Characterization of a Bioactive Compound from *Tinospora cardifolia* Having Activity Against Wide Range of Bacteria and Fungi

Prachi Gupta and Saurabh Kulshrestha*

Faculty of Applied Sciences and Biotechnology, Shoolini University of Biotechnology and Management Sciences, Bajhol, Solan, India.

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Considering the vast potentiality of Tinospora cardifolia as a source for antimicrobial drugs with reference to antibacterial, antifungal and anticandida agents, a systematic investigation was undertaken to screen 17 samples of T. cardifolia accessions collected from different Districts of Himachal Pradesh for its activity against gram positive and gram negative bacteria, Fungus and Candida strains. The zone of inhibition varied with the plant extract, the solvent used for extraction and the organism tested. Acetonic leaf extract was found to be more potent being capable of exerting significant inhibitory activity than other extracts. Highest antimicrobial activity was observed with the acetonic leaf extract of T. cardifolia collected from Baijnath, District Kangra. FT-IR analysis of the acetonic leaf extract revealed the presence of different functional groups at wave numbers corresponding to the functional groups of a number of compounds including Berberine. Thin layer chromatography and HPLC of acetonic leaf extract of T. cardifolia indicated alkaloid as a major active compound which was further confirmed to be Berberine. Active component was isolated and purified by recrysyallization method and tested for its antibacterial activity. The isolated compound was characterized by HPLC, LCMS, GCMS, H1 and C13 NMR. HPLC peak of isolated Berberine at retention time 4.024 mins was observed which was same as that of standard Berberine. Molar mass observed by LCMS and GCMS was 336.1 which were same as that of standard Berberine. NMR also confirms the presence of 18 H atoms as that present in standard Berberine.

Keywords: *Tinospora cardifolia,* Antimicrobial, Berberine, HPLC, NMR.

Medicinal plants are a source of great economic value all over the world. Herbal medicines in Asia represent a long history of human interaction with the environment (Vedhanarayanan *et al*., 2013). The medicinal value of plant lies in some chemical substances that produce a definite physiologic action on the human body. The most important of these phytocompounds are alkaloids, flavanoids, tannins and phenol. Himachal Pradesh in India contains vast number of medicinal plants and local people are utilizing these medicinal plants since years ago (Jogi and Akkewar, 2012). Plants are nature's "chemical factories" providing richest source of organic chemical on earth. In Indian scenario it has been recognized that 2,500 plants have been found to have medicinal value out of 17000 and Himachal Pradesh, one of the pioneer western Himalayan States is a rich repository of medicinal flora and have a great wealth of traditional and folklore medicinal knowledge (Jain *et al*., 1991). The World is looking forward to India for new drugs to manage various challenging diseases because of its rich biodiversity of medicinal plants to cure diseases (Cohen *et al*., 1991; Dennis *et al*., 1998; Salahuddin *et al*., 1998). *Tinospora cordifolia* which is also known by the common name Guduchi, is an herbaceous vine

* To whom all correspondence should be addressed.
Tel:- +91-1792-308000 Ex. 2022, +91-9459241393
Fax:- +91-1792-308000;
E-mail: saurabh_kul2000@yahoo.co.in

of the family *Menispermaceae* indigenous to the tropical areas of India, Myanmar and Sri Lanka. In Ayurveda, *T. cordifolia* is considered one of the most divine herbs. Present investigation was designed to study the antimicrobial potentiality of different accessions of *T. cardifolia* collected from different districts of Himachal Pradesh. Phytochemical screening, TLC and HPLC was carried out to identify major biologically active phytoconstituents. Further, the active compound was isolated and confirmed by HPLC, LCMS, GCMS, H^1 NMR and C^{13} NMR.

MATERIALS AND METHODS

Collection of plant materials

The leaf material has been collected from 17 villages of seven districts i.e Kangra, Solan, Una, Mandi, Bilaspur, Shimla and Hamirpur of Himachal Pradesh for antibacterial assay. The samples were labelled and collected in ice box from the fields. A total of 17 accessions representing different geographical locations like Jaisinghpur, Baijnath, Daladaghat, Daulatpur, Lahla, Berthin, Sulatanpur, Basantpur, Daldi, Rati, Arohi, Ghanati, Kandaghat, Ghumarvi, Dhamsal, Una, Badsal were included in the present study. The botanical identity of plant was identified taxonomically and authenticated from literature available in Department of Botany, Shoolini University, Solan.

