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Characterization of the bioactive constituents of *Nymphaea alba* rhizomes and evaluation of anti-biofilm as well as antioxidant and cytotoxic properties

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Anti-biofilm represents an urge to face drug resistance. *Nymphaea alba* L. flowers and rhizomes have been traditionally used in Ayurvedic medicine for dyspepsia, enteritis, diarrhea and as an antiseptic. This study was designed to identify the main constituents of *Nymphaea alba* L. rhizomes and their anti-biofilm activity. 70% aqueous ethanolic extract (AEE) of *N. alba* rhizomes was analyzed by liquid chromatography, high resolution, mass spectrometry (LC-HRMS) for its phytoconstituents in the positive and negative modes in addition to column chromatographic separation. Sixty-four phenolic compounds were identified for the first time in *N. alba* rhizomes. Hydrolysable tannins represent the majority with identification of galloyl hexoside derivative, hexahydroxydiphenic (HHDP) derivatives, glycosylated phenolic acids and glycosylated flavonoids. Five phenolics have been isolated and identified as gallic acid and its methyl and ethyl ester in addition to ellagic acid and pentagalloyl glucose. Minimum inhibitory concentrations (MIC) and anti-biofilm activity for the extract and the major isolated compounds were determined. Radical scavenging activity using 2.2Di (4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH) assay as well as cytotoxic activity using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay have also been evaluated. MIC of *N. alba* rhizomes against *Staphylococcus aureus* was 0.25 mg/mL compared with 0.1 mg/mL for methyl gallate. The best reduction in biofilm formation (84.9%) as well as the best radical scavenging (IC_{50} 3 µg/mL) and cytotoxic (IC_{50} 9.61 ± 0.3 µg/mL) activities were observed with methyl gallate. This is the first study for in-depth characterization of phenolic compounds in *N. alba* rhizomes revealing it as a valuable source of phenolic compounds and promising anti-biofilm forming agent of natural origin.

Key words: Hydrolysable tannins, Nymphaeaceae, LC-HRMS.

INTRODUCTION

Bacterial illnesses are caused by many virulence factors. Biofilm forming capacity is an additional virulence factor

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that assists the persistence of pathogens in harsh environmental conditions (Upadhyay et al., 2014). Cells in biofilms grow as communities, surrounded by a self-produced thick layer of extracellular polymeric substances (EPS, also known as matrix or slime) (Sauer and Camper, 2001). The extracellular matrix of biofilm-embedded microorganisms is capable of sequestering and concentrating environmental nutrients such as carbon, nitrogen and phosphate. In addition, they can evade multiple clearance mechanisms produced by host and synthetic sources such as antimicrobial and anti-fouling agents, shear stress, host phagocytic elimination and host radical and protease defenses (Archer et al., 2011).

Anti-biofilms have attracted the attention of scientists for the last forty years to combat biofilms that are involved in a wide range of infections and antibiotic-resistant infections, in a trial to develop new and effective antimicrobial agent with high efficiency.

Gram-positive bacteria are the commonest cause of nosocomial infections with predominance of *Staphylococcus aureus* (Valentino et al., 2014). *Staphylococcus* is the most common infectious agent in skin, mucous commensal and indwelling medical devices (Otto, 2009). *S. aureus* biofilm-associated infections are difficult to treat with antibiotics and devices need to be replaced more frequently than those infected with *Staphylococcus epidermidis* (Jones et al., 2001).

Traditional medicine attracted the attention of traditional healers and scientists thousands of years ago. World Health Organization (WHO) estimated that about three-quarters of the world population living in developing countries relied upon traditional remedies (mainly herbs) for the health care of its people (Gilani and Rahman, 2005).

Tannins are polyphenolic compounds with wide range of biological activities. The mode of antimicrobial action of tannins is potentially due to the inactivation of microbial adhesins and cell envelope transport proteins (Saura-Calixto and Pérez-Jiménez, 2009).

Nymphaea alba L. (Nymphaeaceae), also known as the European White Waterlily, White Lotus or Nenuphar, is an aquatic flowering plant with perennial rhizomes or rootstocks anchored with mud (Wiersema, 1987). There are approximately 50 species in this genus. The flowers are white and they have many small stamens inside. Water-lilies have extensive rhizome systems from which leaf and flower stalks emerge each year. The root of the plant was used by monks and nuns for hundreds of years as an aphrodisiac, being crushed and mixed with wine. The dried roots and rhizomes of the white water lily have been used orally to treat gastrointestinal, genital, and bronchial conditions (Khan and Sultana, 2005). Interest on rhizomes of *N. alba* has increased. Bose et al. (2013) proved its possible sedative as well as powerful uterotonic effects (Bose, 2014). Moderate antioxidant activity, analgesic and anti-diarrheal activities have been also proved (Bose, 2012a, b). Although of promising results, to the best of our knowledge, no scientific reports have been found concerning chemical characterization of rhizomes as well as its antimicrobial activity. Therefore, the aim of this study was to get in-depth identification of phenolic constituents of *N. alba* rhizome extract using LC-MS and X calibur software, in addition to the isolation of its main constituents and evaluation of their antimicrobial as well as cytotoxic activities.

