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Abstract

Nymphoides indica (L.) Kuntze (Menyanthaceae) is traditionally used in the Indian subcontinent. However, scientific data reporting its constituents are poor. This study aimed at evaluating its phytochemical constituents and various biological activities. Phytochemical investigations of the extracts and fractions resulted in the isolation of 5 lipophilic compounds, i.e. azelaic **(**nonanedioic) acid (**1**) and 4-methyl-heptanedioic acid (**3**), hexadecanoic (**2**) and stearic acid (**5**) and the fatty alcohol hexadecanol (**4**); 3 seco-iridoids, i.e. 7-epiexaltoside (**6**), 6",7"-dihydro-7-epiexaltoside (**7**) and menthiafolin (**8**); 3 flavonoids, i.e. 3,7-di-*O*-methylquercetin-4'-*O*-β-glucoside (**9**), 3-*O*-methylquercetin-7-*O*- β-glucoside (**10**) and 3,7-di-*O*-methylquercetin (**11**); scopoletin (**12**) and ferulic acid (**13**); and the monoterpenoids foliamenthoic acid (**14**) and 6,7-dihydrofoliamenthoic acid methyl ester (**15**). Compounds 1-5 showed moderate antimicrobial activities, whereas compound **9** presented mild antiprotozoal activities against *Trypanosoma brucei (*IC_{50} 8 µM), *Leishmania infantum (*IC_{50} 32 µM*) and Trypanosoma cruzi (*IC_{50} *30* µM*).* Antiglycation activity was *shown by compound*s *7 (*IC_{50} 0.*36* mM), **10** (IC_{50} 0.*42* mM) *and 15 (*IC_{50} 0.61 mM). *Finally* α-glucosidase inhibition was shown by compounds **7**, **9**, **11** and **13 - 15**. It could be concluded that *N. indica* leaf extracts possess mild to moderate antimicrobial, antiprotozoal, antioxidant and antidiabetic activities.

Key words: *Nymphoides indica*; Menyanthaceae; aquatic herb; traditional medicine; Pakistan

Introduction

Nymphoides indica (L.) Kuntze (Menyanthaceae) is a perennial rhizomatous free floating leaved aquatic herb (Schmidt, 2005). The genus *Nymphoides* is represented worldwide by about 50 species that are not only very diverse but also differ considerably in a number of biological features (Tippery *et al*., 2008). In Pakistan only three species occur, i.e *Nymphoides cristata* (Roxb.), *Nymphoides peltatum* (S.G. Gmel.) (Qaiser, 1977) and *Nymphoides indica* (Marwat *et al*., 2009). These species are primarily reported in the districts of Attock, Thatta, Kashmir and D. I. Khan. *N. indica* is consumed as a vegetable (Cruz-Garcia and Price, 2011; Swapna *et al*., 2011; Boro and Sarma, 2013) and has a consistent folklore usage in the Indian subcontinent for the management of numerous health indications by local healers (called hakeem or herbal doctors). A number of ethnomedicinal surveys can be accessed that principally emphasise on traditional use of this plant species for treatment of dysentery (Das *et al*., 2006; Kensa, 2011), scabies (Panda and Misra, 2011), snake bites (Coea and Anderson, 2005), jaundice (Jain *et al*., 2007), as an antipyretic (Scarp, 2004), anticonvulsant (Madhavan *et al*., 2009), aphrodisiac (Yumnam and Tripathi, 2012), and for antiproliferative purposes (Kitdamrongtham *et al*., 2013). Moreover, it has been reported that it is a part of some important herbal formulations in India (Madhavan *et al*., 2009), Nepal (Siwakoti, 2006), Thailand (Manosroi *et al*., 2012) and Pakistan (Marwat *et al*., 2011) that are used for various therapeutic reasons locally. In spite of the multimedicinal usage of *Nymphoides indica*, no comprehensive published data are available to date concerning its phytochemical constituents and pharmacological properties. Therefore the aim of the present work was to carry out a detailed phytochemical investigation without focusing on a particular activity, and to evaluate extracts and isolated constituents in a series of *in vitro* assays, as a part of running programmes on antimicrobial, antioxidant and antidiabetic properties (inhibition of alpha-glucosidase activity; inhibition of Advanced Glycation Endproducts – AGEs) of medicinal plants and natural products (Upadhyay *et al*., 2014a, Upadhyay *et al.*, 2014b; Mesia *et al*., 2008).

Materials and Methods

General experimental procedures

All solvents were analytical grade and obtained from Fisher Scientific (Leicestershire, UK) and Acros Organics (Geel, Belgium). Methanol and acetonitrile used for HPLC were HPLC grade and purchased from Fisher Scientific. RiOS water was prepared by reverse osmosis and water for HPLC was dispensed by a Milli-Q system, both from Millipore (Bedford, MA, USA). Water was passed through a 0.22 mm membrane filter before usage. All reagents were purchased from Acros Organics or Sigma-Aldrich (St. Louis, MO, USA).

Analytical plates for thin layer chromatography (TLC) were silica gel 60 F_{254} plates (20 x 20 cm) for normal phase (Merck, Darmstadt, Germany). Developed TLC plates (in an appropriate mobile phase, see below) were visualized at UV 254 and 366 nm using Win-Cats software. The spraying reagent *p*-anisaldehyde was prepared by mixing 0.5 mL *p*-anisaldehyde (Sigma-Aldrich) with 10 mL glacial acetic acid, 85 mL methanol and 5 mL sulphuric acid. Developed plates were heated at 105°C until optimal appearance.

Flash column chromatography was carried out on a Reveleris iES system from Grace (Columbia, MD, USA) using the Reveleris Navigator software. The system contained a binary pump equipped with four solvent selection, an ultraviolet (UV) and evaporating light scattering detector (ELSD) and a fraction collector. The column used was a pre-packed Flash Grace Reveleris silica cartridge (80 g) with a particle size of 40 mm. The ELSD carrier solvent was isopropyl alcohol.

