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Cholinesterase Inhibitory Assay and Antimicrobial Effect of Four Constituents of *Lonicera quinquelocularis* Against Gram Positive and Gram Negative Bacteria

[1]Kamran Tahir, [1]Sadia Nazir, [2]Naijin Wu, [2]Arifullah Khan, [2]Qin Jing and [1,2]Shafiullah Khan

[1]Institute of Chemical Sciences, Gomal University, D.I.Khan, Pakistan
[2]State Key Laboratory of Organic-Inorganic Composites,
Beijing University of Chemical Technology, Beijing 100029, China

Abstract: A phytochemical study on the chloroform soluble fraction of *Lonicera quinquelocularis* led to the first time isolation of four known compounds (1-4). These compounds were tested against two human Gram-positive bacteria (*Staphylococcus aureus*, *Micrococcus luteus*) and four Gram-negative ones (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumoniae*). The agar well diffusion method was used to check the antibacterial activity of these four compounds. The antifungal activity against *Aspergillus niger* was determined by the agar well diffusion method. Compound (1) showed maximum antimicrobial activities. The MIC of the compound 1 was determined by agar dilution method while the MBC was carried out by viable cell count method. Two different antibiotics (ampicillin and ofloxacin) were used as positive controls. For antifungal activity an effective antifungal "Itraconazole" was used as a control standard. In addition the isolated compounds were assayed for their Acetyl cholinesterase (AChE) and Butyryl cholinesterase (BChE) inhibitory activities. Moreover, the IC_{50} (50% inhibitory effect) values of compounds 1 and 2 against AChE were determined to be 2.12 and 1.65 μM while the values obtained against BChE were 4.65 and 5.98 μM respectively.

Key words: *Lonicera quinquelocularis* · Antimicrobial Activity · Cholinesterase Inhibition

INTRODUCTION

The genus *Lonicera* belongs to family *Caprifoliaceae*, which includes about 12 genera and 450 species [1], occurring mostly in moderate region of Northern Hemisphere. There are four genera and twenty seven species of *Caprifoliaceae* found in Pakistan [2]. A variety of species of this genus are used for the treatment of various diseases like acute fever, headache, respiratory infections [3], antibacterial [4], antioxidant [5], cytoprotective [6], hepatoprotective [7, 8], antiviral [9], antitumor [10, 11] and anti-inflammatory activities [12]. Previously several compounds were isolated from this genus including iridoids, bisiridoids, sulphur containing monoterpenoids, alkaloidal glycosides, triterpenoids, saponins, coumarin glycosides and flavones glycosides [13-15].

L. quinquelocularis is a part of this genus usually distributed in dry sunny places between 750-3000 m in many countries of Asia. In Pakistan, its habitat is in Baluchistan, Kurram, Chitral, Swat, Astor, Hazara, Murree hills, Poonch and Kashmir [16]. Previously a number of compounds were isolated from this plant including triterpenoid, benzoates, lonicerin, coumarin and iridoide glycosides [5, 17]. It is broadly used in the treatment of hypotensive, sedative, antipyretic and antioxidant activities [5, 18,19]. The diverse medicinal importance of genus *Lonicera* has encouraged us to investigate the constituents of *L. quinquelocularis*.

Keeping in view the previous study on several compounds isolated from *L. Quinquelocularis* which have good biological activity i.e cytotoxic activity and enzyme inhibition activity [20] but here we report the isolation and antimicrobial and enzyme inhibition

Corresponding Author: Dr. Shafiullah Khan, Institute of Chemical Sciences, Gomal University, D.I.Khan, Pakistan.
Tel: +92 966 750359, Fax: +92 966 750250.

Table 1: Zone of inhibition of reference antibiotics

Reference	Micro organisms and their zone of inhibition(mm)					
Antibiotics	Ec	Kp	Ps	Ent	Ml	Sta
Ofloxacin	15.1(±0.02)	14(±0.01)	11.9(±0.01)	13(±0)	10(±0.11)	9.6(±0.05)
Ampicillin	14.1(±0.05)	12.3(±0.5)	11(±0)	14(±0.11)	13(±0.05)	14(±0.05)

Ec: *Escherichia coli*; Kp: *Klebsiella pneumoniae*; Ps: *Pseudomonas aeruginosa*; Ent: *Enterobacter cloacae*; Ml: *Micrococcus luteus*; Sta: *Staphylococcus aureus* (methicillin resistant); mm: millimeter.

activities of four constituents which are first time isolated from *L. Quinquelocularis* i.e *Quercetin*-3-β-D-glucoside *(1), Malvidin* (2), *Peonidin* (3), *Sweroside (4)*.

