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New antioxidant and cholinesterase inhibitory constituents from *Lonicera quinquelocularis*

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A phytochemical investigation on the ethyl acetate soluble fraction of *Lonicera quinquelocularis* (whole plant) led to the isolation of three new and two known compounds. The crude extract and the various soluble fractions were screened for cytotoxic activities. The ethyl acetate soluble fraction was found to be more cytotoxic. The chloroform soluble fraction showed moderate activity while the other fractions and crude extract were weakly cytotoxic. The isolated compounds were tested for antioxidant, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities. Almost all the compounds showed profound antioxidant activities in DPPH free radical scavenging assay. The 50% inhibitory effect (IC_{50}) values of compounds 3 and 5 against AChE were determined to be 1.65 and 3.43 µM, while the values obtained against BChE were 5.98 and 9.84 µM, respectively. Compounds 2 and 4 showed weak inhibition profile.

Key words: *Lonicera quinquelocularis,* phthalates and benzoates, cholinesterase inhibition, antioxidant activity.

INTRODUCTION

The genus *Lonicera* belongs to the family Caprifoliaceae. It contains about 12 genera and 450 species (Mabberley, 1997), and occurs mainly in temperate region of Northern Hemisphere. In Pakistan, it is represented by 4 genera and 27 species (Akhter, 1986). Various species of this genus are used for the treatment of acute fever, headache, respiratory infections (Houghton et al., 1993), bacterial infections (Puupponen-Pimia et al., 2001) and also used as an antioxidant (Kahkonen et al., 2001; Ali et al., 2013), cytoprotective (Chang and Hsu, 1992), heapto-protective (Liu et al., 1992; Shi et al., 1999), antiviral (Chang et al., 1995), antitumor (Wang et al., 2009; Yip et al., 2006) and anti-inflammatory agent (Yoo et al., 2008). Previous studies on this genus reported the isolation of a variety of constituents including iridoids, bisiridoids, sulfur containing monoterpenoids, alkaloids, glycosides, triterpenoids, saponins, coumarin glycosides and flavone glycosides (Machida et al., 1995; Bailleul et al., 1981; Souzu and Mitsuhashi, 1969, 1970). *Lonicera quinquelocularis,* a member of this genus is widely distributed in dry sunny places between 750 and 3000 m in many countries of Asia. In Pakistan, it is found in Baluchistan, Kurram, Chitral, Swat, Astor, Hazara, Murree hills, Poonch and Kashmir (Abdullah, 1972). Previous phytochemical study on this plant resulted in the isolation of triterpenoids, benzoates, lonicerin, loganin, coumarin and iridoid glycosides (Ali et al., 2013; Kumar et al., 2000). It is used as a sedative, hypotensive, anti-pyretic and antioxidant agent (Ali et al., 2013; Calis et al., 1984; Basaran et al., 1988). The diverse medicinal uses of the genus *Lonicera* has prompted the investigate of the chemical constituents of *L. quinquelocularis.*

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MATERIALS AND METHODS

General experimental procedure

Aluminium TLC plates (20 × 20, 0.5 mm thick) pre-coated with silica gel 60 F_{254} (20×20 cm, 0.2 mm thick; E. Merck, Darmstadt, Germany) were used for TLC to check the purity of the compounds. Column chromatography (CC) was carried out using 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

The whole plant of *L. quinquelocularis* was collected from Bara Galli, Hazara Division, District Mansehra, in June 2009. It was identified by Professor Dr. Manzoor Ahmad, Plant Taxonomist, Department of Botany, Government Degree College Abbotabad, Pakistan, where a voucher specimen has been deposited in herbarium (Accession No. C-0013).

Extraction and isolation

The shade dried whole plants (13 kg) was ground and extracted with ethanol at room temperature (3 × 25 L). The combined ethanol extract was evaporated under reduced pressure to obtain a thick greenish gummy material (crude), which was fractionated with *n*-hexane (151 g), chloroform (147 g), ethyl acetate (109 g), and n-butanol (53 g) soluble fractions, respectively.

The ethyl acetate soluble fraction was subjected to column chromatography over silica gel (70-230 mesh) eluted with *n*-hexane (100%), *n*-hexane: EtOAc (1:19–19:1), EtOAc (100%), EtOAc:MeOH (1:19–19:1), MeOH (100%) in increasing order of polarity to obtain 13 fractions (A-M).