Surface sterilization of plant and Drying

The leaves of *T. cardifolia* were thoroughly washed with tap water and then with 1% $HgCl_2$. Final washing was done with autoclaved distilled water for about 2 minutes. The leaves were dried under shade for about ten days. The dried plant sample was crushed well using mortar pestle into a fine powder. The powder was stored in an air sealed polyethylene bag at room temperature before being used for analysis.

Extraction of material

The dried powder (25gms) was extracted with various solvents having different polarity like methanol, acetone and chloroform using a Soxhlet extraction for 10 hours or until the extract was cleared at 5°C less temperature than respective boiling point of the solvents. Solvent solution was poured to the autoclaved petriplates and the solvent was evaporated at 40°C. Dark gummy residue was obtained. Residue was suspended in ethanol giving the concentration of 400 mg/ml for antimicrobial assay. Extract was transferred to autoclaved falcon tubes and was stored at -20°C.

Preparation of Bacterial Culture for Assay

Standard isolates of gram positive and gram negative bacteria i.e *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa* were obtained from the Department of Microbiology, Shoolini University, Solan. All the test strains were maintained on nutrient agar slants (Hi-Media Laboratories Pvt. Limited, Mumbai, India) at 4°C and subcultured on to nutrient broth for 24 h prior to testing. These bacteria served as test pathogens for antibacterial activity assay.

Preparation of Fungal Culture for Assay

Standard isolate of Fungus i.e *Fusarium* was obtained from the Department of Microbiology, Shoolini University, Solan. All the test strains were maintained on potato dextrose agar slants (Hi-Media Laboratories Pvt. Limited, Mumbai, India) at 4°C and subcultured on to potato dextrose broth for 36 h prior to testing. This fungus served as test pathogen for antifungal activity assay.

Preparation of Candida Culture for Assay

Standard isolates of Candida strains i.e *Candida albicans* and *Crytococcus neoformens* were obtained from the Department of Microbiology, Shoolini University, Solan. All the test strains were maintained on YPD slants (Hi-Media Laboratories Pvt. Limited, Mumbai, India) at 4°C and subcultured on to YPD broth for 24 h prior to testing. These strains served as test pathogens for anticandida activity assay.

Antibacterial Assay

Antibacterial activity of solvent extracts was determined by Cup Plate method. Hard agar plates were then prepared and checked for sterility. Inoculum containing 10^6 cfu/ml of each bacterial culture to be tested was mixed with soft agar. Then soft agar was poured on hard agar plates. Subsequently, wells of 8 mm diameter were punched into the agar medium and filled with 100µl (25 mg/ml) of plant extract and allowed to diffuse at room temperature for 2 h. The plates were then incubated in the upright position at 37°C for 24 h. Wells containing the same volume of ethanol served as negative controls while standard antibiotic discs of streptomycin (30µg) were used as the positive controls. After incubation, the

diameters of the growth inhibition zones were measured in mm with zone measuring scale (Hi Media). Three replicates were carried out for each extract against each of the test organism.

Antifungal assay

Antifungal activity of solvent extracts was determined by Cup Plate method. Hard agar plates were then prepared and checked for sterility. Fungal broth was blended in autoclaved plastic beaker with the help of blender to make slurry. Then soft agar was mixed with fungal broth slurry (5ml) and poured on hard agar plates. Subsequently, wells of 8 mm diameter were punched into the agar medium and filled with 100µl (25 mg/ml) of plant extract and allowed to diffuse at room temperature for 2 h. The plates were then incubated in the upright position at 28°C for 36 h. Wells containing the same volume of ethanol served as negative controls while standard antibiotic discs of ciprofloxin (30µg) were used as the positive controls. After incubation, the diameters of the growth inhibition zones were measured in mm with zone measuring scale (Hi Media). Three replicates were carried out for each extract against each of the test organism.

Anticandida activity

Anticandida activity of solvent extracts was determined by Cup Plate method. Hard agar plates were then prepared and checked for sterility. Inoculum containing candida culture to be tested was mixed with soft agar. Then soft agar was poured on hard agar plates. Subsequently, wells of 8 mm diameter were punched into the agar medium and filled with 100µl (25 mg/ml) of plant extract and allowed to diffuse at room temperature for 2 h. The plates were then incubated in the upright position at 25° for 36 h. Wells containing the same volume of ethanol served as negative controls while standard antibiotic discs of flucanazole (30µg) were used as the positive controls. After incubation, the diameters of the growth inhibition zones were measured in mm with zone measuring scale (Hi Media). Three replicates were carried out for each extract against each of the test organism.

