$

where CPM sample is the radioactive count rate of the assay with enzyme and CPM background is the same but without enzyme. CPM total count is the count rate of 25 μl of substrate plus 2 mL of buffer 1. Sildenafil was a positive control.

6. Antimicrobial screening

Antimicrobial activity of *T. triquetrum* has been reported by folk medicine. Bacterial growth inhibition was examined in *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. They are the representative of gram positive, negative bacterium and fungus. The extracts were tested using 6 mm sterilized filter paper discs. Discs were impregnated with 25 μL (100 mg/mL concentration) of the *T. triquetrum* extracts, allowed to dry and placed onto inoculated plates. Plates inoculated with *E. coli*, *Ps. aeruginosa*, *S. aureus* and *C. albicans* were incubated at 37°C for 24 hours, then the diameters of the inhibition zones were measured in millimeters. Each antimicrobial assay was performed in triplicate and mean values were reported. Standard antibiotics, clindamycin (10 μg/ disc), ampicillin (10 μg/ disc) and amphotericin-B (10 μg/ disc) served as positive controls of antimicrobial and antifungal activities respectively. Filter discs impregnated with 10 μL of distilled water were used as a negative control. Solvent control disc (ethanol) was also placed with the test, positive and negative control.

The minimum inhibitory concentration (MIC) of the plant extracts was determined by using sterile 2 mL (96-well plates). Clindamycin (0.1 mg/mL) and ampicillin (0.1 mg/mL) were used as controls for the *S. aureus*, *Ps. aeruginosa* and *E. coli* assays respectively. The deep-wells were incubated for 24 hours at 37 °C. The resulting turbidity was observed, and after 24 hours, MIC was determined to be where growth was no longer visible by assessment of turbidity by optical density readings at 600 nm with a Thermo Scientific Evolution 200 series UV-Vis Spectrophotometer. At least three replications were run for each assay.

Results

We examined the bioactivity of extracts of *T. triquetrum* prepared by different solvents. Then we examined those results to suggest which types of extracts may be appropriate to develop into health applications.

1. Cytotoxicity testing

First, we examined the cytotoxic effects of *T. triquetrum* extracts drawn out by different solvents on human fibroblasts. We determined cytotoxic effect on fibroblast cells by using MTT assay. We measured the survival

of fibroblast cells by measuring the purple color of formazan crystals that are produced by mitochondria enzyme. Low relative cell viability implies that extracts have a high cytotoxic effect on fibroblast cells.

Figure 1 revealed that there were no cytotoxic effect on fibroblast cells for all concentrations of *T. triquetrum* extracts tested (0.2-100 μg/mL). Relative cell viability measurements were 80% or greater for all concentrations and among each of the extracts tested. Although *T. triquetrum* extracted by hexane showed the lowest cell viability compared to other extracts, the relative cell viability of *T. triquetrum* extracted by hexane was still more than 80%.[15]

2. Antioxidant activity

Testing antioxidant activity is the primary screening tool used to identify interesting herbs. DPPH and ABTS assays are usually used for screening antioxidant activity in medicinal plants. In Table 1, antioxidant activity measured in DPPH and ABTS assays were described using the summary measures of IC50 and TEAC, respectively.

T. triquetrum extracted by water showed the highest antioxidant activity with IC50 value of 12.45 ± 0.14 μg/mL and TEAC value of 0.85 ± 0.11 μM. While hexane extract of *T. triquetrum* showed the lowest antioxidant activity with IC50 value of 180.20 ± 1.79 μg/mL and TEAC value of 5.50 ± 0.86 μM.[16]

3. Phosphodiesterase-5 inhibitory activity

The phosphodiesterase-5 inhibitory activity of *T.triquetrum* extracts is shown in Table 2. We present the % inhibitory activity at 50 μg/mL concentrations of the extract. The PDE-5 inhibitory activity of *T. triquetrum* extracts depended on the type of solvent that was used. Water extract of *T. triquetrum* presented the highest inhibition of PDE-5