MATERIALS AND METHODS

Chemicals and Culture Media: Aluminium Thin Layer Chromatography (TLC) plates (20 × 20, 0.6 mm thick) pre-coated with silica gel 60 F_{254} (20x20 cm, 0.2 mm thick; E. Merck, Darmstadt, Germany) were used for TLC in order to check the purity of the compounds. Column chromatography (CC) was done with the help of silica gel of 230-400 mesh (E. Merck, Darmstadt, Germany). Ceric sulphate and potassium permanganate solutions were used as visualization reagents. The UV spectra (λ_{max} nm) were recorded on Shimadzu UV-2700 spectrophotometer (Shimadzu, Japan) in EtOH. Mass Spectra was recorded on Bruker TOF Mass spectrometers (Billerica, USA) using electrospray ionisation (ESI). The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 NMR spectrometer (Billerica, USA) (400 MHz for ^{1}H and 100 MHz for ^{13}C-NMR), using $CDCl_3$ as solvents.

Plant Material: The Plant materials of *L. quinquelocularis* were collected from Hazara division, District Mansehra, in May 2011. The plant was identified by Professor Dr. Manzoor Ahmad Plant Taxonomist on the basis of its morphological features and the database present in the library at the Department of Botany, Government Degree College Abbotabad, Pakistan, where a voucher specimen has been deposited in herbarium (Accession No. C-044).

Extraction and Isolation: The shade dried powdered material of *L. quinquelocularis* (3 kg) was extracted with methanol (70 L) at room temperature for 10 days (3 ×70 L). The resulting extract was filtered and evaporated with the help of rotary evaporator to obtain blackish gummy crude (83.7 g). Four fractions were formed from the methanolic crude i.e *n*-hexane (Y1, 16 g), chloroform (Y2, 17 g), ethyl acetate (Y3, 19.6 g) and *n*-butanol (Y4, 11.3 g).

The chloroform soluble fraction was subjected to column chromatography over silica gel (70-230 mesh) eluting with *n*-hexane (100 %), *n*-hexane: chloroform (1:19□19:1), chloroform (100%), chloroform: MeOH (1:19□19:1), MeOH (100 %), with increasing order of polarity to obtain 12 fractions A-L.

Fraction C (6 g) was again uploaded to a series of silica gel column chromatography eluting with *n*-hexane, n-hexane-chloroform and chloroform in increasing order of polarity give compound 1 with 100% $CHCl_3$ and to a preparative TLC using n-hexane: chloroform (1:4) as solvent system to afford compounds 2 and 3 respectively.

Fraction E (6 g) was re-chromatographed over silica gel eluting with *n*-hexane, n-hexane: chloroform and chloroform in increasing order of polarity followed by preparative TLC eluted with *n*-hexane: $CHCl_3$ (4:1) and isolated compound 4.

Microorganisms: Six bacterial species, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* (clinical strain/PIMS), *Enterobacter cloacae* (clinical strain/PIMS), *Staphylococcus aureus* (MRSA, clinical strain/PIMS) and *Micrococcus luteus* (clinical strain/PIMS) were selected for antimicrobial test. *Aspergillus niger* was used for antifungal assay. Strains were obtained from Microbiology Research Lab (MRL) Beijing University of chemical technology, China where their identification and characterization taken place. These strains were kept on agar slants at 4°C for antimicrobial tests. Microorganisms were incubated overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.3. The ofloxacin (10 µg) and ampicillin (10µg) (Oxoid) were used as reference antibiotics (Table 1).