Phytochemical analysis

Acetonic leaf extracts was subjected to phytochemical analysis to ascertain the presence of metabolites such as steroids, alkaloids, proteins, carbohydrates, flavanoids, tannins, saponins and terpenoids.

FT-IR Analysis of Plant Extract

FT-IR was done to confirm the functional groups present in the acetonic extract of plant collected from Baijnath district (Kangra), which gave the maximum activity against bacteria, Fungi and Candida. Plant extract was boiled in 20 ml methanol till the volume of methanol was reduced to half and then poured into the sterilized petriplate. Methanol was allowed to evaporate. Acetonitrile was added dropwise to the plate and the gummy extract in the plate was scrapped with the spatula and collected in the eppendroff 5 ml of ethanol was added to the extract to dissolve it completely. The extract was applied on the sensory plate in IR reader with the help of capillary tube. Peaks corresponding to various functional groups were obtained in the form of graph.

Thin layer chromatography

The phytocompounds of the acetonic leaf extract of *T. cardifolia* collected from Baijnath of District Kangra showed significant antimicrobial activity were analysed using thin layer chromatography (TLC). About 10µl of each extract was applied on precoated aluminium silica gel 60 F 254 pre coated plates. Developing solvent system used was butanol: Acetic acid: water (50:10:40). The plates were activated for one hour at 110 C and allowed to cool to room temperature and humidity. The developed chromatograms were examined by spraying the plate with draggendroff reagent. Individual R_f for each spot was measured.

HPLC of plant extract

A Shimadzu model HPLC (Kyoto, Japan) with C-18 reverse phase column was utilized for establishing phytochemial profile. The column and HPLC system were kept at ambient conditions. The injection volume used was 20µl. The elution was done with acetonitrile–water (60:40, v/v), at a flow rate of 0.5 mL/min and the elute was analyzed at a wavelength of 265 nm.

Isolation and purification of active compound

TLC Plate was developed and required fraction was scrapped and collected in eppendroff tube. Mixture was dissolved in HPLC grade methanol and shaked. Centrifuged at 6000 rpm for 5 minutes. Supernatant was collected and filtered. Supernatant was dried under reduced pressure. Filterate was dissolved in methanol for further analysis.

Antimicrobial Determination of active compound

The purified compound was tested for its antimicrobial activity against gram positive and gram negative bacteria i.e. *Staphylococcus aureus* and *Eschreichia coli* by cup plate method as described above.

Characterization of Purified Compound

Chromatography analysis

HPLC Analysis of the purified compound was done. Same parameters were followed as in the HPLC of plant extract.

Spectroscopic analysis

Multi Component Analysis (MCA) was done with different spectroscopic methods. Characterization and quantification of purified compound was done by LC/MS, NMR (H1 and C13) and GC/MS.

RESULTS AND DISCUSSION

Out of 51 extracts (acetone, chloroform and methanol) of 17 accessions of *T. cardifolia* collected from 17 villages of 7 districts i.e Kangra, Solan, Una, Mandi, Bilaspur, Shimla and Hamirpur of Himachal Pradesh, screened for potential antimicrobial activity against gram positive and gram negative bacteria, Fungus and Candida strains, acetonic leaf extract of *T. cardifolia* collected from Baijnath village of District Kangra provided most consistent and prominent antimicrobial activity as compared to other two solvent extracts (Table1). The methanol extracts showed the least antibacterial activity as compared to other two solvents. The reason for minimal antimicrobial activity in methanol extract could be a low concentration of antibacterial compounds in these extracts. None of the chloroform extracts were found to be effective against any of the assayed pathogen. Chloroform extract may contain a low concentration of antimicrobial compounds or may not have antimicrobial compounds. All the identified components from plants which are active against microorganisms are obtained through initial acetonic extraction.