Fraction C (4 g) was again subjected to a series of silica gel column chromatography eluted with *n*-hexane, *n*-hexane-EtOAc and EtOAc in increasing order of polarity to afford compound 1 and to a preparative TLC using *n*-hexane:EtOAc (4:1) as solvent system to afford compounds 2 and 3, respectively.

Fraction D (4 g) was re-chromatographed over silica gel eluted with *n*-hexane, *n*-hexane-EtOAc and EtOAc in increasing order of polarity followed by preparative TLC eluted with *n*-hexane:CH_2Cl_2 (2:3), and afforded compounds 4 and 5, respectively.

Identification of compounds

Compounds 1 and 2 were isolated as colourless oils and were identified as bis (2-ethylhexyl) phthalate (Nair et al., 2012) and dioctyl phthalate (Sultan et al., 2010). Identification of compounds 3, 4 and 5 were based on spectroscopic data analysis.

Bis (7-acetoxy-2-ethyl-5-methylheptyl) phthalate (3)

Colourless oil: UV (MeOH) λ_{max} nm (log ε): 296 (4.26) and 232 (5.18); IR (dry film) ν_{max} cm^{-1}: 1725 (Ester -C=O), 1610 (aromatic C=C) and 1130-1200 (C-O); HREIMS-TOF m/z 562.7642 $[M+H]^+$ (calcd. for $C_{32}H_{50}O_8$, 562.3603); ^{1}H NMR (400 MHz, $CDCl_3$). δ 7.73 (dd, *J* = 5.8, 3.3, H-3/H-6), δ 7.56 (dd, *J* = 5.8, 3.3, H-4/H-5), 4.15 (dd, *J* = 11.8, 6.1 Hz, H-1'a), 4.25 (dd, *J* = 12.1, 6.2 Hz, H-1'b), δ 4.09 (t, *J* = 6.8 Hz, H-7'), δ 2.07 (s, H-10'), 1.70 (m, H-2'), 1.64 (m, H-5'), 1.48 (1H, m, H-6'b), 1.32 (1H, m, H-6'a), 1.45 (m, H-11'), 1.35 (1H, m, H-4'b), 1.24 (1H, m, H-4'a), 1.29 (m, H-3'), 0.96 (d, *J* = 6.4, H-13'), 0.89 (t, *J* = 6.5, H-12'). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.22 (C-1/C-8), 132.49 (C-2), 128.80 (C-3/C-6), 130.86 (C-4/C-5), 132.49 (C-7), 68.15 (C-1'), 38.76 (C-2'), 31.60 (C-3'), 28.94 (C-4'), 34.67 (C-5'), 30.39 (C-6'), 64.35 (C-7'), 167.75 (C-9'), 22.65 (C-10'), 23.76 (C-11'), 10.95 (C-12'), 14.09 (C-13').

Neopentyl-4-ethoxy-3, 5-bis [3-methyl-2-butenyl] benzoate (4)

Amorphous powder: UV (EtOH) λ_{max} (log ε): 210 (4.3), 260 (4.5) nm; IR (KBr) ν_{max}: 2940, 1705, 1600, 1440, 1300, 1230, 1010, 780 cm^{-1}; HREIMS-TOF *m/z* $[M]^+$ 372.3742 (calcd for $C_{24}H_{36}O_3$, 372.2156); ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.39 (1H, d, *J* = 1.8 Hz, H-2/H-6), 5.69 (2H, t, *J* = 6.8 Hz, H-2'), 4.38 (2H, q, *J* = 7.8 Hz, OCH_2), 4.15 (s, 2H, H-3''), 3.27 (4H, d, *J* = 6.8 Hz, H-1'), 1.83 (6H, d, *J* = 1.1 Hz, H-4'), 1.74 (6H d, *J* = 1.1 Hz, H-5'), 1.36 (3H, t, *J* = 7.8 Hz, OCH_2CH_3), 0.96 (9H, s, H-5''); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 137. 2 (C-1), 129.3 (C-2), 124.8 (C-3), 154.6 (C-4), 124.8 (C-5), 129.3 (C-6), 27.6 (C-1'), 121.5 (C-2'), 133.5 (C-3'), 19.4 (C-4'), 24.7 (C-5'), 163.0 (C-1''), 83.2 (C-3''), 29.4 (C-4''), 23.1 (C-5''), 68.3 ($O-CH_2$), 15.5 (O-C-CH3).