HPLC analysis was carried out on an Agilent 1200 series system with degasser, quaternary pump, automatic injection, thermostatic column compartment and a diode array detector (DAD) (Agilent Technologies, Eindhoven, The Netherlands). A silica-based Gracesmart C18 column (250 x 4.6 mm, 5 µm) (Grace Vydac, USA) was used together with a suitable precolumn using acetonitrile/H_2O + 0.1 % formic acid as the mobile phase.

The isolation of compounds was carried out on a semi-preparative HPLC-DAD-MS system (Waters) using a Luna 5µ (C18) 100A 250 x 10.0 mm column (Phenomenex) and Masslynx 4.1 software. The mobile phase used was acetonitrile/H_2O + 0.1 % formic acid. The system was equipped with HPLC pump 515 (Waters 2767), make-up pump (Waters 511), system fluid organiser (SFO), diode array detector (DAD) (Waters 2998) and mass detector (MS Micromass Quattromicro).

GC experiments were performed on a TRACE® 2000 Ultra GC (Thermo Scientific) on a column AT-5MS (Grace): length 30m; ID: 0.25mm; film thickness: 0.25um using helium gas as carrier gas. Derivatisation was performed on dried samples using 20 µL of BSTFA + TMCS, 99 : 1 (Supelco, USA) in pyridine (dry) (Seccosolv) in a molar ratio 2-1, by heating for 1 h at 70°C.

NMR spectra were recorded on a Bruker DRX-400 instrument (Rheinstetten, Germany), operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C, using a 3-mm broadband inverse (BBI) probe or a 5-mm dual $^{1}H/^{13}C$ probe using standard Bruker pulse sequences. Additional DEPT-135, DEPT-90 and 2D-NMR (COSY, HSQC and HMBC) experiments were performed if necessary for structure confirmation. Structure elucidation was assisted by a ^{13}C-NMR database (NMR Predict version 4.8.57, Modgraph). Deuterated solvents including $CDCl_3$ (99.8% D) and CD_3OD (99.8% D) were purchased from Sigma-Aldrich.

UV-VIS (ultraviolet – visible light) absorbance was measured on a Perkin Elmer Lambda 11 spectrophotometer. Fluorescence (excitation 335 nm, emission 385 nm) was measured on a Tecan Infinite M200 spectrofluorometer.

Plant material

N. indica leaves were collected in June and July 2012 from wetlands of the river Indus near Dera Ismail Khan (KPK) Pakistan. The collected plant leaves were identified by the Islamabad Herbarium at the Taxonomy Department, Quaid I Azam University, Islamabad, Pakistan, where the voucher specimen was deposited (5298-GA, Accession No. ISL-120019). The leaves were shade dried, powdered and sieved through a 20 mesh filter, and stored below 20°C till further use.

Extraction and fractionation

Powdered leaves (1.92 kg) were extracted with 90% (v/v) methanol by double cold maceration. The extract was instantaneously filtered through Whatman No.1 filter paper using a vacuum pump. The filtrates were combined together and evaporated on a rotary evaporator under reduced pressure below 40°C. The resultant semisolid material was lyophilised with a final yield 88.61 g, and stored below 20°C. Further liquid–liquid

partitioning of the crude extract was performed according to a standard extraction scheme (Supporting Information, Fig. S1). After partitioning with different solvents as shown in the scheme, *n*-hexane (1.8 g), methanol 90% (11.2 g), chloroform (2.03 g), ethyl acetate (1.2 g) and *n*-butanol (19.58 g) fractions were obtained. The collected fractions were dried under reduced pressure at 40 °C and stored below 20 °C. Then each fraction was subjected to TLC analysis (normal phase) using various solvent systems as mobile phase, including $CHCl_3$ / MeOH (75 : 25) with a few drops of NH_4OH for the methanolic fraction; $CHCl_3$ / MeOH (80 : 20) for the chloroform and ethyl acetate fractions; *n*-hexane / $CHCl_3$ (95 : 5) for the *n*-hexane fraction; and $CHCl_3$ / MeOH (25 : 75 or 37 : 63) the for *n*-butanol fraction. Developed TLC plates were examined under UV at 254 nm and 366 nm, after spraying with *p*-anisaldehyde reagent.

Phytochemical analysis

Phytochemical screening for various compound classes was carried out on every fraction using published methods with slight modifications, i.e. sterols and triterpenes (Connolly *et al*., 1970), carbohydrates and saponins (Wolf *et al*., 1962), flavonoids and alkaloids (Egwaikhide, 2007) and tannins (Farnsworth, 1966).

The total phenolic content (TPC) of all fractions was estimated using Folin-Ciocalteu's reagent according to Bursal and Gulcin (2011) with slight modifications. Briefly, 10 µL of each sample (0.2 mg/mL) or reference compound (gallic acid 0.015–0.5 mg/ml) was mixed with 100 µL of Folin-Ciocalteu's reagent, and incubated at room temperature and after 5 min, 90 µl of 10% Na_2CO_3 was added to the above mixture. These mixtures were then incubated at room temperature for 40 min. The absorbance was measured at 765 nm. TPC was expressed as gallic acid equivalents (GAE). Based on findings of Dudonne *et al.* (2009), who have compared 30 plant extracts, TPC were categorized as very high (> 300 mg GAE/g), high (200 -300 mg GAE/g), moderate (50-200 mg GAE/g), low (15-50 mg GAE/g) or very low (<15 mg GAE/g).

The total flavonoid content (TFC) of all fractions was assessed according to Sun *et al*. (2011). Briefly, 20 µL of each sample (1 mg/mL) or reference compound (rutin) was mixed with 30 µL of 5% sodium nitrite. After 6 min, 50 µL of a 10% $AlCl_3$ solution was added, and the mixture was kept untouched for 5 min. Next, 100 µL of a 10% NaOH solution was added, the mixture was incubated at 25°C for 15-20 min, and the absorbance was measured at 510 nm.

TFC was expressed as rutin equivalents (RUE). TFC was expressed as rutin equivalents (RUE). TFC were categorized as very high (>300 mg RUE/g), high (200-300 mg RUE/g), moderate(50-200 mg RUE/g), low (15-50 mg RUE/g), very low (<15 mg RUE/g).