MATERIALS AND METHODS

General experimental procedures

For column chromatography, microcrystalline cellulose (E. Merck, Darmstadt, Germany) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden) were used. For paper chromatography; Whatman no. 1 sheets, Whatman no. 3 sheets (for Preparative Paper Chromatography, PPC) (Whatman Ltd, Maidstone, Kent, England) were used. The pure compounds were visualized under UV light (254 and 365 nm) with exposure to NH_3 vapor or spraying with $FeCl_3$ (1% in ethanol). Solvent systems S_1 (*n*- BuOH/HOAc/H_2O; 4:1:5 v/v/v top layer), and S_2 (15% aqueous HOAc) were used. The NMR spectra were recorded at 300 (^1H) and 75 (^{13}C) MHz on a Varian Mercury 300. The results were reported as δ ppm values relative to TMS in the convenient solvents. UV analysis for pure samples was recorded on MeOH solutions and with different diagnostic UV shift reagents on a Shimadzu UV spectrophotometer (1800 UV probe). LC-HRESI-MS-MS was performed on a Bruker micro-TOF-Q Daltonics (API) Time-of-Flight mass spectrometer (Bremen, Germany), coupled to 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with a high performance autosampler, binary pump, and PDA detector G 1314 C (SL). Chromatographic separation was performed on a Superspher 100 RP-18 (75 × 4 mm i.d.; 4 μm) column (Merck, Darmstadt, Germany).

Preparation of plant extract

Rhizomes of *N. alba* L. were collected from AL Orman garden, Giza, Egypt in November 2012 during the flowering stage. Authentication of the plant was performed by *Dr*. Therese Labib Youssef (consultant of plant taxonomy, Ministry of Agriculture). A voucher specimen (RS006) was deposited at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, October University for Modern Science and Arts (MSA), Egypt.

N. alba rhizome (300 g) was dried, reduced, and sieved to obtain the powdered rhizome, extracted with 70% ethanol under reflux. The aqueous ethanolic extract (AEE) was filtered, concentrated using a rotary evaporator and dried in vacuum at 40°C, to yield 40 g (13.3% yield).

Identification of phenolic compounds of aqueous ethanol extract of *N. alba* rhizomes by LC-HRMS

N. alba AEE was investigated according to Hassaan et al. (2014). The mobile phase consisted of (A) 2% acetic acid (pH 2.6) and (B) 80% methanol. Gradient elution at a flow rate of 100 μL/min was used from 5 to 50% B at 30°C. Pneumatically assisted electrospray ionization was used. Spectra were recorded in positive and negative ion mode between m/z 120 and 1,500 with 4000V capillary voltage. Heated dry nitrogen gas at temperature 200°C and flow rate 10 L/min was used. The gas flow to the nebulizer was set at pressure 1.6 bar. For collision-induced dissociation (CID) MS-MS

measurements, the voltage over the collision cell varied from 20 to 70 eV and Argon was used as collision gas. Data analysis software was used for data interpretation. Sodium formate was used for calibration at the end of LC-MS run. Interpretation for ESI-MS was performed by Xcalibur 2.1 software from Thermo Scientific (Berlin, Germany).

Total phenolic and flavonoid content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu's reagent. Concentration of phenolic content was expressed as gallic acid equivalent (GAE) (Sellappan and Akoh, 2002). Flavonoid content (FC) was estimated using aluminum chloride colorimetric assay. Concentration of flavonoid content was expressed as quercetin equivalent (QE) (Kosalec et al., 2004).

Extraction and isolation

AEE (25 g) was fractionated on a cellulose column (375 g, 110 × 7 cm) using a step-gradient from 10% MeOH in H_2O to 100% MeOH, to yield 80 fractions of 100 mL each, which were further collected into 5 major collective fractions (I-IV) monitored using paper chromatography and solvents S_1 and S_2 visualized using UV-light. Fraction I (2.55 g) was found to be polyphenolic-free ($FeCl_3$ spray reagent/PC). Fraction II (10% MeOH, 1.5 g) was applied on Sephadex LH-20 eluted with 50% methanol to afford **1** (15 mg each). Fraction III (70% MeOH, 400 mg) was chromatographed on PPC using S1 as solvent followed by further purification using Sephadex LH-20 affording **2** and **3**. Fraction IV (100% MeOH, 2.5 g) was chromatographed on a microcrystalline cellulose column using saturated butanol as a solvent then further purified on Sephadex LH-20 affording **4** and **5.** All separation processes were followed up by Comp-PC with S_1 and S_2 solvents.

Bacterial and mycotic strains and growth conditions

Standard strains of *Bacillus subtilis (*ATCC 6633), *Sarcina lutea* (ATCC 9341), *Proteus vulgaris* (ATCC 6899), *Salmonella typhimurium* (ATCC 13311), *Shigella sonnei* (ATCC 9290), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231). In addition to four multi-drug resistant clinical isolates of *S.aureus* from wound infection (identified by microscopic, macroscopic and biochemical tests) (Cheesbrough, 2005).