Antimicrobial Screening

Screening for Antibacterial Activity *(Agar Well Diffusion Method):* In order to determine the antibacterial activity of the isolated compounds the agar well diffusion method [21] was carried out. All bacterial cultures were first grown in nutrient broth at 37°C for 24 h incubated till turbidity became correspondent to McFarland 0.5 turbidity standard was obtained. The inocula of the

respective bacteria were splashed on to the Muller Hinton agar (Oxoid) plates using a sterile swab in order to make sure a uniform thick lawn of growth following incubation. With the help of sterile cork borer wells of 6 mm in diameter were formed on to nutrient agar plates. The test agents (100 µl) were put to the wells and the plates were then allowed to stay for 2 hours at 25°C. Finally, the plates were incubated at 37°C for 24 h and the resulting diameters of zones of inhibition were measured. The compound 1 showed highest antibacterial activity while all the other compounds also showed reasonable antibacterial activity.

Determination of Minimum Inhibitory Concentration (MIC)

Agar Dilution Method: Agar well diffusion protocol was followed to carry out Minimum inhibitory concentration (MIC) of the compound 1 [22-24]. The Muller Hinton Agar (Oxoid) was sterilized and was allowed to cool to 50°C. About 19 ml of this was mixed with 1ml of different concentrations of compound 1 in sterilized test tubes. This mixture was carefully mixed and poured into pre-labeled sterile Petri dishes. Petri dishes having only growth media were prepared in the same way so as to serve for assessment with petri plate containing compound. 2000 µg/ml to 0.156 µg/ml concentrations of the compound were used in this assay. The density of the suspensions of the respective microorganisms was adjusted to 0.5 McFarland turbidity standards. By using sterilized standard loop these were inoculated onto the series of agar plates. The incubation of these plates was taken place at 37°C for 24 hours. The lowest concentration of the compound which inhibited the growth of the respective organisms was considered as MIC. All assays were carried out in triplicate.

Determination of Minimum Bactericidal Concentration (MBC)

Viable Cell Count Method: Viable cell count method was performed for the determination of Minimum Bactericidal Concentration (MBC) of the compound 1 [25, 26] and the results were expressed as number of viable cells as a percentage of the control.

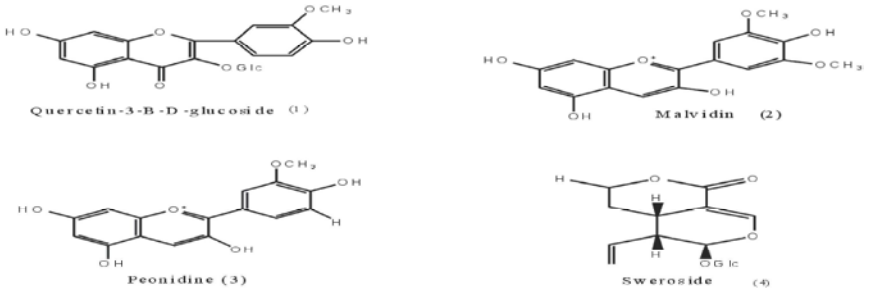
Screening for Antifungal Activity: The required amount of the fungal strain was suspended in 2 ml of Sabouraud dextrose broth. Using sterile cotton swabs, this suspension was homogeneously spread on Petri plates having Sabouraud dextrose agar media. Samples were poured into wells using same technique for bacteria but these were incubated at 25°C for 72 hours. The plates were then checked for the presence of inhibition zones and the results were recorded. A potent antifungal "Itraconazole" was used as a standard or positive control.

Cholinesterase Inhibition Assay and Determination of Ic_{50} Values: Acetyl cholinesterase (EC 3.1.1.7) acetyl thiocholine iodide, butyryl thiocholine chloride, butyryl cholinesterase (horse-serum E.C 3.1.1.8), galanthamine and DTNB (5, 5-dithiobis [2-nitrobenzoic-acid]) were purchased from Sigma. All other chemicals used were of analytical grade. By following the modified spectrophotometric assay used by Ellman [27], the Acetyl cholinesterase (AChE) and butyryl cholinesterase (BChE) inhibiting activities were performed. Protocol and assay conditions were the same as described by Rocha [28].

