Phytochemical Evaluation and Antimicrobial Activity of Stem of *Tinospora sinensis* (Lour.) Merr.

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Abstract

The Magars in Bulingtar rural municipality, Nawalpur district, Gandaki province have been using *Tinospora sinensis*for the treatment of urinary tract infection (UTI). Phytoconstituents present in 70% methanolic extract and hexane extract of stem of the species were evaluated by phytochemical screening, and gas chromatography and mass spectrometry(GC-MS) analysis. Antimicrobial activitiy screenings of the extracts were carried out against *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa*which are UTIcausing bacteria along with*Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella enterica* sub-sp. *enterica*serovarTyphi and *Klebsiella pneumoniae*.Antibiotics such as ampicillin and gentamicin were used as positive controls.A total of 35 compounds were identified in the extracts with high percentage of steroids, fatty acids along with triterpene, vitamins etc by GC-MS analysis. The extracts showed antimicrobial activities against *E. coli, P. aeruginosa, B. subtilis* and *S.* Typhi. The extracts showed moderate to weak antimicrobial activity against UTI causing bacterial species supporting the local use. The antimicrobial activity may be due to the presence of phytoconstituents such as: 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one; 2-methoxy-4-vinylphenol;hexadecanoic acid, methyl ester; octadecanoic acid; stigmasterol and vitamin E.

Keywords: Bacteria, GC-MS analysis, Magars, Phytoconstituents, Urinary tract infection

Introduction

Tinospora sinensis (Lour.) Merr. is known as Malabar gulbel (English name), Gurjo-ko-laharaa (Nepali), chini lahara (Magar) and Amrta (Sanskrit). *T. sinensis*has been used in Ayurvedic and Homeopathic medicines, particularly in jaundice, fever, rheumatism, gonorrhea, diabetes and several other ailments as an alternative drug for *Tinospora cordifolia* (Willd.) Miers (Hegde & Jayaraj, 2016). Traditionally, the stem is used to treat debility, dyspepsia, fever, inflammation, ulcer, jaundice, urinary disease andliver disease (Akhtar et al., 2000; Devi et al., 2014). Leaves, roots and shoots are used by the Tharus of Nawalparasi district for urinary complaints, dysentery, gastric, stone, fracture and sprain (Ghimire, 2000). Stem is used for stomach problem (Joshi et al., 2011; Manandhar, 2002),whereas leaves are used for stomach problem by the Darai tribe of Chitwan distrct (Dangol & Gurung, 2000). In traditional Chinese medicine, *T. sinensis* is used for relieving rigidity of muscles and activating collaterals (Lam et al., 2018). Compounds from stem of *T. sinensis* were purified and examined for inhibition of superoxide anion generation and elastase release, thereby evaluating their in vitro anti-inflammatory potentials. From the experiment it was concluded that the extracts and purified compounds of the stems of *T. sinensis* have the potential to be developed as novel anti-inflammatory lead drugs or health foods (Lam et al., 2018).

Antimicrobial activity of *T. sinensis* was reported previously. Its roots and leaves exhibit marked antimicrobial activity against *Staphylococcus aureus* (Sandhyarani & Praveen, 2014; Hegde & Jayaraj, 2016). Devi et al. (2014) evaluated antimicrobial activities of ethanolic, methanolic, aqueous and chloroform extracts of the leaves, stems and flowers against Gram-negative as well as Gram-positive bacteria.

During our ethnobotanical survey, we came to know that the juice of the stem of *T. sinensis* has been used for the treatment of urinary tract infection (UTI) by

the Magar community in Nawalpur district, Gandaki province, Nepal. To validate the ethnomedicinal knowledge, the present research was focused on evaluation of antibacterial activities of stem extracts of *T. sinensis*. The phytochemicals present in the plant material were also investigated by chemical tests and gas chromatography and mass spectrometry (GC-MS) analysis to identify the biologically active phytochemicals.

Materials and Methods

Sampling site

The study site, Bulingtar rural municipality of Nawalpur district, Gandaki Province, Nepal, was visited in April 2016. Ethnomedicinal data of *T. sinensis* in the Magar community was collected through questionnaires, structured and un-structured interviews among healers and knowledgeable people. The plant sample was collected keeping in mind the conservation of local genetic diversity. Voucher specimen was identified by Prof. Dr. Ila Shrestha, Patan Multiple Campus Patan Dhoka, Lalitpur.

Preparation of the extracts

The collected plant samples were dried in shade at room temperature. Air dried samples were ground and successively extracted with hexane and 70% methanol in water using a Soxhlet extractor until a clean solution was noticed. The extracts were concentrated using a rotary evaporator and vacuum dried. The dried extract was stored in a refrigerator at 4^{o}C for further use.