All the plant extracts tested showed antimicrobial activity, however, the plants from different locations differ in their activities against the assayed pathogens. Highest antibacterial activity was observed with the acetonic leaf extract of *T. cardifolia* collected from Baijnath village of District Kangra (H.P), against gram positive and gram negative bacteria i.e *Bacillus subtilis* (14mm), *Staphylococcus aureus* (18mm), *Escherichia coli* (16mm) and *Pseudomonas aeruginosa* (15mm) respectively. Highest antifungal activity against

Table 1. Antibacterial activity of acetonic leaf extract of *Tinospora cardifolia* determined by agar well diffusion

S.	Place of	Zone of inhibition (mm)						
No.	Collection	*B.bubtilis*	*S.aureus*	*E.coli*	*P.aeuroginosa*	*Fusarium*	*C.albicans*	*C.neoformens*
1	Baijnath	14	18	16	15	16	15	16
2	Daldaght	12	12	13	13	14	13	11
3	Daulatpur	13	12	13	14	12	nil	11
4	Jaisinghpur	11	10.6	14	13	Nil	13	13
5	Lahla	nil	13	12	12	14 .5	nil	13.5
6	Ghumarvi	10	12	11	12	14	12	11
7	Sultanpur	12	12	10	12	Nil	10	nil
8	Dhamsal	10	12	14.5	13	Nil	13	10
9	Arohi	10	10	10	12	12	12	10
10	Basantpur	Nil	12	10	10	13	10	11
11	Berthin	10	11	11	13	nil	13	10.5
12	Barsal	10	13	10	10	14	10	nil
13	Ghanati	nil	10	nil	13.5	11	10	12
14	Kandaghat	11.5	10	nil	10	12	nil	11
15	Una	nil	11	11	12.5	nil	nil	11.5
16	Daldi	10	13	10	10	14	10	nil
17	Rati	nil	10	13	12	nil	12	nil

Fusarium (16mm) was also observed in acetonic leaf extract of *T. cardifolia* collected from Baijnath village of District Kangra (H.P). In the present investigation the acetonic leaf extract of *T. cardifolia* collected from Baijnath District Kangra (H.P) showed the promising broad spectrum anticandida activity agains *Candida albicans* and *Cryptococcus neoformans* with the zone of inhibition of 15mm and 16mm respectively. Results obtained in the current study revealed that studied herbal extracts possess potential antimicrobial activity against entire tested organisms, but the acetonic leaf extract of *T. cardifolia* collected from Baijnath, District Kangra (H.P) was found to have shown the strongest and broadest spectrum. First report of highest antimicrobial activity from acetonic leaf extract of *T. cardifolia* collected from Baijnath village of District Kangra, Himachal

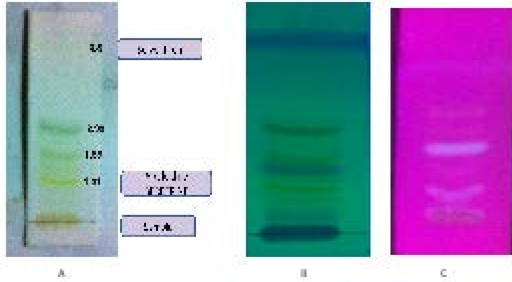
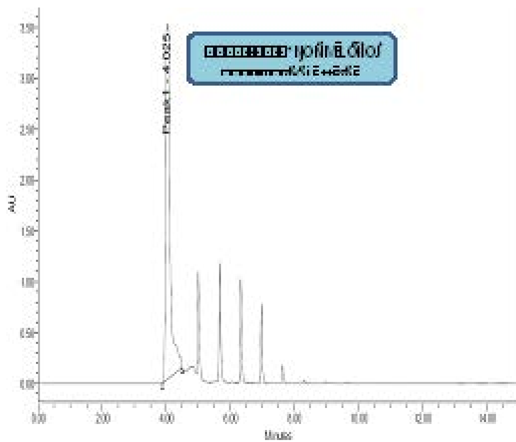


Fig. 1. Thin layer chromatography (TLC) of plant extract: Chromatogram in (A) visible light, (B) Short UV light and (C) Long UV light