Butyryl thiocholine chloride and Acetyl thiocholine iodide were used as substrates to assay acetyl cholinesterase and butyryl cholinesterase, respectively. 5,5´-Dithiobis[2-nitrobenzoic-acid] (DTNB) was used for the measurement of cholinesterase activity. 0.2 mM DTNB in 62 mM sodium phosphate buffer (pH 8.0, 880 µL), test compound solution (40 µL) and acetyl cholinesterase or butyryl cholinesterase solution (40 µL) were mixed and incubated for 15 minutes (25 ºC). By the addition of acetyl thiocholine or butyryl thiocholine (40 µL) respectively, the reaction was initiated. The hydrolysis of acetyl thiocholine and butyryl thiocholine were monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetyl thiocholine and butyryl thiocholine, respectively, at a wavelength of 412 nm (15 min). All the reactions were performed in triplicate in a BMS spectrophotometer (USA). The concentrations of test compounds that inhibited the hydrolysis of substrates (acetyl thiocholine and butyryl thiocholine) by 50% (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The final DMSO concentration in the reaction mixture was 6%.

RESULTS

Compound 1 has high antibacterial activity as compared to other compounds which is cleared from (table 2) and (figure 1). Hence it was further considered for determination of MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) respectively. The MIC values ranged from 0.156 mg/ml to > 10 mg/ml for all tested strains while the MBC values reported were many times higher than



Structures of compounds 1-4 from *loniceocularisra quinquel*

Table 2: Inhibition zones of compounds 1-4

	Zone of inhibition (mm)					
Compounds	Ec	Kp	Ent	Ps	Ml	Sta
1	16(±0.4)	14(±0.3)	15(±0)	15(±0.3)	16(±0.7)	15 (±0.4)
2	13(±0.4)	13(±0.6)	12(±0.4)	11(±0.4)	12(±0.5)	14(±0.2)
3	7(±0.3)	8(±0.4)	5(±0.2)	7(±0.2)	6(±0.5)	7(±0.5)
4	3(±0.2)	3(±0.1)	5(±0.2)	4(±0.6)	4(±0.3)	3(±0.5)

Ec: *Escherichia coli*; Kp: *Klebsiella pneumoniae*; Ps: *Pseudomonas aeruginosa*; Ent: *Enterobacter cloacae*; Ml: *Micrococcus luteus*; Sta: *Staphylococcus aureus* (methicillin resistant); mm: millimeter.

Table 3: MIC and MBC of *Lonicera quinquelocularis* compound 1.

Microorganisms	MIC mg/ml	MBC mg/ml
E. coli	>10	N.d
K. pneumonia	>10	N.d
P. aeruginosa	>10	N.d
E. cloacae	5	15
M. luteus	0.625	1.877
S. aureus	0.156	1.260

N.d = not detected

Table 4: Antifungal activities of compounds 1-4

Compounds	Zone of inhibition (mm)
1	7(±0.5)
2	7(±0.11)
3	5(±0.17)
4	2(±0.14)
Standard	8(0)

MIC (table 3),(figure 2).The MBC values for *Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa* was not detected.

In the same way nearly similar pattern of susceptibility was reported against fungal strain *Aspergillus niger*. The widest zones of inhibition (maximum antifungal activity) were presented by compounds 1 and 2. Compounds 3 has reasonable, while the compound 4 has lowest zone of inhibition was represented in (table 4) (figure 3).

We were interested to identify AChE and BChE inhibiting small molecules from herbal medicinal plants, so performed bioassay-guided search for acetyl cholinesterase (AChE) and butyryl cholinesterase (BchE)