Determination of microbial sensitivity to *N. alba* rhizome extract by disk diffusion method

Bacteria and *Candida albicans* were grown in nutrient broth and Sabarouds' dextrose broth, respectively, overnight and adjusted to a concentration of 10^8 CFU/mL by comparing it with McFarland standard 0.5.

For the disk diffusion assay, 1 mL of each bacterial suspension was uniformly spread on a solid growth medium in a Petri-dish. Four sterile paper disks (6 mm in diameter; Becton, Dickinson & Co.) were placed on the surface of each agar plate and were impregnated with 10 µL of the diluted plant extract (250 mg/mL). Plates were incubated for 24 h under appropriate cultivation conditions. Antimicrobial activity was measured as a diameter of inhibition zone around a disk following the 24 h incubation. Susceptibility was estimated by measuring the inhibition zone according to Valgas et al. (2007). Inhibition zones of 16-21 mm indicates strong activity, 12-16 mm denotes good activity, while 10-11 mm and <10 mm indicate intermediate and no activity, respectively. Disks impregnated with sterile distilled water and ethanol served as negative controls and a disk with an antibiotic (ofloxacin or Amphotericin B, Sigma-Aldrich GmbH, Steinheim, Germany) served as a positive control. Replicas at each concentration were performed.

Determination of minimum inhibitory concentrations (MIC) of *N. alba* rhizome extract and main constituents by microbroth dilution method

MIC was determined according to Klancnik et al. (2010), MIC of each antimicrobial was performed in flat-bottomed 96-well microplates (Greiner Bio-one, Stuttgart, Germany). This test was carried against standard strains of *B. subtilis (*ATCC 6633), *S. aureus* (ATCC 6538), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 27853) and *C. albicans* (ATCC 10231). The antimicrobial activity of *N. alba* rhizome extract against standard *Staphylococcus aureus* was further confirmed by testing its activity against four clinical isolates of *S. aureus* (staph1-4). These clinical isolates were recovered from wound infection. To determine MIC of each, plant extract, and isolated compounds, dilution range was prepared one step higher than the final dilution range required from 0.15 to 2 mg/mL in Müeller Hinton broth.

The inoculum was prepared by adjusting the turbidity from an overnight microbial culture by comparing it to McFarland 0.5 and then diluting it to reach a final concentration of 10^6 CFU/mL. A volume of 75 µL of inoculum was added to equivalent volumes of the two-fold serially diluted plant extract or isolated compound in a microplate. Control wells were prepared with culture medium, bacterial suspension only, plant extracts only and ethanol in amounts corresponding to the highest quantity present. The plate was incubated for 24 h at 37°C and the MIC was recorded as the lowest concentration of antimicrobial which gave no visible growth. The average value of three replicates was taken.

Effect of *N. alba* rhizome extract and main constituents on bacterial biofilm

Strains, which showed sensitivity for antimicrobials of 1 mg/mL for extract were used in the next experiments as this might show promising activity (Rios and Recio, 2005). The effect of *N. alba* rhizome extract and its main constituents was evaluated on biofilm synthesis and the percentage reduction of biofilm was estimated. The concentration used was the MIC value for the plant extract and plant constituents against each isolate. This test was done by adding tested product, in desired concentration, after distribution of bacterial inoculum in microplate wells so that final concentration of bacteria was 5×10^5 cell/mL. The plates were incubated for 24 h at 37°C. The plates were then aspirated, washed, fixed and stained with crystal violet as described by Peeters et al. (2008). Readings of optical density at 545 nm, using microplate plate reader (Stat Fax®2100), in the presence of different concentrations of antimicrobials was compared to the positive control wells without antimicrobials (Yassien and Khardori, 2004).

$$\text{Percent reduction in biofilm formation} = \frac{A_c - A_t}{A_c} \times 100$$

Where Ac is OD_{545nm} for positive control wells and A_t is OD_{545nm} for biofilm in the presence of antimicrobials.