Isolation of compounds

An aliquot of 0.8 g from the *n*-hexane fraction was dissolved in 2 mL methanol, mixed with 1.1 g silica and dried with nitrogen gas. The dried extract was subjected to flash chromatography with a gradient from *n*-hexane over methylene chloride to methanol. Based on UV and ELSD detection different subfractions were collected. All fractions were analysed by TLC and similar fractions were combined. In this way 21 subfractions were obtained. The crude extract, the *n*-hexane fraction and all obtained subfractions were analysed by HPLC using an acetonitrile / H_2O + 0.1% formic acid gradient, ranging from 5% acetonitrile to 100% in 60 min at a flow rate of 1 mL/min. Samples were prepared in a concentration range from 1-10 mg/ mL in methanol. Based on TLC and HPLC profiling, subfractions 4 (190 mg) and 7 (150 mg) were selected for isolation of pure compounds by semi-preparative HPLC-DAD-MS using the same gradient at 3 mL/min, yielding compounds **1** (3.5 mg)**, 2** (4.2 mg)**, 3** (4.2 mg), **4** (3.6 mg) and **5** (5.2 mg).

An aliquot of 0.79 g from the methanolic fraction was subjected to flash chromatography; 19 subfractions were obtained and analysed by HPLC as described above. Based on TLC and HPLC profiling, subfractions 4 (250 mg), 6 (180 mg) and 7 (150 mg) were selected for isolation of pure compounds by semi-preparative HPLC-DAD-MS using a gradient ranging from 15% acetonitrile to 100% in 40 min, yielding compounds **6** (5.2 mg), **7** (5.7 mg), **8** (7.5 mg), **9** (4.2 mg), **11** (4.9 mg), **12** (2.6 mg).

Similarly an aliquot of 0.9 g from the ethyl acetate fraction was subjected to flash chromatography as described above; 15 subfractions were obtained and analysed by HPLC using previously mentioned conditions. Based on TLC and HPLC profiling subfractions 4 (126 mg) and 7 (154 mg) were selected for isolation of pure compounds by semi-preparative HPLC-DAD-MS, using a gradient ranging from 35% acetonitrile to 70% in 45 min, yielding compounds **9** (4.5 mg), **10** (3.2 mg) and **13** (3.8 mg).

Similarly an aliquot of 0.7 g from the chloroform fraction was subjected to flash chromatography as previously explained; 10 subfractions were obtained and analysed by HPLC as described previously. Based on TLC and HPLC profiling subfraction 2 (130 mg) was selected for isolation of pure compounds by semi-preparative HPLC-DAD-MS, using a gradient ranging from 39% acetonitrile to 65% in 45 min, yielding compounds **14** (3.2 mg) and **15** (3.8 mg).

Finally an aliquot of 1.5 g from the *n*-butanol fraction was subjected to flash chromatography as described above; 18 subfractions were obtained and analysed by HPLC as explained previously. Based on TLC and HPLC profiling subfraction 2 (126 mg) and 5 (154 mg) were selected for isolation of pure compounds by semi-preparative HPLC-DAD-MS, using a gradient ranging from 30% acetonitrile to 65% in 50 min, yielding compounds **9** (7.3 mg), **10** (4.6 mg), **14** (7.2 mg), **15** (6.3 mg).

Structure elucidation

The preliminary NMR data of compounds **1** – **5** from the *n*-hexane fraction indicated the possible occurrence of fatty acids and fatty alcohols, and were therefore subjected to GC-MS analysis after derivatisation (as explained previously). They could be identified as azelaic acid **(**nonanedioic acid**)** (**1**), hexadecanoic acid (**2**), 4-methyl-heptanedioic acid (**3**), hexadecanol (**4**) and stearic acid (**5**) (Table 1).
Structures of the remaining compounds were elucidated using ^{1}H and ^{13}C NMR as well as 2D NMR spectroscopy. The molecular ion was derived from mass spectral data obtained from the semi-preparative HPLC-DAD-MS system. Detailed mass and NMR spectral data and assignments are added as Supporting Information.

7-Epiexaltoside (**6**): Yellow powder; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Junior, 1991) (Fig. S2, S3); ESI-MS (positive ion mode): *m/z* 563 $[M+Na]^{+}$.

6",7"-Dihydro-7-epiexaltoside (**7**): Yellow powder; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Fig. S4, S5); ESI-MS (positive ion mode): *m/z* 565 $[M+Na]^{+}$.

Menthiafolin (**8**): Yellow powder; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Junior, 1989) (Fig. S6, S7); ESI-MS (positive ion mode): *m/z* 563$[M+Na]^{+}$

3,7-Di-O-methylquercetin-4'-O-β-glucoside (**9**): Yellow powder; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Agarwal and Rastogi, 1981; Guvenalp and Demirezer, 2005) (Fig. S8, S9); ESI-MS (positive ion mode): *m/z* 493 $[M+H]^{+}$.

3-O-Methylquercetin-7-O-β-glucoside (**10**): Yellow amorphous powder; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Krenn *et al*., 2003) (Fig. S12, S13); ESI-MS (positive ion mode): *m/z* 479 $[M+H]^{+}$

3,7-Di-O-methylquercetin (**11**): Yellow amorphous powder; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Guerrero *et al*., 2002) (Fig. S14, S15); ESI-MS (positive ion mode): *m/z* 331 $[M+H]^{+}$

Scopoletin (**12**): Colourless needles; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Darmawan *et al*., 2012) (Fig. S16, S17); ESI-MS (positive ion mode): *m/z* 193 $[M+H]^{+}$.

Ferulic acid (**13**): White needles; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Sajjadi *et al*., 2012) (Fig. S18, S19); ESI-MS (negative ion mode): *m/z* 193 $[M-H]^{-}$.

8-Hydroxy-2,6-dimethyl-(2E,6E)-octadienoic acid; foliamenthoic acid (**14**): Yellowish powder; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Iwagawa *et al*., 1990) (Fig. S20, S21); ESI-MS (positive ion mode) *m/z* 207 $[M+Na]^{+}$.

