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ANTIMICROBIAL ACTIVITY FROM LEAVES AND STEMS OF TRIPHASIA TRIFOLIA (BURM. F.) P. WILSON

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Abstract: Air dried leaves and stems of *Triphasia trifolia* were macerated with hexane, dichloromethane and ethanol, respectively. Preliminary chemical screening results of these crude extracts from its leaves and stems found alkaloids, flavonoids, tannins and triterpenoids. Antimicrobial activity of the crude extracts was tested by agar diffusion method. Tetracycline was used as a positive control. The crude ethanolic extract from its stems showed the strongest antimicrobial activity against both gram positive (*S. aureus*, *S. epidermidis*, *B. subtilis* and *M. luteus*) and gram negative bacteria (*E. coli*) while the crude dichloromethane and ethanolic extracts from its leaves showed strong inhibition of *B. subtilis* and *M. luteus*. In conclusion, the crude ethanolic extract from the stems of *T. trifolia* exhibit strong antimicrobial activity. Therefore, it might be used for potential source for novel antimicrobial drugs in the future.

Keywords: *Triphasia trifolia*, antimicrobial activity, agar diffusion method

บทคัดย่อ : นำใบและลำต้นแห้งของมะนาวเทศมาแช่ใน hexane, dichloromethane และ ethanol ตามลำดับ ผลการตรวจสอบองค์ประกอบทางเคมีเบื้องต้นของสารสกัดหยาบจากใบและลำต้นของมะนาวเทศพบสารกลุ่ม alkaloid, flavonoid, tannin และ terpenoid จากนั้นนำมาทดสอบ ฤทธิ์ต้านจุลชีพโดยวิธี agar diffusion method โดยใช้ tetracycline เป็นสารควบคุมแบบให้ผลบวก ผลการทดสอบพบว่า สารสกัดหยาบ ethanol จากลำต้นมีฤทธิ์ต้านเชื้อจุลชีพทั้งเชื้อแกรมบวก (*S. aureus*, *S. epidermidis*, *B. subtilis* และ *M. luteus*) และเชื้อแกรมลบ (*E. coli*) ดีที่สุด ในขณะที่ สารสกัดหยาบ dichloromethane และ ethanaol จากใบมีฤทธิ์ยับยั้งเชื้อ *B. subtilis* และ *M. luteus* ซึ่งสรุปได้ว่าสารสกัดหยาบ ethanol จากลำต้นของมะนาวเทศมีฤทธิ์ต้านเชื้อจุลชีพที่ดี ดังนั้นจึงอาจใช้มะนาวเทศเป็นแหล่งของยาปฏิชีวนะชนิดใหม่ในอนาคตได้

คำสำคัญ: มะนาวเทศ ฤทธิ์ต้านจุลชีพ agar diffusion method

INTRODUCTION

Triphasia trifolia (Burm. F.) P. Wilson or limeberry belongs to the family Rutaceae. This plant was used as ethnomidicine for treatment of dandruff, cough, diarrhea, dysentery, influenza, parasitic diseases and lung disorders (Abaul *et al*., 1994; Jain and Srivastava, 2005; Dondon, Bourgeois and Fery-Forgues, 2006; Samoisy and Mahomoodally, 2016).

Several genera of the family Rutaceae have been reported to their antimicrobial activity against diverse microorganisms such as *Aegle marmelos* (Jaishree and Kumar, 2011; Abirami *et al*., 2014; Meena, Pareek and Meena, 2016), *Citrus paradise* (Cvetnic and Vladimir-knezevic, 2004), *Coleonema album* (Esterhuizen, Meyer and Dubery, 2006), *Murraya koenigii* (Vats, Singh and Sardana, 2011; Salomi and Manimekalai, 2016), *Ruta graveolens* (Benazir *et al*., 2011; Kumar *et al*., 2014; Amabye and Shalkh, 2015), *Teclea afzelii* (Kuete *et al*., 2008). However, there are no reports on antimicrobial activity of *T. trifolia*. Therefore, the aims of this study were to investigate the chemical constituents and antimicrobial activity of *T. trifolia* crude extracts. Thus the data obtained from this study may

provide useful information on phytochemistry, chemotaxonomy and biological activities of the genus *Triphasia*.