Phytochemical screening

Phytoconstituents present in the stem extracts were analyzed using different specific reagents. Braymer, Dragendorff, Shinoda, Liebermann-Burchard, Salkowski andfroth tests were carried out to detect polyphenols, alkaloid, flavonoids, steroids, terpenoids and saponins respectively.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses of the hexane and 70% methanolic extracts of *T. sinensis* were performed using an Agilent 7890A GC system coupled with an Agilent 5975 C mass selective detector, equipped with a HP-5MS GC column (5% phenyl methyl siloxane, Agilent 19091S- 433, 30 m × 250 μm internal diameter, 0.25 μm film thickness). Helium was used as a carrier gas at flow rate of 1.21 mL/min. The instrument was operated in the electron impact (EI) mode at 70 eV and ion source temperature 230°C in the scan range of 50-500 m/z. The initial column temperature was set at 40°C held for 2 min, ramped at a rate of 4°C/min to 270°C and held for 5.5 min (total run time 65 min). Dilute sample solutions of the extracts were prepared in HPLC grade hexane or methanol, and a volume of 2 μL was injected. The constituents were identified by comparing the mass spectra available in a MS database of National Institute Standard and Technology (NIST 08).

Antibacterial susceptibility assay

Leading etiological agents of UTIs include *Escherichia coli, Enterococcus faecalis, Pseudomonas. aeruginosa* (Svanborg & Godaly, 1997; Shankar, et al., 2001). Hence, the bacterial strains *Esherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27263) along with *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6051), *Salmonella enterica* subsp. *enterica*serovar Typhi (Clinical isolate) and *Klebsiella pneumoniae* (ATCC 700603) were used for antimicrobial assays.The cultures of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *K. pneumoniae* were collected from Shukraraaj Tropical and Infectious Disease Hospital (STIDH), while those of *Enterococcus faecalis*, *B. subtilis* and *Salmonella enterica* subsp. *enteric* serovar Typhi were collected from Department of Plant Resources, Thapathali Kathmandu, Nepal. These bacteria were sub-cultured in sterile Mueller-Hinton agar (MHA) media.

Agar well diffusion assay: Inoculum was prepared by suspending 3-4 isolated colonies in 5 ml of sterile Mueller-Hinton broth (MHB) and standardized by comparing with McFarland 0.5 standard. Thus preparedinoculums were swabbed on sterile Mueller-Hinton agar (MHA) plates. The hexane and 70% methanolic extracts weredissolved in dimethyl sulfoxide (DMSO) to prepare sample

solutionsof 0.1 g/mL concentration. Wells were bored on the MHA plates with of 6 mm diameter cork borer. The wells were loaded with 50 µL of the samples prepared.Ampicillin and gentamicin (Mast Diagnostics) discs of 10 µg/disc wereused as standards. For negative control DMSO was used. The loaded plates were incubated at 37°C for 18–24 hours following Clinical and Laboratory Standards Institute (2012). Zone of inhibition (ZOI) was measured in mm.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

50 µL of the prepared extract solutions of 0.1 g/mL concentration were mixed with Mueller-Hinton broth (MHB) (50 µL) and then the content was serially double diluted in wells of 96 well microplate. The standardized suspension was further diluted to 1:100 using MHB and then 50 µL of the suspension was inoculated. Thus prepared microplate was incubated for 24 h at 37°C. The minimum inhibitory concentration (MIC) value was taken as the lowest concentration at which there was no turbidity seen by naked eye. Minimum bactericidal concentration (MBC) values were then determined as no colony growth (or growth by 10^{-1}%) by direct streaking the content of the wells inhibiting bacterial growth (MIC and concentrations higherthan the MIC value) on sterile MHA plates.

Statistical analysis

Statistical analysis was done using Microsoft Excel. Experiments were performed in triplicates (n = 3) and the results are presented as mean ± standard error of mean (SEM).

Results and Discussion

Phytoconstituents

Successive Soxhlet extractions of the stem of *Tinospora sinensis* (100 g) yielded hexane extract (0.5g, 0.5%, greenish color) and 70% methanolic extract (9.7g, 9.7 %, dark brown). Phytochemical screening of the extracts revealed that the stem of *T. sinensis* constituted terpenoids, polyphenols, alkaloids, steroids and saponins. Similarly alkaloids, phenolics, steroids, tannins and saponins were reported from various extracts of leaves and stem of *T. sinensis* on preliminary phytochemical screening (Jain et al., 2010; Vijaya & Aruna, 2014; Hegde et al., 2015a; Hegde et al., 2015b; Khandelwal, 2000).