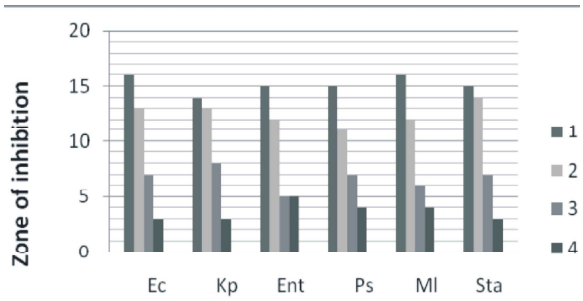


Fig 1: Inhibition Zones of Compounds 1-4

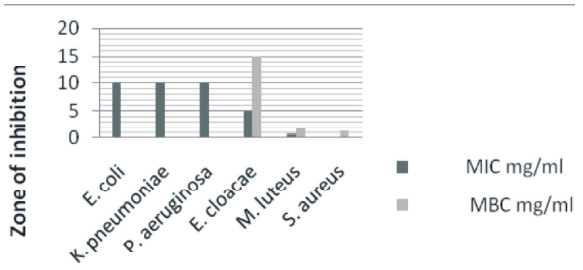


Fig 2: MIC and MBC of Ranunculus Muricatus Compounds 1

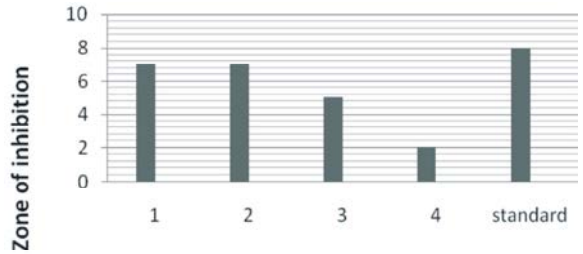


Fig 3: Antifungal Activities of Compounds 1-4

Table 5: AChE and BChE inhibitory activities of compound 1-4 from *L. quinquelocularis* (IC_{50}, µM)

S.No	Compounds	AChE ± SEM[a]	BChE ± SEM[a]
1	1	Nil	Nil
2	2	2.12 ± 0.07	4.65 ± 0.079
3	3	1.65 ± 0.03	5.98 ± 0.079
4	4	5.27 ± 0.04	14.76 ± 0.087
5	Allanzanthane b	2.94± 0.45	12.96 ± 0.053
6	Galanthamine [b]	1.79 ±0.061	7.98 ± 0.01

[a] Standard error of mean of five assays

[b] Positive control used in the assays.

Note: Data showed are values from triplicate experiments.

inhibitors from this medicinal plant. Compounds 1-4 isolated from *L. quinquelocularis* were tested against AChE and BChE, which show the most eye-catching target for drug treatment of neurone design and discovery of mechanism-based inhibitors for the degenerative disorders such as Alzheimer's disease [29]. The percentage of inhibition was first determined at 0.1 mM.Those compounds which were having enzyme inhibition greater than 50% were consequently assayed for IC_{50} (50% inhibitory effect) determination. Among the isolated compounds, 2 and 3 showed most effective inhibition activity against AChE and BChE as compared to standard drugs; allanzanthane and galanthamine in a dose dependent manner. The IC_{50} values of compounds 2 and 3 against AChE were determined to be 2.12 and 1.65 µM, while against BChE, were measured as 4.65 and 5.98 µM respectively. Compounds1 has no inhibition and 4 showed weak inhibition profile against AChE and BChE (Table 5).

DISCUSSION

The antimicrobial activities of four compounds isolated from chloroform fraction were tested against six bacteria species *E. coli, K. pneumoniae, P. aeruginosa, E. cloacae, S. aureus and M. luteus.* Nearly all compounds exhibited more or less antimicrobial activity against the test strains. Compound 1 exhibited best activity against these bacteria. Besides that, compounds 2 and 3 have moderate and compound 4 have lowest activity against these bacteria. Nearly all the constituents from *L. quinquelocularis* were primarily reported as active against the *Aspergillus niger*. Compounds 1, 2 showed highest antifungal activities, while compound 4 showed least antifungal activity.

The MIC of the compound 1 was taken as 0.156 mg/ml. It is important that the MIC value is too high to be taken in susceptible ranges [30]. The MBC value of compound 1 is many times higher than MIC. The antibacterial and antifungal assay were done by agar well diffusion method.The MIC was carried out by using Agar well dilution method while MBC was performed by viable cell count method. The MBC values for *E. coli, K. pneumonia* and *P. aeruginosa* were not detected.

Nearly all the four compounds isolated from the chloroform fraction of *L. quinquelocularis* are polar and were primarily as antimicrobial reagents. Compounds 2 and 3 have highest Acetyl cholinesterase and butyryl cholinesterase inhibitory effects. This investigation is probably the first to explore the antimicrobial and enzyme inhibitory activities of compounds 1-4 of *L. quinquelocularis*, as a comprehensive literature review to the best of our knowledge there is no information about the antimicrobial and enzyme inhibitory activities of these isolated constituents from this plant.

CONCLUSION

From the present studies it is clear that four compounds were isolated from chloroform fraction of *L. quinquelocularis* which showed high antimicrobial and enzyme inhibitory activities. Compound 1 showed highest antimicrobial activity. The MIC of compound 1 was taken as 0.156µg/ml. Consequently the other three fractions *n*-hexane, ethyl acetate and *n*-butanol are further recommended for investigations to explore the potential medicinal compounds.

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