MATERIALS AND METHODS

Plant material

T. trifolia plant material was purchased from Chatuchak Market, Bangkok, Thailand. The plant sample was identified by comparing with Tree flora of Malaya (1972) and confirmed by Asst. Prof. Dr. Thaya Jenjittikul (Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand). The voucher specimen of this plant sample was deposited at Faculty of Pharmacy, Rangsit University, Thailand.

Chemicals

Iodine, potassium hydroxide, potassium iodide, sodium hydroxide and Magnesium ribbon were purchased from Ajax Finechem Pty Ltd. (USA); hydrochloric acid, sulphuric acid and mercuric chloride from J.T. Baker (USA); dichloromethane from Fisher Scientific (USA); sodium chloride, 3, 5-dinitrobenzoic acid and Zinc dust from Sigma Aldrich (USA); gelatin, tryptic soy broth and tryptic soy agar from Difco (USA); dimethyl sulfoxide (DMSO), ethanol and picric acid from Merck (Germany); bismuth nitrate and ferric sulphate from Farmitalia Carlo Erba (Italy); hydrogen peroxide from Chem supply (Australia); glacial acetic acid from RCI. Labscan (Thailand). All chemicals are of reagent grade, except extraction solvents (hexane, dichloromethane and ethanol) were commercial grade.

Extraction procedure

Leaves and stems of the plant samples were washed with tap water, air dried and ground in to fine powder. The crude extracts were prepared by macerating 300 g of ground sample with hexane, dichloromethane and ethanol (500 ml x 3 times x 3 day each), respectively. The extracts were filtered and concentrated using rotary evaporator.

Preliminary phytochemical screening

Preliminary phytochemical screening of the crude extracts was performed by following the methods of Evans, 2009, Tiwari *et al*., 2011 and Gul *et al*., 2017 with a few modifications.

Detection of alkaloids

The crude extracts (1 g) were dissolved in 1% HCl (5 ml) and filtered to obtain the testing filtrates.

Dragendroff's Test: Filtrates (1 ml) were treated with few drops of Dragendroff's reagent. Formation of red precipitate indicates the presence of alkaloids.

Mayer's Test: Filtrates (1 ml) were treated with few drops of Mayer's reagent. Formation of a yellow precipitate indicates the presence of alkaloids.

Wagner's Test: Filtrates (1 ml) were treated with few drops of Wagner's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

Hager's Test: Filtrates (1 ml) were treated with few drops of Hager's reagent. Formation of yellow precipitate indicates the presence of alkaloids.

Detection of anthraquinones

Modified Borntrager's Test: The crude extracts (1 g) were dissolved in 1% HCl (2 ml), added with 30% H_2O_2 (2 ml) and boiled in water bath for 5 min. The mixture was cooled and extracted with equal volume of CH_2Cl_2. The CH_2Cl_2 layer was separated and treated with conc. ammonia solution. Formation of rose-pink color in the ammonical layer indicates the presence of anthraquinone glycosides.

Detection of saponins

Froth Test: The crude extracts (1 g) were diluted with distilled water (5 ml) and shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Haemolysis Tests: The crude extracts (0.5 g) were diluted with distilled water (2 ml) and added with RBC solution in 0.9% NaCl (2 ml). Formation of clear solution indicates the presence of saponins.

Detection of cardiac glycosides

Kedde Test: The crude extracts (0.5 g) were added with few drops of Kedde reagent and followed with 1 M KOH (0.5 ml). Formation of reddish purple solution indicates the presence of cardiac glycosides (cardenolides).

Keller-Kiliani Test: The crude extracts (0.5 g) were dissolved in CH_2Cl_2 (2 ml) and then added with Keller reagent (1 ml). The reaction mixture was mixed ~~it~~ very well and then added with few drops of conc. H_2SO_4. The reddish brown colorization at interface of the two liquid layers indicates the presence of deoxysugar that is the characteristic of cardiac glycosides.

Detection of phytosterols and triterpenoids

Libermann's test: The crude extracts (0.5 g) were dissolved in distilled water (2 ml) and followed with acetic acid (2 ml) and dichloromethane (2ml). The reaction mixture was cooled and then added with a few drops of conc. H_2SO_4. Formation of green color indicates the presence of phytosterols.