6,7-Dihydrofoliamenthoic acid methyl ester (**15**): Yellowish powder; ^{1}H and ^{13}C NMR assignments: Supplementary Information (Otsuka, 1994) (Fig. S22, S23); ESI-MS (positive ion mode) *m/z* 201 $[M+H]^{+}$.

Biological Evaluation

DPPH radical scavenging activity. The ability of the crude extract or fractions to scavenge DPPH radicals was evaluated according to Szabo *et al*. (2007) with some modifications. An aliquot (50 μL) of test sample (prepared in a concentration range (3.84 – 0.12 mg/mL in

methanol) or quercetin (reference compound with stock concentration ranging from 1.08 – 0.0312 mg/mL in methanol) was mixed with PBS (450 µL, 10 mM, pH 7.4) and 1.0 mL of a methanolic solution of DPPH (0.1 mM), yielding a series of test samples in a final concentration range of 128 – 4 µg/mL) and a series of quercetin samples in a final concentration of (1 – 36 µg/ml). After 30 min the absorbance was recorded at 517 nm and % inhibition was calculated using the following formula:

DPPH radical scavenging activity (%) $= [(OD_{control} - OD_{sample}) / OD_{control}] \times 100$

Based on findings of Dudonne *et al.* (2009), DPPH inhibition was categorized as very low (1-10%), low/week (10%-30%), moderate (30-60%), high (61-69%) or very high (70-100%).

Inhibition of α-glucosidase. α-Glucosidase inhibitory activity was assayed according to method adopted by Choudhary *et al*. (2010) with some modifications. Briefly, 50 µL of a solution of α-glucosidase from *Saccharomyces cerevisiae* (0.2 U/mL in 0.1 M phosphate buffer at pH 6.8, Sigma-Aldrich, St. Louis, MO, USA) was incubated with 20 µL of test compounds or extracts at different concentrations (ranging from 6 mM – 0.372 mM in 25% DMSO for isolated constituents or from 5 mg to 0.039 mg/ml for extracts) at 37 °C for 10 min. Then 50 µl of the substrate, *p*-nitrophenyl-α-D-glucopyranoside (0.7 mM stock, final concentration 0.29 mM) was added to the reaction mixture and incubated again for 30 min at 37 °C. The final concentrations of test substances in the reaction mixture ranged from 1 – 0.062 mM for isolated constituents, and from 834 µg to 6.5 µg/ml for extracts. The reaction was stopped by adding Na_2CO_3 (100 µl, 200 mM stock) solution and the absorbance was measured at 400 nm. Final DMSO concentration remained below 7.5% during the assay. Acarbose (1 – 0.031 mM final concentration) was used as reference compound. The % inhibition was calculated using the following formula:

% Inhibition $= 100 - [OD_{(test\ sample)} / OD_{(control)}] \times 100$

Antiglycation assay. The antiglycation assay was performed according to the method developed by Matsuura *et al.* (2002) with minor modifications. The reaction mixture (300 µL) contained bovine serum albumin (BSA) (10 mg/ml, 135 µL), D-glucose (500 mM, 135 µL) dissolved in phosphate buffer (50 mM, pH 7.4, containing sodium azide (0.02%, added

to prevent bacterial growth) and test compounds (30 µL), at different final concentrations (0.6 – 0.021 mM for isolated constituents or 100 – 1.17 µg/ml for extracts) dissolved in 100% DMSO. The mixtures were incubated at 60 °C for 48 h. After incubation, the reaction mixture was allowed to cool down at room temperature. Then 100 µL reaction mixture was transferred to a new plastic tube (1.5 mL) and the reaction was stopped by adding 10 µL of 100% (w/v) trichloroacetic acid (TCA) to precipitate proteins. The TCA-added mixture was kept at 4 °C for 10 min, followed by centrifugation (14000 rpm, 4 °C, 4 min). The supernatant containing unreacted D-glucose, test sample and interfering substances was discarded, whereas the precipitate containing AGEs-BSA was redissolved with 0.8 mL alkaline PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 10). The change in fluorescence intensity (excitation 335 nm, emission 385 nm) due to formation of AGEs was monitored by spectrofluorometry (Tecan® Infinite M200, Giessen, The Netherlands). In order to eliminate interference by autofluorescence of the test compounds, a parallel incubation of test substance with BSA at 60°C without D-glucose was performed for all samples. Quercetin (0.5 – 0.0312 mM final concentration) and aminoguanidine (3 - 0.046 mM final concentration) were used as reference compounds.

The AGEs inhibition was calculated as

$$\% \text{ inhibition} = \{1- (F_{BSA + glucose + test substance} - F_{BSA + test substance}) / (F_{BSA + glucose} - F_{BSA})\} \times 100$$

where F is the fluorescence intensity.

Antimicrobial activity. Fractions and isolated compounds were evaluated for antimicrobial activity in an integrated screening panel against the bacteria *Staphylococcus aureus* and *Escherichia coli*, the fungi *Microsporum canis* and *Aspergillus fumigatus*, the yeast *Candida albicans*, and the protozoa *Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania infantum*, and *Plasmodium falciparum* strain K1. In order to evaluate selectivity, cytotoxicity against MRC-5 cells (human foetal lung fibroblasts) was evaluated as well. The IC_{50} values were determined from five 4-fold dilutions. The selectivity index (SI) was calculated as the ratio of the cytotoxicity on MRC-5 (IC_{50}) to the antimicrobial activity (IC_{50}) (Cos *et al*., 2006; Balde *et al*., 2008; Mesia *et al*., 2008). For the different tests, appropriate reference drugs were used as positive control: Erythromycin for *S. aureus* (IC_{50} 11.2 µM), trimethoprim for *E. coli* (IC_{50} 11.2 µM), terbinafin for *M. canis* (IC_{50} 0.1 µM) and *A. fumigatus* (IC_{50} 0.79 µM), miconazole for *C. albicans* (IC_{50} 5.9 µM), benznidazole for *T. cruzi* (IC_{50} 3.27 µM), suramine for *T.*

brucei (IC_{50} 0.03 µM), fungizone for *L. infantum* (IC_{50} 1.15 µM), chloroquine for *P. falciparum* K1 (IC_{50} 0.16 µM) and tamoxifen for MRC-5 cells (C_{50} 11.4 µM).