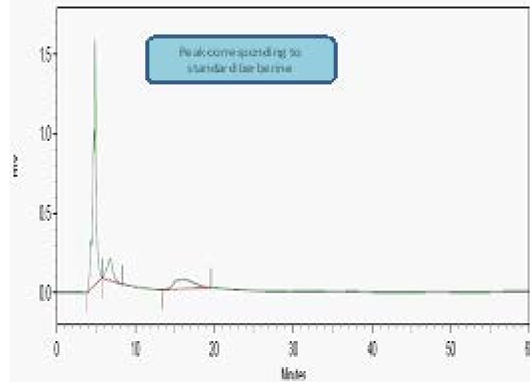
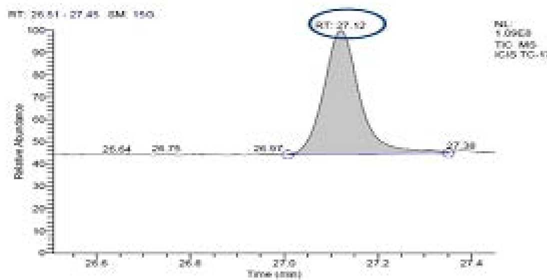
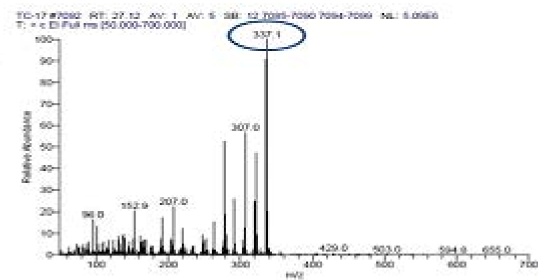


Fig. 2. (A) HPLC graph of standard Berberine; (B) HPLC peak no.1 corresponds to the isolated and purified Berberine and smaller peaks corresponds to the degradation products





Library Search Results Table

Compound Name	RT	Probability	Molecular Formula
Berbine, 13,13a-didehydro-9,10-dimethoxy-2,3-(methylenedioxy)-	27.12	85.35	C20H19NO4
Berberine hydrochloride	27.12	7.60	C20H18ClNO4
5,5-Dimethyl-6,6a-dihydro-3-methoxy-11-oxoisoindolo[2,1-a]quinoline-10-carboxylic acid	27.12	2.54	C20H19NO4

Fig. 3. Peaks showing molar mass of (A) purified Berberine (B) compound search table

Pradesh. Present investigation also demonstrated that environmental factors and geographical location also contributed to the variation in concentration of secondary metabolite which leads to variations in antimicrobial activity as has been seen in the present investigation.

Results of phytochemical analysis (Table 2) revealed the presence of alkaloids, carbohydrates, tannins, saponins, phenols and terpenoids but the absence of flavanoids and proteins in the acetonic leaf extract of *T. cardifolia* collected from Baijnath, District Kangra (H.P). It is well known that these phytochemicals have already exhibited antimicrobial activity (Cowan, 1999). The results are different to those reported by Mishra and coworkers (Mishra *et al*., 2014) who reported the presence of proteins and phenolics in the hydroacetonic extract of *T. cardifolia* but in line with other phytoconstituents reported i.e the presence of alkaloids, steroids and phenolics and absence of flavanoids in the hydroacetonic and ethanolic extract of the plant. The absence of phenols in the petroleum ether and water extract of *T. cardifolia* was in contradiction to the present results but the presence of alkaloids, tannins, steroids, carbohydrates and terpenoids were in line with the present study (Grover *et al*. 2013). It is because of variation in polarity of extraction solvent that leads to the solubilisation of only particular type of metabolites.

Table 2. Antibacterial activity of Berberine shown as zone of inhibition produced

Active	Microorganism	Zone of inhibition
compound	*Staphylococcus aureus*	
	50 µg/ml	14mm
Berberine	80 µg/ml	15mm
	Escherichia coli	
	50 µg/ml	11mm
	80 µg/ml	14mm
	100 µg/ml	15mm

TLC analysis revealed the presence of alkaloid in the acetonic leaf extract of *T. cardifolia*. The acetonic leaf extract of *T. cardifolia* showed different Rf values ranging from 0.08-0.78 in visible, short UV light and long UV light. A total

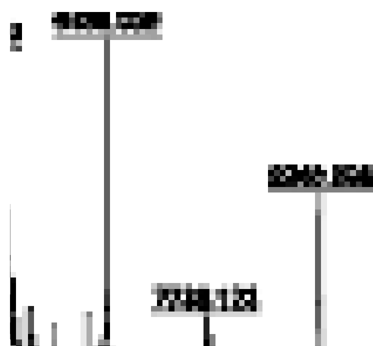


Fig. 4. (A) structure of Berberine; (B) peaks showing number of Protons as singlet, doublet and triplet (C) library and NMR chromatogram showing full Range of peaks observed in purified Berberine

no. of 10, 12 and 2 spots were observed in the chromatogram in visible light, short UV and long UV light respectively (Fig. 1). Out of 12 spots one Rf value calculated from the spot observed at the distance of 1.31 cm which corresponds to the Rf value of 0.551 i.e Berberine (alkaloid) was found to be universaly present in the plants and this was used as a chemical marker of the plant. These findings corroborated with the observations of (Gahlaut *et al*., 2012; Choudhary *et al*., 2014) who reported Berberine at the Rf value of 0.55 in the ethanolic extract of *Tinospora cardifolia* which may be responsible for the antimicrobial efficacy of the plant.