Radical scavenging activity

The activity of 1, 1-diphenylpicrylhydrazil (DPPH) was estimated

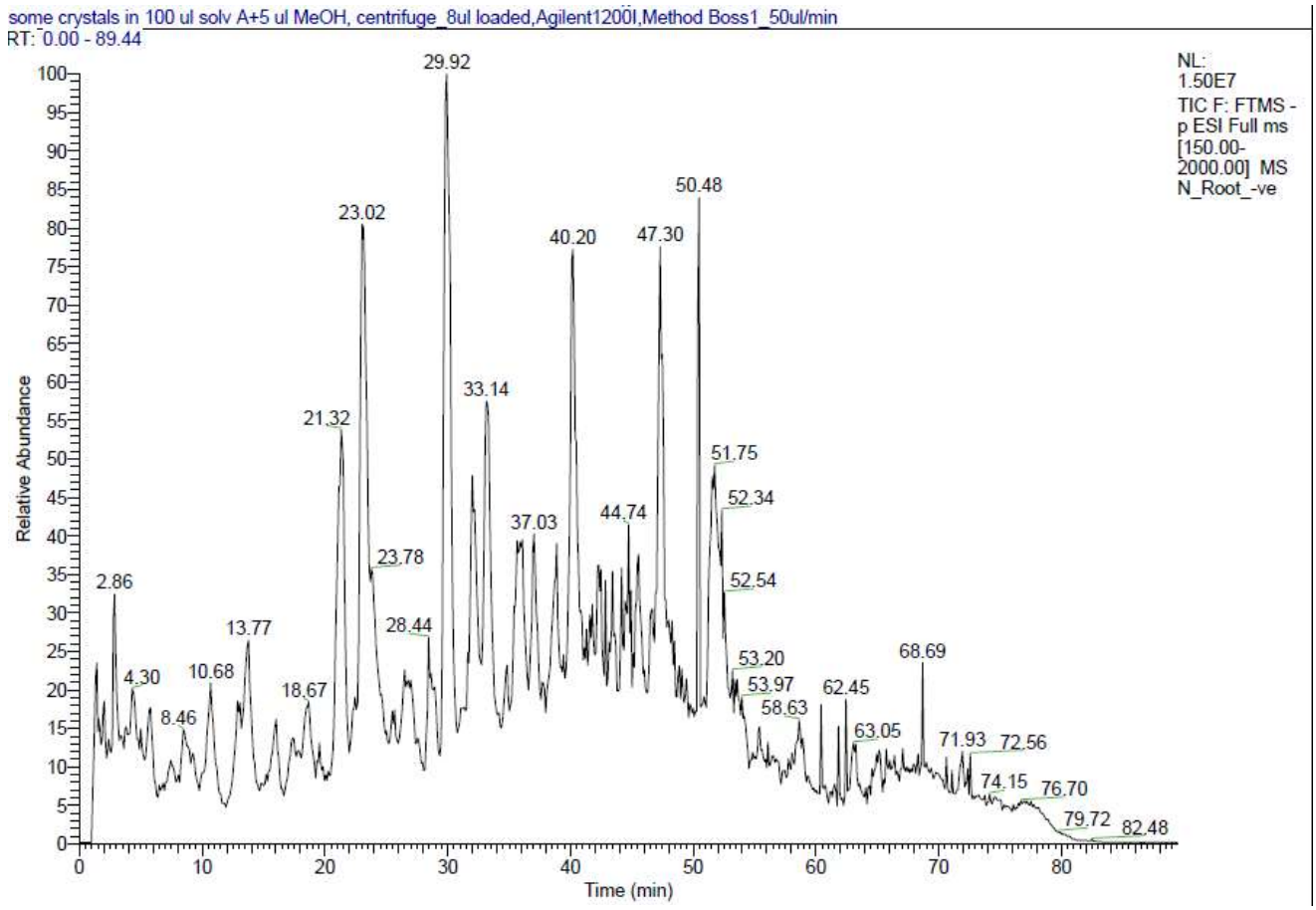

Figure 1. HPLC chromatogram of *N. alba* rhizomes.

according to the method described by Shimada et al. (1992). Radical scavenging activity was measured at 517 nm.

DPPH scavenging effect (%) = [(A0 - A1) / A0) × 100]

Where: A0 is the absorbance of the control reaction (DPPH) and A1 is the absorbance of the test extract. Ascorbic acid was used as standard (Oktay et al., 2003).

Cytotoxic activity

The viability of control and treated cells were evaluated at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University using the MTT assay in triplicate. Liver carcinoma cell line (HepG2) was used to test the cytotoxic activity according to Fotakis and Timbrell (2006). Doxorubicin was used as the positive control drug while the untreated cells whose absorbance was considered as 100% represented the negative control. Serial two-fold dilutions of the tested compound and reference compound were estimated. The results were determined by three independent experiments (Wilson, 2000). Percentage cell viability was calculated as follows:

% Cell viability = (Mean Abs control – Mean Abs test metabolite) × 100/Mean Abs control

Where: Abs is absorbance at 570 nm.

The graphic plots were used for estimation of the 50% inhibitory concentration (IC_{50}). STATA statistical analysis package was used for the dose response curve drawing in order to calculate IC_{50}.

RESULTS AND DISCUSSION

As part of ongoing effort to investigate plants from traditional medicine, *N. alba* represents an interesting field of study where the rhizomes have not been previously studied and preliminary testing of flavonoid and phenolic contents showed high concentration, reflecting probably promising biological activities.

Phytochemical investigation

The TPC of *N. alba* AEE was estimated as 32.96 ± 0.86 mg/g GAE (standard curve equation: y = 0.0011x+ 0.0009, r2 = 0.9867) while the FC was evaluated as 0.43 ± 0.59 mg/g QE (standard curve equation: y = 0.005x-0.0198, r^2 = 0.9774). The considerable high phenolic and moderate flavonoid contents have a great impact on biological activities.

LC-HRMS

HPLC-MS-MS provides a powerful tool for phytochemical analysis in crude plant extracts. It provides useful structural information and allows for tentative compound identification when standard reference compounds are unavailable (Seeram et al., 2006). HPLC-MS-MS analysis of AEE of *N. alba* rhizomes revealed the identification of sixty-four phenolic compounds reported for the first time (Figure 1 and Table 1).