Neopentyl-4-hydroxy-3, 5-bis [3-methyl-2-butenyl] benzoate (5)

Amorphous white solid: UV (EtOH) λ_{max} (log ε): 217 (4.2), 265 (4.5), 295 (4.3) nm; IR (KBr) ν_{max}: 2940, 1705, 1600, 1440, 1300, 1230, 1010, 780 cm^{-1}; HREIMS-TOF *m/z* $[M]^+$ 344.6403 (calcd. for $C_{22}H_{32}O_3$, 344.2549); ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.48 (1H, d, *J* = 1.6 Hz, H-2/H-6), 5.72 (2H, t, *J* = 7.1 Hz, H-2'), 5.31 (1H, bs, OH), 4.19 (2H, s), 3.33 (4H, d, *J* = 7.1 Hz, H-1'), 1.85 (6H, d, *J* = 1.2 Hz , H-4'), 1.78 (6H, d, *J* = 1.2 Hz, H-5'), 0.98 (9H, s, H-5''); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 135.0 (C-1), 129.3 (C-2), 124.2 (C-3), 152.5 (C-4), 124.2 (C-5), 129.3 (C-6), 27.3 (C-1'), 123.1 (C-2'), 133.5 (C-3'), 19.1 (C-4'), 25.4 (C-5'), 165.4 (C-1''), 81.9 (C-3''), 29.7 (C-4''), 21.8 (C-5'').

Cytotoxic activity

Cytotoxic activities of the extract and fractions were determined using brine-shrimp (*Artemia salina*) lethality bioassay (Meyer et al., 1982). Artificial sea water was prepared by dissolving 3.7 g of sea salt per liter of double distilled water and was filtered. “Sea” water was placed in small tank, and brine shrimp eggs (1 mg) was added and was darkened by covering with aluminium coil. Twenty milligrams of concentrated sample was dissolved in 2 ml of $CHCl_3$ (20 mg/2 ml (10 mg/ml)) and transferred to 500, 50 and 5 µl vials corresponding to 1000, 100 and 10 µg/ml, respectively. Three replicates were prepared for each concentration making a total of nine vials. The vials containing the sample were concentrated, dissolved in DMSO (50 µl) and 5 ml “sea water” was added to each. Then, ten shrimps were added per vial. All the vials were incubated at 37°C for 24 h and the brine shrimps that survived were counted. The activities of these extract and fractions were compared with standard drugs Ampicillin (Mc Laughlin and Anderson, 1988; Rashid et al., 2002). The data was analyzed with a Finney computer program to determine LD_{50} values with 95% confidence interval.

Cholinesterase inhibition assay and determination of IC_{50} values

Acetylcholinesterase (EC 3.1.1.7), butyrylcholinesterase (horse-

Table 1. Cytotoxic activities of crude extract and fractions.

Concentration (µg/ml)	Fractions and crude extract (% deaths at doses)					
	n-hexane (F1)	$CHCl_3$ (F2)	EtOAc (F3)	*n*-BuOH (F4)	Ethanol crude	Standard drug (Ampiciline)
10	15	40	50	30	20	40
100	25	50	60	40	30	80
1000	30	60	80	60	40	120
ED_{50}	650.55	320.60	120.25	450.40	540.65	70.50

serum E.C 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, galanthamine and 5, 5´-dithiobis [2-nitrobenzoic-acid] (DTNB) were purchased from Sigma. All other chemicals were of analytical grade. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibiting activities were measured according to a modified spectrophotometric method used by Ellman (Ellman et al., 1961). Protocol and assay conditions were the same as described by Rocha (Rocha et al., 1993).

Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay acetylcholinesterase and butyrylcholinesterase, respectively. 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 acetylcholinesterase or butyrylcholinesterase solution (40 µl) were mixed and incubated for 15 min (25°C). The reaction was then initiated by the addition of acetylthiocholine or butyrylthiocholine (40 µl), respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine 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 acetylthiocholine and butyrylthiocholine, 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 (acetylthiocholine and butyrylthiocholine) 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%.