Statistical analysis

IC_{50} calculations were performed using regression analysis [% inhibition vs. log(concentration)] using Sigma plot 13.0.

Results and Discussion

Phytochemical screening of the different extracts and fractions revealed that flavonoids, tannins, saponins and triterpenes are present with varying concentrations in the different fractions of the leaves extract (Table 2). No alkaloids were detected. Fractions of medium polarity contained flavonoids and terpenes. The residual aqueous fraction was positive for tannins and saponins. These finding are consistent with previous reports (Madhavan *et al*., 2009; Kitdamrongtham *et al*., 2013).

All fractions were analysed for their total phenolic, total flavonoid content (TPC and TFC, respectively) and DPPH scavenging activity (Table 3). The TFC of the crude extract was 70.28 mg RUE/g extract, as calculated from the rutin calibration curve**.** In the different fractions the highest flavonoid contents were observed in the ethyl acetate fraction (73.44 mg RUE/g) followed by the methanol fraction (62.34 mg RUE/g). The chloroform (17.52 mg RUE/g), *n*-butanol fractions (10.57 mg RUE/g) had comparably low flavonoid contents. Similarly, the total phenolic content (TPC) of the total extract was 28.88 mg GAE/g as calculated from the gallic acid calibration curve, whereas the ethyl acetate (34.1 mg GAE/g) and methanol 90% (31.87 mg GAE/g) subfractions contained comparably high amounts. The comparably high phenolic and flavonoid contents were indicative of possibly high antioxidant activities in the corresponding fractions. Indeed the ethyl acetate fraction presented comparably better antioxidant potential (IC_{50} 81 µg/ml), then methanol fraction (IC_{50} 97 µg/ml) and chloroform fractions (IC_{50} 119 µg/ml). These finding were in accordance with previous reports that correlate the antioxidant activities of plant extracts with high flavonoid and phenolic contents (Yoo *et al*., 2008; Pathirana and Shahidi, 2005; Goulas *et al*., 2014). Furthermore, in an attempt to quantitatively estimate the TPC level, these findings were compared with a broader investigation that involved a wide range of

medicinal plants (30 in total) (Dudonne *et al.*, 2009). Upon comparison with the present TPC findings, it could be stated that the total phenolic contents of *Nymphoides indica* should be considered as very low to low. Likewise, based on the findings in the same study for antioxidant (DPPH scavenging) activity, the *N.indica* leaves fractions was considered as moderately active.

The different fractions were further tested for potential antiglycation and α-glucosidase inhibitory activity (Table 4). A mild to moderate antiglycation activity was observed for the crude extract at 100 µg/mL (33%). Likewise all tested fractions presented moderate (24-36%) inhibition of the formation of AGEs. A positive correlation has been reported before between antiglycation activities and DPPH scavenging activity in a study focusing on phenolic constituents (Harris *et al*., 2011). However, the ethyl acetate extract of *N. indica* showed the highest DPPH scavenging activity (IC_{50} 81 µg/ml), but its antiglycation activity was lower than the MeOH 90% extract. This indicates that *N. indica* contains antiglycation constituents with a non-phenolic structure, active through non-oxidative pathways.

Similarly mild α-glucosidase inhibitory activity was observed for the total extract at 834 µg/mL (13%), whereas the highest inhibition was shown by the methanol 90% (31%) and *n*-butanol fractions (25%) fractions at the same concentration. At higher test concentrations the inhibitory activity did not further increase and the 50% inhibition level could not be reached.

Finally the crude extracts were analysed for antimicrobial and antifungal activities. The highest levels of antimicrobial and antifungal activities were observed for n the *n*-hexane fraction, i.e. an IC_{50} of 19.5 µg/mL against *S. aureus* and 32 µg/mL against *M. canis*. Similarly the methanol 90% fraction was active (IC_{50} 36.4 µg/ml) against *S.aureus* and cytotoxic (IC_{50} 38.9 µg /mL) against MRC-5 cells (Table 5).

A total of 16 compounds were isolated from the different active fractions. From the *n*-hexane fraction 5 lipophilic constituents were obtained. Their ^{1}H- and ^{13}C-NMR spectra showed the characteristic features of fatty acids and fatty alcohols, and after derivatisation and GC-MS analysis they were identified as the dicarboxylic acids azelaic acid **(**nonanedioic acid**)** (**1**) and 4-methyl-heptanedioic acid (**3**), the monocarboxylic acids hexadecanoic acid (**2**) and stearic acid (**5**) and the fatty alcohol hexadecanol (**4**). In view of the antimicrobial activity of the *n*-hexane fraction, compounds **1** – **5** were evaluated in an integrated screening panel. Only

azelaic acid (**1**) was active against *S. aureus* with an IC_{50} value of 55.1 µM (vs. 11.2 µM for erythromycin) (Table 6). It also showed weak antiplasmodial activities against *P. falciparum* K1, *T. brucei* and *T. cruzi*, but it was also cytotoxic against MRC-5 cells in the same concentration range (IC_{50} 32.2 µM). Compounds **2** – **5** showed comparable activity against some of the protozoal parasites. Lipophilic compounds such as medium and long chain fatty acids have been previously reported to contribute to antiprotozoal and antifungal activities (Krugliak *et al*., 1995; Wang & Johnson, 1992; Ozcelik 2005a; Orhan, 2009). It was therefore concluded that the activity of the *n*-hexane fraction of *N. indica* was mainly due to presence of long chain mono and dicarboxylic fatty acids and fatty alcohols that may act synergistically.