Identified compounds include, caffeic acid hexoside, syringic acid hexoside, p-coumaroyl quinic acid and protocatechuic acid. Hydrolysable tannins including, gallotannins and ellagitannins, in addition to epicatechin, flavone and flavonol aglycone and glycosides have also been identified (Table 1).

The main fragmentation pattern from gallotannins involved the loss of one or more galloyl groups (152 amu) and/or gallic acid (170 amu) from the deprotonated molecule $[M-H]^-$. However, the fragmentation pattern of ellagitannins was less clear than that of gallotannins as ellagitannins display enormous structural variability because of different linkages of HHDP residues with the glucose molecule and their strong tendency to form C-C and C-O-C linkages (Khanbabaee and Vanree, 2001).

Gallic acid (Pk **33**) was tentatively identified with [M-H] at m/z 169.01 and a characteristic daughter ion at m/z 125.06. While ellagic acid was identified with a precursor ion peak at [M-H] at m/z 301 and characteristic fragments at m/z 257, 229 and 185.

Digalloyl hexoside (pk **4**, [M-H] at m/z 483.08), trigalloyl (pk **21**, [M-H] at m/z 635.09), tetragalloyl (pk **42**, [M-H] at m/z 787.1), pentagalloyl (pk **17**, [M-H] at m/z at 939.02), hexagalloyl (Pk **59**, M-H at m/z 1091.12), as well as heptagalloyl (pk **43**, [M-H] at 1243.1) hexose were tentatively identified by sequential losses of galloyl moieties and appearance of daughter ion peaks at m/z 169 and 125 in addition to comparison with literature.

Pk **1** was tentatively identified as HHDP hexoside with a precursor ion at [M-H] at m/z 481.06 and daughter ions at m/z 301.12 and 275.16. Pk **8** was tentatively identified as galloyl HHDP-hexose with [M-H] at 633.07 and daughter ion at m/z 463.19 [M-H-170] and 301.13 [M-H-170-162]. Isomer with same molecular weight appeared at Pk **13** with different fragmentation pattern and daughter ion at m/z 451.12 and identified as isostrictinin (Galloyl HHDP hexose). The presence of a compound with the same molecular weight at different retention times illustrated one of its isomeric forms. Different isomeric forms of hydrolysable tannins were observed and have been reported previously in eucalyptus (Barry et al., 2001).

Pk **7** was tentatively identified as digalloyl HHDP-hexose (pedunculagin II) with [M-H] at m/z 785.11 and daughter ion at m/z 633.29 [M-H-152], 481.19 [M-H-152-152], 301.13 [HHDP]. Isomer appeared at Pk **38** with [M-H] at m/z 785.08 and daughter ion at m/z 483.28 [M-H-HHDP], 633.28 [M-H-152], 615.05 [M-H-152-H_2O] and base peak at m/z 301.11 was tentatively identified as tellimagrandin I isomer. Pk **51** was tentatively identified as trigalloyl HHDP hexose (tellimagrandin II) with a precursor ion at m/z 937.09 and daughter ion at 633.22 [M-H-digalloyl], 785.18 [M-H-152] and 301.15. Pk **46** with a precursor ion peak at 935.08 [M-H] was tentatively identified as casuarinin or galloyl–bis-HHDP-hexose, with daughter ions at m/z 633.23 [M-H-HHDP] and 301.14.

Flavonoids have been also tentatively identified as myricetin hexoside (Pk **6**) with [M-H] at m/z 479.05 and characteristic peak at 317.17; its pentoside (Pk **10**) with a precursor ion at m/z 449.04, while the aglycone was observed at Pk **52** with typical fragmentation pattern (Table 1). Quercetin pentoside (Pk **47**) was tentatively identified with [M-H] at m/z 433.04 while Pk **50** with [M-H] at m/z 463.05 and daughter ions at 343.16 [M-H-120], 373.17 [M-H-90] and characteristic base peak 301.15 was tentatively identified as quercetin hexoside. Kaempferol (Pk **53**) was identified with its hexoside derivative (Pk **58**). Pk **52** was tentatively identified as kaempferol glucuronide hexoside with m/z 623.13 [M-H], base peak 285.17 and daughter ion at 447.29 [M-H-176]. Apigenin (Pk **56**) and its glycosylated derivative have been also identified (Pk **57**, Pk **64**).

Isolated compounds

The 2D-PC screening of the AEE of *N. alba* rhizomes revealed the presence of phenolic compounds (color properties under UV-light and responses to NH_3). The isolated compounds were identified on the basis of their spectral data (UV, 1D NMR), co-chromatography, in addition to comparison with published references of phenolics or in family Nymphaeaceae, genus Nymphaea and *N. alba* flowers (Nonaka et al., 1987; Jambor and Skrzypczak, 1991; Nawwar et al., 1994; Li et al., 1999; Elegami et al., 2003). This study is the first report for isolation of phenolics from *N. alba* rhizomes.

Five main phenolics have been isolated and identified including, gallic acid and its methyl and ethyl ester, ellagic acid and pentagalloyl glucose. Methyl and ethyl gallate as well as pentagalloyl glucose, were tested for the differences in their response for antibiofilm, radical scavenging and cytotoxic activities.