DPPH radical scavenging activity

The DPPH assay was performed according to the standard procedure with little modifications (Gymfi et al., 1999). The fresh stock solution was prepared by dissolving 3 mg DPPH with 100 ml of methanol and then stored at 20°C. The working solution was obtained by diluting DPPH solution with methanol to obtain an absorbance of about 0.980 (± 0.02) at 517 nm using the spectrophotometer. A 900 µl aliquot of this solution was mixed with 100 µl of the compounds (10 µg/ml). The same was repeated with positive reference, that is, ascorbic acid (10 µg/ml). The solution in the test tubes were shaken well and incubated in dark for 30 min at room temperature. Then, the absorbance was taken at 517 nm. The scavenging activity was calculated from the percentage of DPPH radical scavenged as the following equation:

Scavenging effect (%) = [(control absorbance-sample absorbance) / (control absorbance)] ×100.

RESULTS AND DISCUSSION

The crude ethanol extract (430 g) was successively extracted with *n*-hexane (F_1), chloroform (F_2), ethyl acetate (F_3) and *n*-butanol (F_4). All these fractions and the crude extract were tested for their cytotoxic activities (Brine shrimp lethality assay). The F3 was found to be more effective and showed high activities, F2 and showed optimum while F1 and F4 showed low lethality in brine shrimp assay (Table 1).

In the present investigation, phytochemical study on the ethyl acetate extract of whole plant of *L. quiquelocularis* afforded bis (2-ethylhexyl phthalate (1), dioctyl phthalate (2), bis (7-acetoxy-2-ethyl-5-methylheptyl phthalate (3), neopentyl-4-ethoxy-3, 5-bis (3-methyl-2-butenyl benzoate (4) and neopentyl-4-hydroxy-3, 5-bis (3-methyl-2-butenyl benzoate (5) (Figure 1).

Compounds 1 to 5 from *L. quinquelocularis* were tested against AChE and BChE, which represent the most attractive target for drug design and discovery of mechanism-based inhibitors for the treatment of neurone degenerative disorders such as Alzheimer's disease (Zhang, 2004). The percentage of inhibition was first determined at 0.1 mM. Compounds for which enzyme inhibition was greater than 50% were subsequently assayed for IC_{50} (50% inhibitory effect) determination. Among the isolated compounds, 3 and 5 showed most effective inhibitory activity against AChE and BChE as compared to standard drugs; allanzanthane and galanthamine in a dose dependent manner. The IC_{50} values of compounds 3 and 5 against AChE were determined to be 1.65 and 3.43 µM, while against BChE, were measured as 5.98 and 9.84 µM, respectively. Compounds 2 and 4 showed weak inhibition profile against AChE and BChE (Table 2).

The DPPH radical scavenging activity assay was performed to evaluate the antioxidant property of the isolated compounds with positive reference ascorbic acid (10 µg/ml) according to the standard procedure with some modifications (Gymfi et al., 1999). All isolated compounds in (Figure 2) showed higher antioxidant property than ascorbic acid. Furthermore, the compound 5 was found more active than others. It may be due to different structure of the compounds.

Conclusion

Several antioxidant and anticholinesterase active compounds were isolated from *L. quinquelocularis*, which

Table 2. AChE and BChE inhibitory activities of compound **1**-**5** from *L. quinquelocularis* (IC_{50}, µM).

Compound	AChE ± SEM[a]	BChE ± SEM[a]
1	Nil	Nil
2	8.74 ± 0.07	20.12 ± 0.079
3	1.65 ± 0.03	5.98 ± 0.079
4	5.27 ± 0.04	14.76 ± 0.087
5	3.43 ± 0.02	9.84 ± 0.037
Allanzanthaneb	2.94± 0.45	12.96 ± 0.053
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. Data shown are values from triplicate experiments.

which showed good activity. The ethyl acetate soluble fraction was found to be more cytotoxic. In the present work, several new compounds from ethyl acetate soluble fraction which showed good activities in cholinesterase inhibition and DPPH radical scavenging assays were isolated. This species is as one of the ingredient of the traditional medicine in some part of the world.

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