Three seco-iridoid glucosides (**6** – **8**) were isolated from the methanolic fraction. Based on its ^{1}H-, ^{13}C-NMR and mass spectra compound **6** was found to be identical to 7-epiexaltoside, originally obtained from *Villarsia exaltata* (Menyanthaceae) by Junior (1991). This compound contains a geranyl-like side chain, with two double bonds having an *E*-configuration. The carbonyl group of the ester moiety showed a ^{13}C-NMR resonance at 167.1 ppm, which is typical for an α, β-unsaturated ester. The NMR spectra of compound **7** were very similar to those observed for compound **6**, but it contained one double bond less, which was confirmed by the $[M+Na]^+$ signal observed in MS at *m/z* 565, rather than *m/z* 563 for compound **6**. Since the signals assigned to the seco-iridoid moiety in compound **6** were also observed in compound **7**, it was concluded that the saturation was located in the geranyl-like side chain. Because the ester carbonyl was observed at δ 166.1, as expected in case of an α,β-unsaturation, it was concluded that, compared to compound **6**, the 6",7"- double bond was saturated rather than the 2",3"-double bond. A compound named dihydrofoliamenthin, containing a geranyl-like side chain saturated in position 2",3" and a *Z*-configuration at the 6",7"-double bond, has been reported before from *Villarsia exaltata* by Junior (1991). Hence, compound **7**, for which the name 6",7"-dihydro-epiexaltoside was adopted, is reported here for the first time from nature. Finally, all spectral data of compound **8** were in good agreement with those reported for menthiafolin, containing a C_{10} side chain with a terminal double bond from *Menyanthes trifoliata* by Junior (1989). This is the first report on the occurrence of these seco-irodoids in *N. indica*. Remarkably, *Villarsia exaltata*, *Menyanthes trifoliate* as well as *Nymphoides indica* belong to the Menynathaceae family.

Interestingly, both monoterpenoid substituents were isolated as pure compounds from the chloroform fraction. Compound **14** was identified as 8-hydroxy-2,6-dimethyl-(2*E*,6*E*)-octadienoic acid or foliamenthoic acid, reported before from *Radermachia sinica* (Bignoniaceae) by Iwagawa *et al.* (1990), which is in fact the monoterpenoid side chain present in 7-epiexaltoside (**6**). Similarly, compound **15** could be identified as 6,7-dihydrofoliamenthoic acid methyl ester, the acyl part of which is the ester moiety occurring in 6",7"-dihydro-epiexaltoside (**7**). 6,7-Dihydrofoliamenthoic acid has been reported before as a structural component of e.g. monoterpene diglucosides from *Linaria japonica* (Scrophulariaceae) by Otsuka et al. (1994). The same authors have reported the ^{1}H- and ^{13}C-NMR assignments for 6,7-dihydrofoliamenthoic acid methyl ester, obtained by methanolic 0.1 M NaOH hydrolysis followed by enzymatic hydrolysis of its parent glycosidic compound.

From the methanol, ethyl acetate and *n*-butanol fractions 3 compounds were obtained that showed the typical NMR spectroscopic features of flavonoids. Compound **9** was identified as 3,7-di-*O*-methylquercetin-4'-*O*-β-glucoside (**9**). Its ^{1}H- and ^{13}C-NMR assignments were based on those reported for related flavonoids by Agarwal and Rastogi (1981) and Guvenalp and Demirezer (2005), and confirmed by 2D-NMR experiments. Two other flavonoids, i.e. 3-*O*-methylquercetin-7-*O*-β-glucoside (**10**) and 3,7-di-*O*-methylquercetin (**11**) were identified based on spectral assignments reported by Krenn *et al*. (2003) and Guerrero *et al*. (2002), respectively. 3,7-Di-*O*-methylquercetin (**11**) and its 4'-glucoside (**9**) were reported before from *N. indica* in a study by Bohm *et al*. (1986) on the occurrence of flavonoids in the Menyanthaceae, in contrast to 3-*O*-methylquercetin-7-*O*-β-glucoside (**10**). Its isomer 7-*O*-methylquercetin-3-*O*-β-glucoside had been reported before by Bohm *et al.* (1986), but the identity of **10** was evident from the ^{13}C-NMR chemical shift of the methoxy-group at δ 59.1, where it was rather expected around δ 55 for a methoxyl in at C-7. Finally, compounds **12** (from the methanolic fraction) and **13** (from the ethyl acetate fraction) were identified as scopoletin and ferulic acid, respectively, by comparison with published data (Darmawan *et al*., 2012; Sajjadi *et al*., 2012).

Compounds **6** – **15** were evaluated in the same *in vitro* pharmacological assays as the crude extracts and fractions. With regard to antimicrobial activities (Table 6), the seco-iridoids **6** – **8** were not active. Nevertheless, the possibility cannot be excluded that after oral intake the

glycosidic moiety is hydrolysed, and that the resulting aglycones and their metabolites are active. Flavonoid **9** showed weak activity against *T. brucei* and *T. cruzi*; weak activity against *L. infantum* was accompanied by PMM (peritoneal mouse macrophages) cytotoxicity. A number of reports (Muzitano *et al*., 2006a; Muzitano *et al*., 2006b) covering the anti-leishmanial activities of flavonoids support our findings. It is well known that flavonoids are major contributors of many plants to a wide array of activities including antimicrobial (Lovkova *et al*., 2001; Cushnie and Lamb 2005). Compounds **10** – **15** were devoid of antimicrobial activity.

In the antidiabetic assays the seco-iridoid **7** presented moderate antiglycation (54% inhibition at 0.67 mM, IC_{50} 0.36 mM) and mild α-glucosidase inhibitory (37% at 1 mM) activities (Table 7). Flavonoids **9** – **11**, ferulic acid (**13**) and the monoterpenoids **14** and **15** showed moderate anti-glycation properties, being less active than the standard inhibitor quercetin, but more active than aminoguanidine. Some of these compounds also presented mild to moderate α-glucosidase inhibitory activities, more in particular flavonoids **9** and **11**, ferulic acid 13 and the monoterpenoids **14** and **15**. As the *n*-butanol fraction showed antiglycation activity (24% inhibition), it seems that the AGEs inhibition potential of this fraction is mainly contributed by flavonoids and monoterpenes as reported earlier (Al-Musayeib *et al*., 2011; Balasubramaniam and Anuradha, 2011; Joglekar *et al*., 2013). These findings were consistent with previous reports (Silvan *et al*., 2011; Srey *et al*., 2010; Adisakwattana *et al*., 2009). Compound **12** was not tested for antiglycation and α-glucosidase inhibitory activity due to insufficient amount available; however, this class of compounds is well known for antiglycation and α-glucosidase inhibition (Mosihuzzman *et al*., 2013). It could therefore be concluded that major compounds that contributed towards the antiglycation activity of the ethyl acetate fraction could be phenolic acids and flavonoids.