1, 2, 3, 4, 6 Penta-O- galloyl β-D-glucose (1), failed to crystallize and was obtained as off-white amorphous powder. It gave violet blue color using short UV light and intense blue color with $FeCl_3$. UV λ_{max} (nm): 272 nm.^{1}H-NMR (300 MHz, DMSO-d_6): δ ppm 6.89, 6.84, 6.83, 6.82, 6.78 (each 2H, *s*, galloyl H-2 & H-6), 6. 32 (1H, *d*, J=8 Hz, glc, anomeric H-1), ^{13}C (75MHz, DMSO-d6): δ-ppm 165.29, 165.2, 164 (-COO-), 146.18, 145.73 (galloyl C-3, C-5), 140.1, 140.15, 140.2,(galloyl C4), 120.5, 120, 119.2, 119.1 (galloyl, C-1), 109.19 (galloyl C-2 & C-6), 92.35 (anomeric glc. C-1), 63.24 (glc. C-6), 76.66 (glc. C-

Table 1. Tentatively assigned structures based on HPLC/ESI-MS.

Peak no.	tR (min)	Identified compound	Negative ionization MS/MS	Positive ionization MS/MS	References
1	1.92	HHDP hexoside	481.06 **301.12** 437.26 275.16	483.08 **465.16** 311.14 309.09 437.09 337.06 291.04	Mena et al., 2012
2	2.44	Galloyl quinic acid	343.07 **169.07** 299.2 191.15 173.1 125.14 328.21	345.08 **152.97** 327.17 171.09 124.99	Wyrepkowski et al., 2014
3	3.13	Ellagic acid	**301.00**, 257, 229, 185		Wyrepkowski et al., 2014
4	3.29	Digalloyl hexoside	483.08 **451**.16 313.16 331.22 301.14 271.18 169.12	485.04 **453**.17	Wyrepkowski et al., 2014
5	3.61	Digalloyl quinic acid	495.08 **343.21** 325.27 247.13 193.11 169.15		Wyrepkowski et al., 2014
6	3.88	Myricetin hexoside	479.05 **461.25** 313.19 317.17 301.14 435.18 277.1 169.09	481.10 **319.13** 463.20 421.03 309.13	Regueiro et al., 2014
7	4.37	digalloyl HHDP hexose Pedunculagin II	785.11 **633.29** 481.19 451.18 301.13 275.19		Dincheva et al., 2013; Fischer et al., 2011
8	4.66	Galloyl HHDP-hexoside	633.07 **301.13** 463.19 275.15	635.09 **617.24** 465.14 446.81 429.28 315.11 303.11 277.20	Mena et al, 2012
9	4.85	Protocatechuiic hexoside	315.07 **300.14** 153.03 270.1	317.12 **185** 155.04 203.12 299.14	Abu-Reidah et al., 2013
10	5.35	Myricetin pentoside	449.04 **317.19** 379.17 301.19 285.20 405.20 433.19 271.17 169.12		Fracassetti et al., 2013
11	10.37	Lagerstannin B derv	967.16 **483.16**	969.18 **951.23** 799.36 483.21 315.19 303.19	Fischer et al., 2011
12	10.67	Brevifolin	247.13 **203.09** 219.15 191.15 175.16	249.04 **207.05** 193.04	Wu et al., 2012
13	11.18	Galloyl HHDP hexose (Isostrictinin)	633.07 **451.12** 301.13	635.09 **617.25** 465.23 321.13 303.16 277.13	Wu et al., 2012
14	11.45	Vescalin or castalin/ tergallagic C-glucoside	631.06 **301.15** 613.17 463.21 451.15 275.17 631.06 613.17 461.13 445.22 301.15	633.07 **615.20** 471.27 427.15 309.12.	Piwowarski and Kiss, 2012
15	12.92	Valoneic acid dilactone	469 **425.15** 393.95 301.18 169.14		Wyrepkowski et al., 2014
16	13.04	Digallic acid	321.03 **169.08** 277.15 233.14 189.03 147.03 125.14		Tian et al., 2009
17	13.22	Pentagalloyl hexose	939.02 **769.23** 787.28 635.29 617.29 469.10 425.17 301.18	941.03 **453.09** 471.01 407.11 922.4	Wyrepkowski et al., 2014
18	13.27	Bis-HHDP-hexose (casuariin)	783.07 **631.22** 451.15 299.15 271.18	785.08 **449.15** 767.28 633.17 615.18 337.07 319.14 303.18 279.12 261.11	Mena et al., 2012; Fischer et al., 2011
19	14.47	Methyl gallate	183.03 **168.01**, 124.07		
20	14.67	Dimers of tergallagic-O-hexoside	631.06 **451.18** 425.17		Mena et al, 2012
21	15.05	Trigalloyl hexose	635.09 **483.23** 465.23 301.15 313.18 275.16	637.10 **466.21** 454.26 303.06	Wyrepkowski et al., 2014
22	15.18	Syringic acid hexoside	359.1 **239.14** 197.2 299.25 269.18 169.12		Abu-Reidah et al., 2013

Table 1. Contd.