Salkowski's Test: The crude extracts (0.5 g) were dissolved in CH_2Cl_2 (2 ml). After addition of a few drops of conc. H_2SO_4, the reaction mixture was shaken and allowed to stand for a while. Formation of a reddish brown color at the interface indicates the presence of terpenoids.

Detection of flavonoids

Pew Test: The crude extracts (0.5 g) were dissolved in 95% ethanol (2 ml), added with zinc powder and followed by 2-3 drops of conc. HCl. Formation of reddish purple or cherry color indicates the presence of flavonoids.

Shinoda Test: The crude extracts (0.5 g) were dissolved in 95% ethanol (2 ml) and followed with addition of few pieces of magnesium ribbon and a few drops of conc. HCl. Formation of magenta color indicates the presence of flavonoids.

Detection of tannins

Gelatin Test: The crude extracts (0.5 g) were dissolved in purified water (2 ml) and followed with 1% gelatin solution in 0.9% sodium chloride (2 ml). Formation of white precipitate indicates the presence of tannins.

Antibacterial activity

Bacterial strains

Three bacterial strains including *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, gram positive, and *Escherichia coli* ATCC 25922, gram negative, were obtained as lyophilized cultures from Department of Medical Science, Ministry of Public Health, Thailand while *Staphylococcus epidermidis* and *Micrococcus luteus* (clinical isolated), gram positive, were obtained from Faculty of Medical technology, Rangsit University, Thailand.

Agar diffusion method

Test microorganisms used for antimicrobial activity assay were freshly cultured on Tryptic Soy Broth (TSB) medium, which was incubated at 37°C for 24 hr. After that, the TSB medium was approximately adjusted to a solution concentration of 0.5 McFarland with 0.9% sterile normal saline solution. The solution mixture was spread on Tryptic Soy Agar (TSA) plate with sterile cotton swab and allowed to dry. Sterilized paper filter discs (diameter 6 mm) were impregnated with 20 µl of the crude extracts (500 µg/ml in 5% v/v DMSO) and placed on the inoculated agar. The plates were left to stand for 30 min at room temperature to allow the diffusion of the crude extract and then they were incubated at 37°C for 24 hr. Antimicrobial activity was evaluated by measuring the clear zone of inhibition against the test microorganisms. In this experiment, paper filter disc without test material was used as a negative control and paper filter disc containing 50 µg/ml tetracycline solution in 5% v/v DMSO dissolved in TSB were used as a positive control. All experiments were performed in triplicate and the developed inhibition zones were compared with that of the positive control.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) for antibacterial activity assay was determined by the broth dilution method (Karaman *et al*., 2003; Daud, Gallo and Riera 2005). All tested bacterial were culture in TSB and incubated at 37°C for 24 hr. After that, the TSB mediums were adjusted to 1 McFarland standard turbidity. The crude extracts dissolved in 5% v/v DMSO in TSB was first diluted to the highest concentration (500 µg/ml) prior to being tested, and then the serial two-fold dilutions were made in a concentration range from 500 to 0.98 µg/ml in 10 ml sterile test tubes containing nutrient broth. Tetracycline and microorganism without the crude extract were used as positive and negative control, respectively. The mixture in each tube was incubated at 37°C for 24 hr. The MIC value of each crude extracts was determined as the lowest concentration of the crude extract that completely prevent any turbidity or growth of the test organisms. All samples were tested in triplicate.

RESULTS AND DISCUSSION

The air dried leaves and stems of *T. trifolia* were macerated with hexane, dichloromethane and ethanol, respectively. The appearances and percent yields of the crude extracts were showed in Table 1.