Conclusions

In conclusion it could be demonstrated that *Nymphoides indica* leaf extracts possess mild to moderate antimicrobial, antioxidant and antidiabetic activities and this may support the traditional use of this plant species for treatment of various health issues in the subcontinent of India and Pakistan. Nevertheless, no pure compounds demonstrating pronounced activities could be obtained. Several constituents may contribute synergistically towards the observed

activities; indeed it has been observed that isolation of compounds from an extract may cause a decrease in activity (Orhan *et al*., 2009). The activity could be due to the combined action of fatty acids, flavonoids, monoterpenoids and coumarins that may act synergistically to produce the different effects.

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Conflict of interest

The authors declare no conflicts of interests.

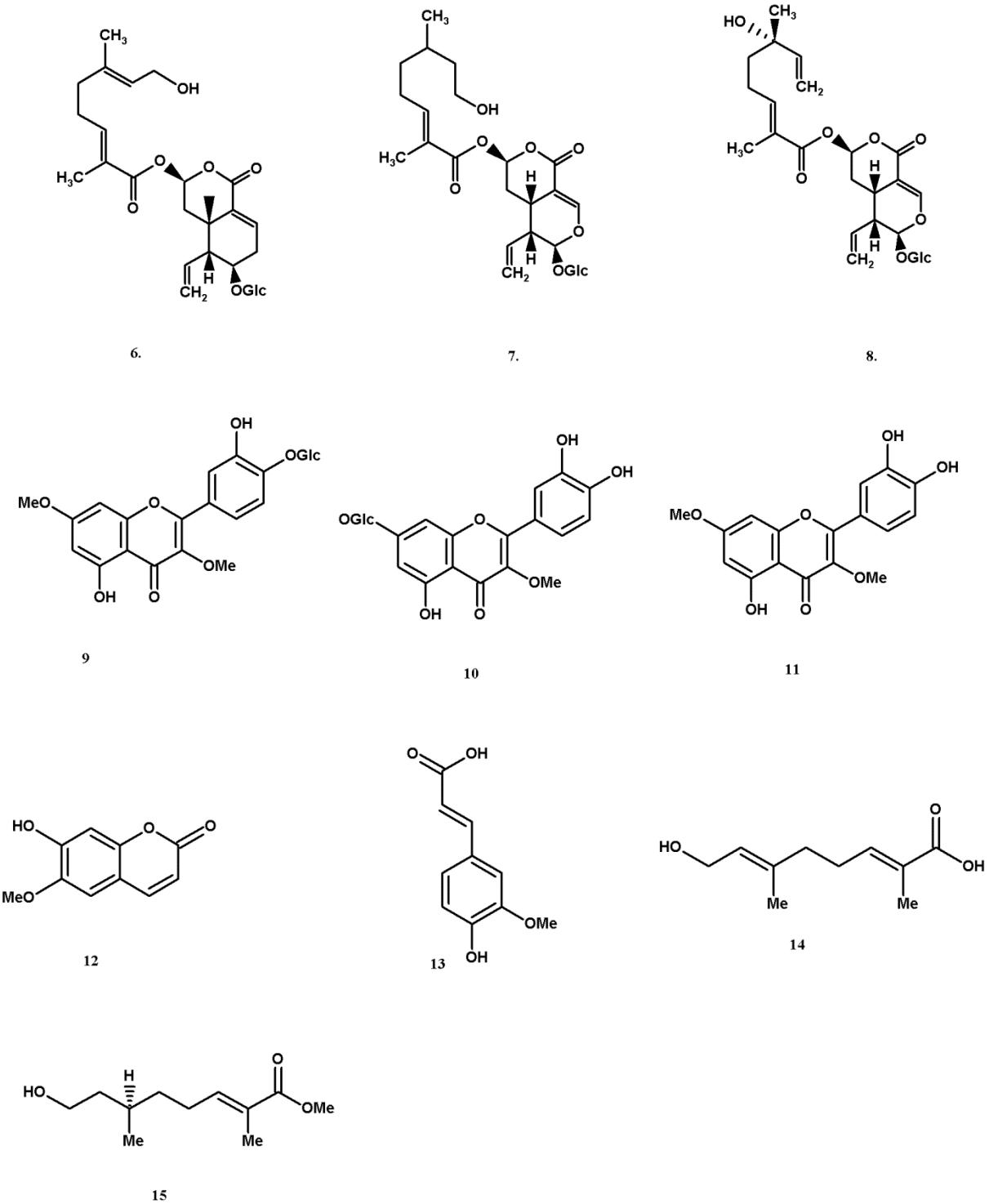

Figure 1. Isolated compounds from *N. indica* leaves.

Table. 1 GC-MS profile of compounds isolated from *n*-hexane fraction.

Comp	RT(min)	MW	Prob.	Formula	Peak name
1	27.73	332	45.24	$C_{15}H_{32}O_4Si_2$	Azelaic acid-bis(trimethylsilyl)ester
2	35.57	328	72.86	$C_{19}H_{40}O_2Si$	Hexadecanoic acid trimethylsilyl ester
3	24.37	318	48.36	$C_{14}H_{30}O_4Si_2$	Heptanedioic acid, 4-methyl (bistrimethylsilylester)
4	39.10	314	17.38	$C_{19}H_{42}OSi$	1-trimethylsiloxyhexadecane
5	39.36	356	48.99	$C_{21}H_{44}O2Si$	Stearic acid trimethylsilylester

Table 2. Phytochemical analysis of the *N.indica* leaves

Extract fraction	Phytochemicals				
	Alkaloids	Flavonoids	Tannins	Steroids/triterpenoids	Saponins
MeoH 90%	-	+++	-	+++	-
Chloroform	-	++	-	++	-
Ethyl acetate	-	+++	-	+	-
n-Hexane	-	-	-	-	-
n-butanol	-	+	-	++	++
Aqueous	-	-	+++	-	+++

profoundly present: high degree of precipitation (dark coloration), moderately present: less degree of precipitation (medium colouration), slightly present: very low precipitation (very little colouration), absent: no change in colour (Senguttuvan et al., 2014)

Phytochemicals: +++ profoundly present, ++ moderately present , + slightly present, - absent.