FT–IR analysis was used to identify the functional groups of the biomolecules found in the plant extract *collected from Baijnath district (Kangra), which gave the maximum activity against antimicrobial activity. Different wave numbers which corresponds to different functional groups are observed in the acetonic leaf extract of Baijnath District Kangra. The peaks present at the wave number 3361 cm^{-1}, 2854 cm^{-1}, 1644 cm^{-1} and 1074 cm^{-1} corresponds to the functional groups i.e O-H, H bonded alcohols, Phenols, C-H, C=C, C=N and C-O are present in IR spectrum of the acetonic leaf extract of the plant. Peak at 1246 cm^{-1} belongs to the plane CH bending and semicircle ring stretching. Peak at 1171 cm^{-1} confirms the ring deformation. CH wag vibrations of $C5H_2$ and $C6H_2$ groups appear at around 1380 cm^{-1}. The band at 1045 cm^{-1} probably corresponds to symmetric OCO stretch of the dioxolane type ring. Lower intensity vibrations at 724 cm^{-1} corresponds to CH twist of C atoms (Fig S1). These functional groups are present in the structure of alkaloid i.e Berberine, which further confirms the presence of Berberine in the acetonic leaf extract of *T. cardifolia* collected from Baijnath. The other wave numbers observed in the IR spectra might correspond to other metabolites present in the acetonic extract of *T. Cardifolia*. The results are in contrast to those reported earlier (Sivasubramanian *et al*., 2010) who showed the bands at 3427 cm^{-1} corresponding to O-H group, carbonyl group at 1705 cm^{-1} , unsaturated carbonyl groups at 1672 cm^{-1} and furan moieties at 2923

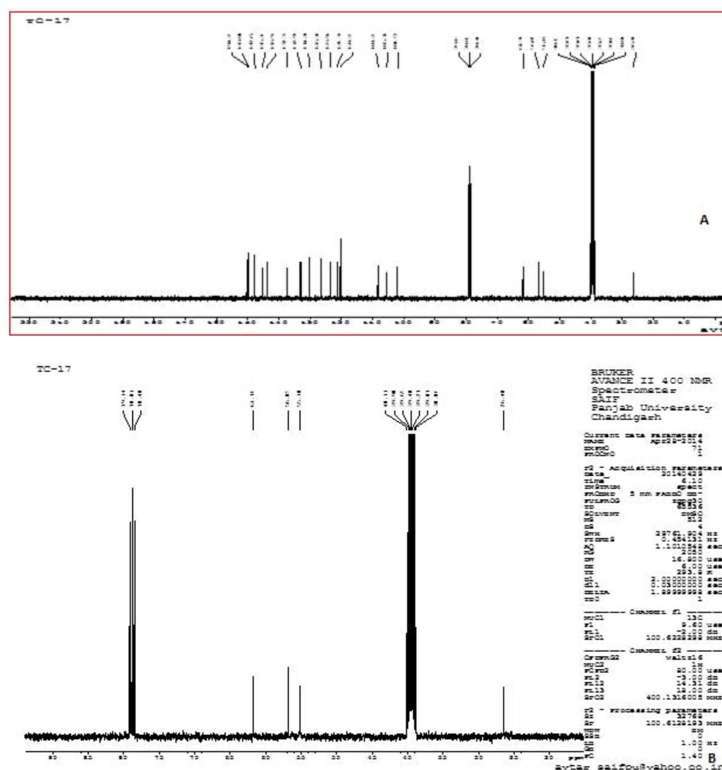


Fig. 5. (A) structure of Berberine; (B) peaks showing number of Carbon atoms in different environment (C) library and NMR chromatogram showing full Range of peaks observed in purified Berberine

cmtract^{-1}. The results of our investigation are different to those reported by (Abbasi *et al*., 2014) who showed medium and strong absorption bands in the FT-IR pattern of stem extract of *T. cardifolia* at the wave numbers 3394 cm^{-1}, 2925 cm^{-1}, 1606 cm^{-1}, 1388 cm^{-1} corresponding to NO_2 group and 1037 cm^{-1}, 3300 cm^{-1}and 1080 cm^{-1} corresponding to OH group.