Table 1: Phytoconstituents identified from 70% methanolic extract of *Tinospora sinensis* stem

S. N.	R. T.	Compound	Peak area %	Compound nature
1	15.229	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	1.41	Flavonoid fraction
2	21.099	2-Methoxy-4-vinylphenol	3.17	Phenol
3	22.359	2,6-dimethoxy phenol	1.13	Phenol
4	23.859	Vanillin	2.11	Aldehyde
5	27.727	Dodecanoic acid, methyl ester	2.21	Fatty acid
6	32.697	2,6-dimethoxy-4-(propenyl)phenol	1.54	Phenol
7	33.662	4-(1E)-3-Hydroxy-1-propenyl -2-methoxyphenol	9.17	Phenol
8	36.985	1,2-Benzenedicarboxylic acid, diphenyl ester	1.39	Ester
9	38.430	Hexadecanoic acid, methyl ester	4.49	Fatty acid
10	39.319	n-Hexadecanoic acid	10.04	Fatty acid
11	42.331	9, 12-Octadecadienoic acid (Z,Z)-, methyl ester	4.28	Fatty acid
12	42.478	9-Octadecenoic acid-, methyl ester, (Z)-	5.56	Fatty acid
13	43.089	Octadecanoic acid, methyl ester	1.69	Fatty acid
14	43.215	9,12-Octadecadienoic acid, (Z,Z)-	6.51	Fatty acid
15	43.345	Oleic acid	15.50	Fatty acid
16	43.880	Octadecanoic acid	2.72	Fatty acid
		Total	72.92	

The only alkaloid detected in the stems of *T. sinensis* is palmatine (Bisset & Nwaiwu, 1983).

Next, the extracts were used for the GC-MS analyses. Total of 35 compounds were identified from the extracts of *T. sinensis* stem. Sixteen compounds, accounting for 72.92%, were identified in the 70% methanolic extract of the stem of *T. sinensis* by GC-MS analysis (Table 1). As a flavonoid fragment, the extract constituted of 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, which was reported as an antimicrobial and anti-inflammatory agent (Kumar et al., 2010). 2-Methoxy-4-vinylphenol exhibits antioxidant, antimicrobial and anti-inflammatory activities (Al-Marzoqi et al., 2016). Oleic acid is reported as anti-inflammatory, anti-androgenic, cancer preventive, etc. (Alagammal et al., 2011). Hexadecanoic acid, methyl ester (Rukshana et al., 2017) and octadecanoic acid was reported to be antimicrobial (Mujeeb et al., 2014).

Phytoconstituents identified in the hexane extract by GC-MS analysis are presented in Table 2. Nineteen compounds (accounting for 93%) were identified in the hexane extract with a higher percentage of steroids (42.23%) and then fatty acids (33.46%) followed by hydrocarbons (11.55%), triterpene (3.35%), vitamin (1.69%), ketone (0.46%) and alcohol (0.26%). Steroids such as stigmasterol are reported as antioxidant, antibacterial, anti-inflammatory, antiarthritic, antiasthma and diuretic (Tyagi & Agarwal, 2017). Sitosterol exhibits strong antifungal, antibacterial and anti-angiogenic activities (Raman et al., 2012). It has been reported that many fatty acids possess antibacterial and antifungal properties (Knapp & Melly, 1986). Vitamin E has beenreported as antioxidant, antimicrobial, analgesic, antidiabetic, anti-inflammatory, antidermatitic, antileukemic, antitumor, anticancer, hepatoprotective and antispasmodic (Mujeeb et al., 2014). Betulin is antiviral, analgesic, anti-inflammatory and antineoplastic agent ("Showing metabocard", n.d.).

Table 2: Phytoconstituents identified from the hexane extract of *T. sinensis*

S. N.	R. T.	Compound name	Peak area %	Compound nature
1	39.368	n-Hexadecanoic acid	10.75	Fatty acid
2	40.061	Hexadecanoic acid, ethyl ester	0.37	Fatty acid
3	42.080	9-Dexadecenoic acid	0.19	Fatty acid
4	42.320	10,13-Octadecadienoic acid, methyl ester	0.26	Fatty acid
5	42.467	6-Octadecenoic acid	0.29	Fatty acid
6	42.740	E-10,13,13-trimethyl-11-tetradecen-1-ol acetate	0.26	Alcohol
7	43.247	9,12-Octadecadienoic acid, (Z,Z)-	5.20	Fatty acid
8	43.389	cis-Vaccenic acid	12.29	Fatty acid
9	43.891	Octadecanoic acid	2.59	Fatty acid
10	43.967	(E)-9-Octadecenoic acid, ethyl ester	0.91	Fatty acid
11	47.961	beta-Sitosterol	32.63	Steroid
12	51.627	3',8,8'-Trimethoxy-3piperidyl-2, 2'-binaphthalene-1,1',4,4'-tetrone	0.46	Ketone
13	52.008	Docosanoic acid	0.61	Fatty acid
14	54.327	Ethyl isoallocholate	0.39	Steroid
15	61.342	17-Pentatriacontene	11.55	Hydrocarbon
16	61.883	Vitamin E	1.69	Vitamin
17	63.465	Betulin	3.35	Triterpene
18	63.579	Campesterol	4.21	Steroid
19	64.397	Stigmasterol	5.00	Steroid
		Total	93.00	