Table 3. Total phenolic, total flavonoid contents and antioxidant activities of *N. indica* fractions.

Sample	TPC	TFC	DPPH	
	(mg GAE/g extract)[a]	(mg RUE/g extract)[b]	% inhibition [c]	IC_{50} µg/ml
Total Extract	28.88 ± 0.4	70.28 ± 1.0	53	121
MeoH 90%	31.87 ± 0.8	62.34 ± 0.9	55	97
Chloroform	9.76 ± 0.9	17.52 ± 1.2	52	119
Ethyl acetate	34.10 ± 0.5	73.44 ± 1.2	60	81
n-butanol	16.38 ± 1.0	10.57 ± 0.3	48	-
Quercetin				6

(a) Total phenolic contents, gallic acid equivalents (b) Total flavonoid contents, rutin equivalents (c) at 128 µg/mL (final concentration).

Table 4. Anti-glycation and α-glucosidase inhibitory effects of *N. indica* fractions.

Sample	Antiglycation % Inhibition[a]	α-glucosidase inhibition % Inhibition[b]
Total Extract	33	13
MeOH 90%	36	31
Chloroform	23	12
Ethyl acetate	27	17
n-butanol	24	25
Standard inhibitor	78	67[b]

[a] at 100 µg/mL. Quercetin IC_{50} 0.11mM,
[b] at 834 µg/mL. Acarbose IC_{50} 0.26mM

Table 5. Antibacterial, antifungal and cytotoxic activities of *N. indica* fractions.

Sample	IC_{50} µg/ml				
	S. aureus	*E. coli*	*C. albicans*	*M. canis*	MRC-5
MeoH 90%	36	>64	>64	>64	39
Chloroform	>64	>64	>64	>64	>64
Ethyl acetate	>64	>64	>64	>64	>64
n-Hexane	20	>64	>64	32	>64
n-butanol	>64	>64	>64	>64	>64
Aqueous	>64	>64	>64	>64	>64

Reference: Tamoxifen(MRC-5) IC_{50} 11.4 µg/mL; *erythromycin (S.aureus)*IC_{50} 11.2 µg/m; trimethoprim (*E.coli*) IC_{50} 0.25 µg/mL; miconazole(*C. albicans*) IC_{50} 5.99 µg/mL; terbinafin (*M.canis*) IC_{50} 0.11 µg/mL.

MRC-5: Human fetal lung fibroblasts; *S. aureus: Staphylococcus aureus; E. coli: Escherichia coli; C. albicans: Candida albicans*

Table. 6. Antimicrobial, antifungal cytotoxic and antiprotozoal activities (IC_{50} µM)of isolated compounds from *N. indica* fractions.

Compound no.	IC_{50} µM									
	MRC5	*Pf-K1*	*T. bruc*	*T. cruz*	*L. inf*	*PMM*	*S. aureus*	*E. coli*	*C. albicans*	*A. fumigatus*
1	32	62	32	35	> 64	> 64	55	> 64	> 64	> 64
2	29	> 64	> 64	34	> 64	> 64	> 64	> 64	> 64	> 64
3	32	> 64	> 64	38	> 64	> 64	> 64	> 64	> 64	> 64
4	> 64	> 64	32	31	51	> 64	> 64	> 64	> 64	> 64
5	33	> 64	> 64	34	32	32	> 64	> 64	> 64	> 64
6	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
7	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
8	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
9	> 64	> 64	8	30	32	32	> 64	> 64	> 64	> 64
10	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
11	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
12	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
13	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
14	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
15	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64

Reference: Tamoxifen (MRC-5), IC_{50} 11.35 µM; suramine (*T.brucei*), IC_{50} 0.03 µM; fungizone (*L. inf.*), IC_{50} 1.15 µM; chloroquine (*Pf-K1*), IC_{50} 0.16 µM; benznidazole (*T. cruzi*), IC_{50} 3.27 µM; erythromycin (*S. aureus*), IC_{50} 11.31 µM; chloramphenicol (*E. coli*), IC_{50} 4.88 µM; miconazole (*C. albicans*), IC_{50} 10.5 µM; terbinafine (*A. fumigatus*), IC_{50} 0.79 µM.

MRC-5: human fetal lung fibroblasts; Pf - K1: *Plasmodium falciparum* K1; *T. bruce: Trypanosoma brucei; T. cruzi*: *Trypanosoma cruzi; L. inf.: Leishmania infantum*; PMM: Peritoneal murine macrophages; *S. aureus: Staphylococcus aureus; E. coli: Escherichia coli; C. albicans: Candida albicans; A. fumigatus: Aspergillus fumigatus*.

Table 7. Anti-glycation and α-glucosidase inhibitory effect of isolated compounds from *N. indica*

Compound	Protein glycation		α-glucosidase	
	% inhibition[a]	IC_{50} (mM)	% inhibition[b]	IC_{50} (mM)
6	49	-	na	-
7	54	0.36	37	-
8	41	-	na	-
9	44	-	45	-
10	54	0.42	na	-
11	39	-	27	-
12	nt	-	nt	-
13	34	-	35	-
14	47	-	38	-
15	51	0. 61	40	-
Standard Inhibitor		0.11[c] 1.75[d]		0.26[e]

na not active
nt not tested (because of low amount available)
[a] (at 0.67 mM)
[b] (at 1 mM)
[c] Quercetin
[d] Aminoguanidine
[e] Acarbose