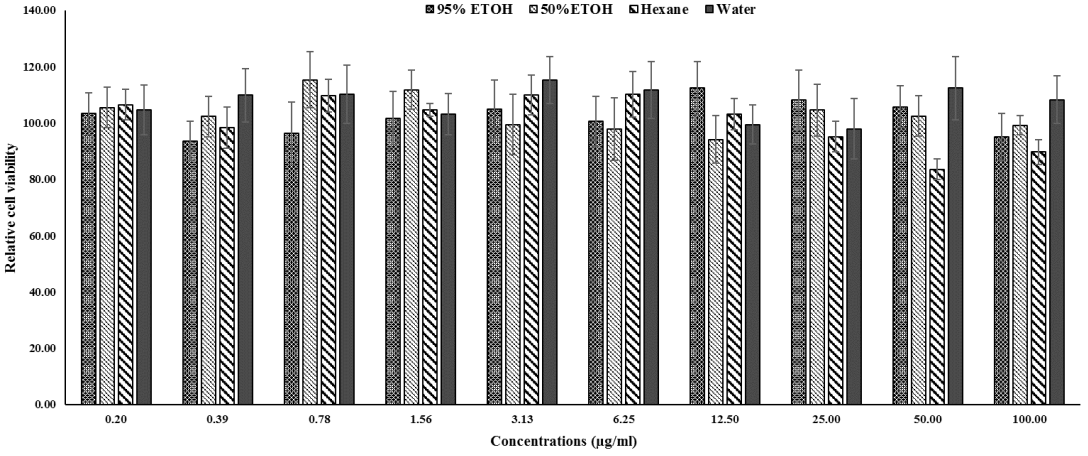

Figure 1 Cytotoxic effect of *T. triquetrum* extracts on human fibroblast cells

Table 1 Antioxidant activity of *T. triquetrum* extracts determined by DPPH and ABTS assays

Extracts	Antioxidant activities	
	IC50 (μg/mL) ± SD	TEAC (μM) ± SD
95% ethanol	123.60 ± 2.78	1.18 ± 0.01
50% ethanol	66.14 ± 2.57	1.20 ± 0.04
Hexane	180.20 ± 1.79	5.50 ± 0.86
Water	12.45 ± 0.14	0.85 ± 0.11
Trolox	0.89 ± 0.04	-

Table 2 PDE-5 inhibitory activity of *T. triquetrum* extracts

Extracts	%Inhibitory activity at 50 μg/mL*
95% ethanol	39.86 ± 3.20
50% ethanol	52.53 ± 2.60
Hexane	11.75 ± 1.98
Water	60.45 ± 0.14

*Data are reported as the mean ± S.D and are derived from three repeats.

activity (60.45% ± 0.14). Hexane extract of *T. triquetrum* showed the lowest inhibition of PDE-5 activity (11.75% ± 1.98).

4. Antimicrobial screening

Antimicrobial activity was determined by using disc diffusion and broth dilution assays. We examined the effects of *T. triquetrum* on various microorganisms including *E. coli*, *S. aureus*, *Ps. aeruginosa* and *C. albicans*.

Table 3 shows that inhibitory zone, or clear zone measurements (mm) for cell cultures treated with *T. triquetrum* extracts in different solvents varied. Larger clear zone measurements indicated that the extract with a particular solvent extract was more effective in stopping microbial growth. Ethanolic and water extracts showed obvious inhibition effect on bacterial growth with moderate clear zone measurements for bacteria. Hexane extract presented growth inhibition of yeast with moderate clear zone measurements for yeast.

The minimal inhibitory concentration (MIC) of each extract shown in Table 4 correlated with the clear zone measurement results presented in Table 3. The MIC is the smallest concentration of the particular extract that was necessary to inhibit microbial growth. The water extract had the lowest MIC compared to other polar extracts. This indicates that the water extract has the highest antibacterial activity of the different types of solvent because only a relatively low concentration was necessary to inhibit bacterial growth. Interestingly, only hexane extract shows the inhibition activity of yeast (*C. Albicans)*.