Table 1. The appearances and percent yields of the crude extracts of leaves and stems of *T. trifolia*

Solvent	Leaves		Stem	
	Appearance	% Yield	Appearance	% Yield
Hexane	Green viscous liquid	6.28	Greenish brown viscous liquid	5.74
Dichloromethane	Dark yellow viscous liquid	4.78	Green viscous liquid	3.63
Ethanol	Dark green viscous liquid	12.86	Yellowish Green viscous liquid	10.44

Preliminary phytochemical screening

In this research, the preliminary phytochemical screening results of the crude extracts of leaves and stems of *T. trifolila* revealed the presence of alkaloids, flavonoids, tannins and triterpenoids (Table 2). Furthermore, Silva *et al*. (1981) has supported that the *T. trifolia* leaves constituented were coumarins, i.e. isomeranzin, umbelliferone and triphasiol and both leaves and stem of *T. trifolia* from France constituented bicoumarin (Dondon, Bourgeois and Fery-Forgues, 2006). The results were also correlated with the previous reported. Preliminary phytochemical screening of several plants in the family Rutaceae such as *Citrus aurantium*, *Glycosmis pentaphylla*, *Murraya koenigii* and *Ruta graveolens* has also found alkaloids, flavonoids, tannin and triterpenoids (Benazir *et al*., 2011; Gayathri and Kiruba, 2014; Murugan and Natarajan 2016; Renugadevi and Meerabai 2016; Salomi and Manimekalai 2016).

Table 2. Phytochemical constituents of leaves and stems of *T. trifolia*

Test	Leaves			Stem		
	Hexane ext.	CH_2Cl_2 ext.	EtOH ext.	Hexane ext.	CH_2Cl_2 ext.	EtOH ext.
Alkaloids	-	-	+	-	-	+
Anthraquinones	-	-	-	-	-	-
Saponins	-	-	-	-	-	-
Cardiac glycosides	-	-	-	-	-	-
Phytosterols	-	-	-	-	-	-
Triterpenoids	+	+	+	+	+	+
Flavonoids	-	+	+	-	+	+
Tannins	-	-	+	-	-	+

+ : Present - : Absent

Antibacterial activity

The antimicrobial activity of the crude hexane, dichloromethane and ethanolic extracts from the leaves and stems of *T. trifolia* was investigated using agar diffusion method. In addition, the MIC values of all the crude extracts were also investigated. At the concentration of 500 mg/ml, the crude ethanolic extract from the stem exhibited high antibacterial activity against *M. luteus* with 25 ± 1.36 mm in diameter of the zone of inhibition as same as the crude ethanolic extract from leaves (Table 3). The crude dichloromethane extract from the leaves and stems showed high antibacterial activity against *M. luteus* with 22 ± 1.68 and 18 ± 1.07 mm in diameter of the zone of inhibition, respectively. Table 4 showed that the crude hexane extract from the leaves and stems showed low antibacterial activity against all tested bacteria while the crude dichloromethane and crude ethanolic extracts from the leaves showed strong inhibition capability of *B. subtilis* and *M. luteus*, gram positive bacteria, with the MIC values of 15.62, 3.91, 7.81 and < 0.98 μg/ml, respectively. The crude ethanolic extract from the stem exhibited the strongest antimicrobial

activity against both gram positive and gram negative bacteria. The MIC values of the crude ethanolic extract against *S. aureus, S. epidermidis, B. subtilis, M. luteus* and *E. coli* were 7.81, 7.81, 1.95, < 0.98 and 7.81 μg/ml, respectively (Table 4).

The results of antibacterial activity for all the crude extracts were in agreement with previous reports for the leaves and stems of the plants in the family Rutaceae (Esterhuizen, Meyer and Dubery, 2006; Khulbe and Sati, 2009; Howlader *et a*l., 2011; Vats, Singh and Sardana 2011; Amabye and Shalkh, 2015). Khulbe and Sati (2009) reported that the crude hexane, mathanolic and aqueous extracts of *B. albiflora* showed strong antimicrobial activity against *B. subtilis*, *S. aureus*, *E. coli* and *P. vulgaris* and the crude acetone and ethanolic extracts of *C. album* exhibited antimicrobial activity against *E. coli*, *P. aeruginosa*, *E. faecalis*, *S. aureus* and *C. albicans* (Esterhuizen, Meyer and Dubery, 2006). The crude methanolic extract of *G. pentaphylla* has been reported for its antimicrobial activity against *E. coli* (Howlader *et a*l., 2011). Antimicrobial activity of *M. koenigii* was investigated by Vats, Singh and Sardana (2011). The results showed the antimicrobial activity of the crude petroleum ether, chloroform, ethyl acetate and ethanolic extracts against *S. aureus*, *B. subtilis*, *M. luteus*, *E. coli* and *P. aeruginosa*. The crude ethanolic, methanolic, chloroform and aqueous extracts of *R. graveolens* showed strong antimicrobial activity against *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* (Amabye and Shalkh, 2015).