23	16.21	Lagerstannin C	649.07 **435.2** 605.23 301.17 497.21	651.08 **633.21** 337.14 481.23 463.2 445.1 355.1 253.07	Fischer et al., 2011; Mena et al., 2012
24	16.53	Caffeic acid hexoside	341.09 **179.08**		Abu-Reidah et al., 2013
25	16.61	Galloyl ellagic acid	453.04 **431.31** 301.07 291.08 247.11 169.04		
26	16.93	Phyllanthusin U	924.11 **301.11** 622.25 604.22 290.21 906.25 879.27 275.16	926.12 **908.27** 606.16 588.16 571.19 774.2 454.28 436.11 303 277.12	Wu et al., 2012
27	17.50	P-coumaroyl quinic acid	337.09 **163.10** 191.15 119.21	339.11 **147.02** 320.09	Abu-Reidah et al., 2013
28	17.54	Isorh derv	445.04 **401.20** 315.25 343.2 311.14 427.22 415.34 287.26 169.12		
29	18.44	Punicalin A	781.11 **691.2** 763.31 479.19 783.34 425.26 301.25	783.07 **723.03** 765.2 553.05 277.13 303.11 463.18 445.22 613.16 631.29	Mena et al., 2012 Fischer et al., 2011
30	21.29	Galloyl shikimic acid	325.06 **173.12** 169.03 125.06		Wyrepkowski et al., 2014
31	21.46	Quinic acid	191.06 **127.07** 173.2 85.03 171.12 111.11 93.04		Simirgiotis 2013
32	21.75	Vesgalagin or castalagin	933.07 **613.21** 301.19 273.15 913.22 631.2 569.3 463.18	935.08 **783.19** 453.05 303.04 337.06	Piwowarski and Kiss, 2012
33	21.85	Gallic acid	169.01 **125.06**	171.03 **126.99** 152.99 109.03	Wyrepkowski et al., 2014
34	21.95	Phyllanthusiin B	969.09 **925.22** 949.14 881.26 633.20 589.24 419.31 301.16	971.09 **303.05** 651 335.07 951.19 479.18 315.10 277.16	Wu et al., 2012
35	23.5	Dehydrated tergallagic *C*-hexoside	613.01 **407.25** 595.18 569.26 523.22 491.32 371.16 301.2 291.22 613.01 559.14 495.24 463.24 301.21	615.06 **453.10** 291.14	Cantos et al., 2003
36	24.15	Lagerstannin B (Flavogalloyl-HHDP-gluconic acid)	949.2 **495.2** 903	951.21 **453.68** 931.45 780.23 701.51 497.13 479.28 589.29 337.15	Fisher et al, 2011
37	24.59	Phyllanthusiin C	925.10 **605.22** 301.11 623.22 551.15 453.17 881.24	927.11 **756.19** 303.10 277.13	Wu et al, 2012
38	24.67	Tellimagrandin I isomer (digalloyl-HHDP-hexose)	785.08 **301.11** 483.28 633.28 741.3 615.05	787.10 **303.06** 769.20 617.22 321.06 725.06	Wyrepkowski et al., 2014
39	24.89	Granatin A/lagerstannin A	799.16 **301.18** 479.22 781.06 635.26	801.11 **303.08** 463.2 783.23 277.11 471.07 481.22 453.21 337.16 321.16	Sentandreu et al., 2013
40	25.27	Monogalloyl hexose	331.07 **169.17** 271.12 313.14 211.23 193.06 125.18	333.08 **171.01** 315.11 153.05 127.06	Wyrepkowski et a.l, 2014
41	25.64	Ethyl gallate	197.05 **169.06** 125.1	199.06 **126.97** 170.99 137.03 152.95	Wyrepkowski et al, 2014
42	27.14	Tetragalloyl hexose	787.1 **635.22** 617..25 301.13	789.11 **771.08** 619.08 449.21 303.08	Wyrepkowski et al., 2014

Table 1. Contd.