Table 3 Antimicrobial activity of *T. quetrum* extracts presented by disc diffusion assay

Microbial species	Clear zone (mm) ± SD						
	T. triquetrum extracts				Positive controls		
	95EtOH	50EtOH	Water	Hex	DA	AMP	AmB
S. aureus	9.00 ± 1.34	10.00 ± 0.22	12.00 ± 0.89	ND	25.00 ± 1.77	33.00 ± 3.89	ND
E. coli	12.00 ± 2.67	12.00 ± 1.56	15.00 ± 3.44	ND	ND	24.00 ± 1.56	ND
C. albicans	ND	ND	ND	6.00 ± 1.29	ND	ND	15.00 ± 3.12
P. aeruginosa	16.00 ± 2.33	15.00 ± 0.22	16.00 ± 1.89	ND	25.00	33.00 ± 4.55	ND

Note: 95EtOH: 95% ethanol, 50EtOH: 50% ethanol, Hex: Hexane, DA: Clindamycin, AMP: Ampicillin, AmB: Amphotericin-B, ND: Not detected

Table 4 Antimicrobial activity of *T. triquetrum* extracts presented by minimal inhibiting concentration (MIC)

Microbial species	MIC (mg/mL)			
	T. triquetrum extracts			
	95EtOH	50EtOH	Water	Hex
S. aureus	12.50	12.50	6.25	-
E. coli	25.00	12.50	6.25	-
C. albicans	-	-	-	25.00
P. aeruginosa	25.00	25.00	12.50	-

Note: 95EtOH: 95% ethanol, 50EtOH: 50% ethanol, Hex: Hexane

Discussion

T. triquetrum leaves were extracted by solvents possessing different polarities. All types of extract did not show any cytotoxic effect on human fibroblast cells. Relative cell viability for cells treated with all types of extracts were more than 80% compared with the control. From these results, we can infer that *T. triquetrum* extracts may be safe for human skin cells at concentrations ranging from 0.20-100.00 μg/mL.[15]

The water extract of *T. triquetrum* showed the most promising result as an inhibitor of PDE-5. In our study, *T. triquetrum* extracted by high polar solvents such as water showed more beneficial effects for PDE-5 inhibition than nonpolar extracts such as hexane. Another study found that *T. triquetrum* extracted by alcohol inhibited phosphodiesterase enzyme activity more than that chloroform extract, which is also a nonpolar solvent.[17] Our result correlates with the traditional Thai

folk medicine recommendation that the decoction from *T. triquetrum* leaves can be used to improve sexual and physical performance. Improved sexual and physical performance may be facilitated by PDE-5 inhibitory activity[14,18-20] experienced by patients taking a decoction of *T. triquetrum* leaves.

Antioxidant activity is often used to screen for promising medicinal compounds or herbs because free radicals are associated with many diseases. We interpret low IC50 and TEAC values as showing that a compound effectively scavenges free radicals.[16] In our study, water extract of *T. triquetrum* presented the highest antioxidant activity with the lowest IC50 and lowest TEAC of all the types of extract. Our finding supports the theory that antioxidant activity of *T. triquetrum* extract may explain the effectiveness of *T. triquetrum* Thai folk medicine formulations.[21] Thai folk medicine practitioners have promoted the boiled leaf of *T. triquetrum* as a health promotion beverage.[1,8] The presence of free radicals is an important risk factor for several non-infectious diseases and organ degeneration.[22-23] Taking *T. triquetrum* and thus reducing free radicals may improve and promote consumer health. Moreover, *T. triquetrum* extract possessing hepatoprotective activity[24] and capability to increase glucose consumption[12] are benefit to patients who expect to promote their health.

In addition, our experimental results support the observations by Thai folk medicine practitioners that *T. triquetrum* extract has antimicrobial activity.[1,8-9] Previous research has also reported pathogenic bacteria growth inhibited by *T. triquetrum* extract.[25] In our study, the polar extracts (water and ethanolic) of *T. triquetrum* inhibited bacterial growth. We also found that non-polar extract (hexane) suppressed fungal growth. Our results suggest that *T. triquetrum* could be a promising antimicrobial agent.

Conclusion

According to the results of the current study, *T. triquetrum* is a promising herb for health care product development. It could be developed as a product for health promotion or an alternative antimicrobial agent. Because our results show the beneficial effects for *in vitro* experiments, we recommend that *in vivo* studies and clinical studies be carried out in the future.

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