In addition, there have been many researches reporting the correlation of phytochemical constituents towards their antimicrobial activities. For instances, flavonoids from *Citrus bergamia* pell exhibited antimicrobial activities against both gram-positive bacteria (*L. innocua*, *B. subtilis*, *S. aureus* and *L. lactis*) and gram-negative bacteria (*E. coli*, *P. putida*, *S. enterica*) whereas flavonoids from *Murraya koenigii* exhibited antimicrobial activities against *S. aureus*, *B. subtilis*, *M. luteus*, *E. coli* and *P. aeruginosa* (Mandalari *et al*., 2007; Vats, Singh and Sardana, 2011). Flavonoids and tannins from *Citrus limon* peel exhibited antimicrobial activities against *S. aureus* and *E. coli* (Ali, Das and Saikia, 2017). Alkaloids from leaves and stem of *Vepris lecomteana* showed antimicrobial activities against *B. subtilis*, *P. agarici*, *M. luteus*, *S. warneri* and *E. coli* (Kouam *et al*., 2018).

On the phytochemical screening testing of the crude extracts of leaves and stems of *T. trifolila* and the antimicrobial activity of the leaves and stems of the plants in the family Rutaceae reported earlier, it might conclude that the antimicrobial activity of *T. trifolia* was caused by their phytochemical constituents of alkaloid, flavonoid, tannin, and triterpenoid types.

Table 3. Antibacterial activity of the leaves and stems of *T. trifolia*

Bacterial Strain	Zone of inhibition (mm)[a]						
	Standard	Leaves			Stem		
	Tetracycline	Hexane ext.	CH_2Cl_2 ext.	EtOH ext.	Hexane ext.	CH_2Cl_2 ext.	EtOH ext.
Gram positive							
S. aureus	10 ± 0.87	7 ± 1.23	8 ± 1.85	9 ± 1.32	7 ± 1.46	9 ± 1.97	15 ± 1.02
S. epidermidis	14 ± 1.02	9 ± 1.06	9 ± 1.22	10 ± 1.51	8 ± 1.03	8 ± 1.62	17 ± 0.33
B. subtilis	12 ± 0.47	8 ± 0.84	12 ± 1.22	13 ± 0.57	7 ± 1.56	8 ± 1.42	14 ± 0.35
M. luteus	23 ± 1.53	15 ± 1.43	22 ± 1.68	25 ± 0.82	12 ± 0.89	18 ± 1.07	25 ± 1.36
Gram negative							
E.coli	18 ± 1.12	7 ± 1.15	8 ± 1.16	10 ± 0.98	7 ± 1.34	10 ± 1.76	22 ± 1.39

Each value is expressed as means ± standard deviation (n=3)

[a] include disc diameter (6.0 mm)

Table 4. Minimal inhibition concentration of the crude extracts from the leaves and stems of *T. trifolia*

Bacterial Strain	MIC (μg/ml)						
	Standard	Leaves			Stem		
	Tetracycline	Hexane ext.	CH_2Cl_2 ext.	EtOH ext.	Hexane ext.	CH_2Cl_2 ext.	EtOH ext.
Gram positive							
S. aureus	1.95	500	500	250	500	250	7.81
S. epidermidis	1.95	500	500	125	500	125	7.81
B. subtilis	1.95	500	15.62	7.81	500	62.50	1.95
M. luteus	< 0.98	125	3.91	< 0.98	125	31.25	< 0.98
Gram negative							
E.coli	3.91	500	250	125	500	125	7.81

CONCLUSION

The preliminary phytochemical screening results of the all crude extracts from the *T. trifolia* leaves and stems found alkaloids, flavonoids, tannins and triterpenoids. The crude ethanolic extract from the stems possessed strong antimicrobial activity against *M. luteus*. Consequentlt, the bioactive compounds present in the stems of *T. trifolia* might be used as a potential source for novel drugs. However, pharmacological activity and toxicity should be further evaluated.

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