Antimicrobial assays

Results of the antibacterial susceptibility assay of both the hexane and 70% methanolic extracts are given in Table 3 showing Zones of Inhibition (ZOI). The 70% methanolic extract showed antibacterial activity against *Pseudomonas aeruginosa* and *Bacillus subtilis*. The results obtained by using 70% methanolic extract showed antimicrobial activity against *Eshcerichia coli* and a moderate activity against *B. subtilis* and *P. aeruginosa*, and no antimicrobial activity against rest of the tested bacteria. Similarly Shakya et al., (2008) reported antimicrobial activity of 50% ethanolic extract of *Tinospora sinensis* stem against *B. subtilis* and *E. coli* but not against *Salmonella enterica* subsp. *enterica* serovar Typhiand *Staphylococcus aureus*.

Devi et al. (2014) reported methanolic extract of leaf, stem and flower of *T. sinensis* had not shown antibacterial activity against Gram-positive and Gram-negative bacterial strains, but it was found effective against *Candida albicans*.

The antimicrobial activities of 70% methanolic extract may be due to presence of 2,3 dihydro 3,5 dihydroxy 6 methyl 4H pyran 4 one and 2-methoxy-4-vinylphenol The hexane extract was also found effective to inhibit the growth of *B. subtilis*, *E. coli* and *Salmonella enterica* subsp. *enterica* serovar Typhi. Perhaps the antibacterial activity observed was mainly due to the presence of fatty acids.

The result of MIC and MBC of the 70% methanolic extract and hexane extract areshown in table 4. The result showed that 70% methanolic extract was bactericidal against *B. subtilis*, and bacteriostatic against *E. coli* and *P. aeruginosa*. The hexane extract also showed bactericidal activity against *B. subtilis*.

Conclusion

The Magar community of Bulingtar rural municipality, Nawalpur district, Gandaki Province, Nepal uses juice of fresh stem of *Tinospora sinensis* for UTI. The result of antimicrobial assay showed antimicrobial activity against UTI causing bacteria supporting the local use of *T. sinensis* for UTI. But the antimicrobial activity was weak to moderate and the extract killed the bacteria only at high concentrations. The result of GC-MS analysis showed presence of antimicrobial compounds as well as high percentage of steroids which have been

Table 3: Antibacterial activity of the extracts of *Tinospora sinensis* stem

Sample	Diameter of ZOI±SEM (in mm)						
	Gram positive bacteria			Gram negative bacteria			
	Sa	***Bs***	***Ef***	***Ec***	***St***	***Kp***	***Pa***
Hexane extract	-	9±1	-	10.5±0.5	9±1	-	-
70% Methanolic extract	-	11±0	-	8±0	-	-	12±0.48
Ampicillin	32.50±0.50	8.50±0.50	17.75±0.25	25.00±1.00	15.50±0.50	8.50±0.50	–
Gentamicin	16.75±0.25	15.50±0.50	18.5±0.50	17.50±0.50	12.66±0.33	11.33±0.88	14.66±0.33
DMSO	–	–	–	–	–	–	–

Note: *Sa = Staphylococcus aureus, Bs = Bacillus subtilis, Ef = Enterococcus faecalis, Ec = Escherichia coli, St = Salmonella enterica* subsp. *enterica* serovar Typhi*, Kp = Klebsiella pneumoniae, Pa = Pseudomonas aeruginsa*

Table 4: MIC and MBC values of the extracts of *Tinospora sinensis* stem

S.N.	Bacterial strain	Hexane extract		70% Methanolic extract	
		MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
1	*Staphylococcus. aureus*	-	-	-	-
2	*Bacillus subtilis*	50	50	25	25
3	*Enterococcus faecalis*	–	–	-	-
4	*Escherichia coli*	50	Na	>50	>50
5	*Salmonella enterica* subsp. *enterica* serovar. Typhi	50	>50	–	–
6	*Klebsiella pneumonia*	–	–	-	-
7	*Pseudomonas aeruginosa*	-	-	12.5	50

reported as showing anti-inflammatory, antiarthritic, antiasthma,diuretic and anti-angiogenic activities. Lam et al. (2018) concluded from their work on *T. sinensis* that the stems of *T. sinensis* have the potential to be developed as novel anti-inflammatory lead drugs or health foods.

Author Contributions

All the authors were involved in the research. C. M. Nemkul visited the study site, collected plant materials, and performed phytochemical screening, GC-MS analysis and antimicrobial assays in the laboratory. G. B. Bajracharya helped in chemical analysis by GC-MS and reviewed the manuscript. I. Shrestha reviewed the ethnobotanical part of the manuscript.

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