Chromatographic analysis of acetonic leaf extracts of the plant, comprising of Acetonitril: water 60:40 (V/V) as mobile phase at a flow rate of 0.5ml/min and 265 nm gives good separation of Berberine at Rt 4.024min. Berberine peaks from the acetonic leaf extract was compared with the Rt values with those obtained by the chromatogram of the standard Berberine under the same conditions. Standard Berberine gave strong peak at the Rt 4.024 min and HPLC of acetonic leaf extract of the plant gave different peaks at different retention times as shown in the figure 3 but the peak 1 corresponds to the Rt of 4.024 min as that of standard Berberine (Fig S2) which further confirms the presence of Berberine in the acetonic leaf extract of *T. cardifolia*. The results are different to those reported by (Ahmed *et al*., 2006) who reported four compounds i.e 20 beta hydroxyecdysone, tinosporoside, cordioside and columbin at Rt 19.872 min, 22.443 min, 24.213 min and 27.67 min respectively in the reverse phase HPLC-UV-diode array detection method of ethanolic extract of *T. cardifolia*, *Tinospora malabrica* and *Tinospora crispa* but in line with those reported by (Sivakumar and Ranjan, 2011) who identified and quantified Berberine from methanolic stem extract of *T. cardifolia* at Rt 4.025 min.

Crytallization method was used to isolate and purify the active compound i.e Berberine from the pure plant extract which was successfully prepared by percolation of fresh green powdered leaves of *T. cardifolia* collected from Baijnath District Kangra. The acetonic extract of *T. cardifolia* leaf sample was chromatographed on silica gel plate and the active compound was isolated by scrapping the fraction from silica gel and elution of the isolated compound was done with methanol. The elutent was further purified. Recrystallization of an active fraction gave a pale yellowish compound i.e Berberine. First report on isolation of active compound from the *T.cardifolia* collected from Baijnath District Kangra i.e Berberine by crytallization method and its antibacterial activity against gram positive and gram negative bacteria.The results are in contrast to those reported by (Ali and Dixit, 2013) who extracted Palmatine from the stem of *T. cardifolia* after 16 hours of extraction time under 40 ^{0}C with four extraction cycles. Makisterone A was isolated and purified from the stems of *T. cardifolia* by soxhlet and repeated washing with predistilled methanol under reflux (Kumar *et al*, 2014).

Results obtained from antibacterial assay of the crystallized and purified active compound i.e Berberine relieved that the compound possesses potential antibacterial activity against gram positive and gram negative bacteria. The tested compound at 50µg/ml, 80µg/ml and 100µg/ml conc. showed a maximum mean value of zone of inhibition ranging from 11-15mm in diameter against *Staphylococcus aureus* and *Escherichia coli* respectively (Table 3). Berberine was tested earlier and the zone of inhibition was reported measuring 13mm and 12mm against *Escherichia coli* and *Staphylococcus aureus* which are very near to the results of the present study (Cernakova *et al*. 2002).

Structure of isolated compound was elucidated by chromatographic and spectroscopic analysis. HPLC Analysis of the purified compound gave strong peak at the Rt 4.025 min (Fig 4). HPLC of acetonic leaf extract of the plant and standard Berberine gave the same peaks at retention time 4.024 min which confirms that the isolated compound is Berberine. The peak of Berberine was determined by (Tsai and Tsai, 2002) in *Coptidis* and *Phellodendri cortex* by HPLC at the retention time 4.023 min which was very close to the results of present study. Berberine in raw herb i.e *Berberis aristata* was reported by HPLC-UV and observed the peak at retention time 11.893 min which is found to be different with the results of present investigation (Pasrija *et al*. 2010).

Multi Component Analysis (MCA) of isolated compound i.e. berberine by LC/MS, GC/MS, H1 NMR and C13 NMR further confirmed that the isolated compound is Berberine. The molar mass of standard Berberine and purified Berberine was found as 336.2 in LC chromatogram (Fig S3). This further confirms that the isolated compound is Berberine. The results are in line with those observed by (Bajpai *et al*., 2014), who isolated

Berberine from *Berberis aristata* and reported molar mass measuring 336.1. Berberine was isolated from *Coptis chinensis*, *Coptis japonica*, *Berberis thunbergii*, *Hydrastis Canadensis* and *Thalictrum lucidum* and molar mass observed found was 336.1 by LCMS which was in line with those observed in the present study (Zhangian *et al*., 2009).

The molar mass of purified Berberine was found to be 337.1 in GC chramotogram (Fig 6) which is very close to the molar mass of standard Berberine i.e 336.1. The structure of compound was established as Berberine by Library search table which shows the structure of Berberine with its molecular formula. This further confirms that the isolated compound is Berberine. The results are very close with those observed by (Ding *et al*., 2007) who calculated m/z ratio of Berberine isolated from *Coptis rhizome* by GCMS measuring 336.3.

The structure of compound was further established as Berberine by both H1 and C13 NMR spectroscopy. Numbers of Hydrogen atoms found in the NMR chromatogram are shown in (Table S1). It is evident from Table 4 that 14 signals in H1 spectra confirmed 18 H atoms in the compound. Two triplet, two doublet and seven singlet peaks are observed in the Chromatogram of Berberine (Fig 7) which corresponds to total of eighteen Hydrogen atoms. The number of Hydrogen atoms found in NMR chromatogram are same as in the structure of Berberine. This further confirms that the isolated and purified compound is Berberine. The results are in line with those observed by (Naika and Krishna 2008), who observed 18 hydrogen atoms in the H1NMR spectrum in the structure of Berberine which was isolated from the medicinal plant *Naravelia zeylanica*. 18 hydrogen atoms were found in the NMR spectrum of Berberine isolated from the urinary metabolites of rats and humans and this is in line with the results of present study (Qui *et al*. 2008).

C13 NMR chromatogram showed a signal which corresponds to the C atoms present in the berberine. The different carbon environment with their ppm values are shown in Table S2). Different Carbon peaks observed in the C13 NMR chromatogram of purified Berberine corresponds to same carbon environment as in the structure of standard Berberine (Fig 8). This analysis further confirms that the isolated and purified compound is Berberine. The results are in line with those observed by (Naika and Krishna 2008), who observed chemical shift of 35ppm, 40ppm, 80ppm, 120ppm and 145ppm corresponding to CH_3CO, R-CH_2, C-O, C=C and C aromatic rings goups respectively in the C13NMR spectrum of Berberine isolated from the medicinal plant *Naravelia zeylanica*. CH_3CO, R-CH_2, R-CH_2OH goups in the C13 NMR spectrum of Berberine was found in the urinary metabolites of rats and humans and this is in line with the results of present study (Qui *et al*. 2008). A C13 NMR spectrum of Berberine isolated from *Arcangelisia gusalung* was observed by (Yu *et al*., 2014) and found CH_3CO, R-CH_2, C-O and C=C groups and this was in line with the results of present investigation.

CONCLUSION

From the above study, it can be concluded that the medicinal plant i.e *T. cardifolia* have great potential as antimicrobial agents against the selected pathogens. Acetonic leaf extract of *T. cardifolia* showed greater inhibitory action than methanolic and chloroformic extracts against gram positive and gram negative Bacteria, Fungus and Candida strains. First report of highest antimicrobial activity from acetonic leaf extract of *T. cardifolia* collected from Baijnath, District Kangra, Himachal Pradesh. The results of present investigation clearly indicate that the antibacterial and antifungal activity vary with the plant material collected from different environmental conditions. Our findings suggest that same plant from different places at varying distance and environments may have altered antimicrobial activities, urging the need to pay attention to substrate, habitat, etc., when collecting plants to test for antimicrobial properties. According to the antibacterial assay done for screening purpose all extracts in general are more effective on Gram-positive bacteria than on Gram-negative bacteria. The results agree with observations of previous researchers and could be explained by the different cell wall structures of these bacteria. Gram-negative outer membrane comprising of phospholipids and lipopolysaccharides act as a barrier to the entrance and reaction of most antibiotics and/or antimicrobial agents through cell envelope (27,

28). The phytochemicals possess various bioactive properties and may be used as external therapeutic supplements. First report on isolation of active compound i.e Berberine by crytallization method and its antibacterial activity against gram positive and gram negative bacteria. Chromatographic and spectroscopic analysis confirmed the presence of Beberine. Hence, this study would lead to the development of some stable and biologically active drugs which can be employed as antimicrobial agents. Thus, the study ascertains the value of plants used in ayurveda, which could be of considerable interest to the development of new drugs.

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