43	28.63	Heptagalloyl hexose	1243.1 **1091.23** 939.26 917.24		Berardini et al 2004
44	30.57	Pedunculagin I isomer	783.07 **481.18** 299.18 301.1 451.15 271.2 613.15 631.23		Mena et al., 2012; Fischer et al., 2011
45	34.14	Castalgin derv	965.09 **933.18** 631.22 301.21 or 965.09 613.28 933.15 631.23 301.15	969.10 **935.2** 647.15 303.15 277.1 795.39 477.10	
46	34.73	Casuarinin/ Galloyl bis-hhdp-glucose	935.08 **633.23** 613.24 917.19 301.14	937.09 **919.17** 862.22 783.19 633.3 303.13	Liberal et al., 2014
47	34.81	Quercetin pentoside	433.04 **405.18** 291.17 303.14 389.21 269.24 301.2 275.23		Regueiro et al., 2014
48	41.16	Granatin B	951.07 **933.16** 613.22 301.22	953.08 **934.18** 783.21 633.23 615.17 465.23	Mena et al., 2012
49	42.13	Catechin or Epicatechin	289 **245.11** 291.01, 291.35		Pérez-Magariño et al.. 1999
50	43.84	Quercetin hexoside	463.05 **301.15** 343.16 373.17 275.17 169.17	465.07 **303.07** 277.09	Kajdzanoska et al., 2010
51	44.15	HHDP-trigalloyl hexose Tellimagrandin II/pterocaryanin C isomer	937.09 **633.22** 785.18 301.15	939.11 **303.07** 770.18 647.26 455.15 321.13	Regueiro et al., 2014
52	44.41	Kaempferol glucuronide hexoside	623.13 **285.17** 605.17 447.29	625.14 **287.12** 449.14	Abu-Reidah et al., 2013
53	44.61	Kaempferol	285.19 **241.16** 217.25 199.15 175.15 151.06	287.12 **269.21** 251.16 209.15 191.15	Martucci et al., 2014
54	46.70	Isorhamnetin hexoside	477.07 **315.12** 300.17	479.08 **317.10** 419.96 361.03 461.16	Hossain et al., 2010
55	46.98	Isorhamnetin	315.01 **300.15** 270.09 299.13 169.15	317.03 **285.07** 302.04 270.37	Kim and Park, 2009
56	48.55	Apigenin	269.16 **225.22** 195.12		Abu Reidah et al., 2013;
57	48.65	Apigenin 8 C-hexoside	431.10 **311.19** 341.16 269.17		Matrucci et al., 2014
58	49.0	Kaempferol 7 hexoside	447.09 **285.16** 169.17	449.11 **287.14** 429.21	Kajdzanoska et al., 2010
59	49.70	Hexagalloyl hexose	1091.12 **939.25** 940.24 787.30		Berardini et al., 2004
60	50.95	Chebulagic acid	953.08 **935.18** 907.16 787.25 739.31 617.35 613.25 301.16	955.10	Huang et al., 2011; Wu et al., 2012
61	51.01	Dehydrogalloyl-HHDP-hexoside	615.06 **463.22** 301.18 257 229	617.08 **303.08** 277.07 455.13 429.01	Mena et al., 2012
62	51.21	Myricetin	317.20 **289.19** 300.12 302.20 243.18 245.18 209.14	319.04 **301.1** 291.09 273.11 245.06	Sójka et al., 2009
63	52.51	Pentagalloyl HHDP hexose	1241.12 **1087.34** 937.19 769.12 787 633.31 469.18		
64	54.03	Apigenin 7 hexoside	431.10 **269.21** 413.13 311.20 275.12		Abu-Reidah et al., 2013

Ions in bold indicate the most intense product ion (100% relative intensity). Ions are arranged according to their relative abundance.

5), 76.65 (glc. C-3), 72.1 (glc. C-2), 67 (glc. C-4), 63.24 (glc.C-6). Methyl gallate (2), cream colored needles, Rf values 0.72 (S1), 0.58(S2) on PC. It

Table 2. *N. alba* rhizome AEE against different standard microorganisms.

Organism tested	Zone of inhibition (mm)			
	***N. alba* AEE**	**Ofloxaciline**	**AMP B***	**AMP B***
Staphylococcus aureus (ATCC 6538)	21±00	32±00	-	-
Bacillus subtilis (ATCC 6633)	10±01	36±00	-	-
Sarcina lutea (*ATCC* 9341)	18±05	30±03	-	-
Escherichia coli (ATCC 8739)	12±00	29±00	-	-
Proteus vulgaris (ATCC 6899)	10±00	38±02	-	-
Salmonella typhimurium (ATCC 13311)	15±00	27±00	-	-
Shigella sonni (*ATCC* 9290)	12±05	31±00	-	-
Pseudomonas aeruginosa (ATCC 27853)	12±02	29±05	-	-
Candida albicans (ATCC 10231)	12±01	-	25±05	25±05

*AMP B: Amphotericin B; No results means test not done.

Table 3. Minimum inhibitory concentrations (MIC) of *N. alba* rhizome AEE against standard microbial strains.

Standard strain	MIC (mg/mL)
S. aureus (ATCC 6538)	0.25
B. subtilis (ATCC 6633)	>2
E. coli (ATCC 8739)	>2
P. aeruginosa (ATCC 27853)	>2
C. albicans (ATCC 10231)	>2

gave violet blue color using short UV light and intense blue color with $FeCl_3$. UV λ_{max} (nm): (MeOH) 218, 272.^{1}H-NMR (300 MHz, DMSO-d_6): δ 6.96 (2H, *s*, H-2 & H-6), δ 3.39 (3H, s, OOCH3).

Ethyl gallate (3), white amorphous powder, Rf values 0.9 (S1), 0.72 (S2) on PC. It gave violet blue color using short UV light and intense blue color with $FeCl_3$. UV λ_{max} (nm): (MeOH) 225, 274. ^{1}H-NMR (300 MHz, DMSO-d_6): δ ppm 6.93 (2H, *s*, H-2 & H-6), 4.20 (2H, *q*, J=7 Hz, CH_2), 1.26 (3H, *t*, J=7 Hz, CH_3). Gallic and ellagic acid have been tentatively identified by Co-chromatography.

Antimicrobial activity by disc diffusion test

The sensitivity of nine standard strains to extracts from *N. alba* rhizomes was tested using disc diffusion method. All standard bacterial strains used were sensitive to ofloxacin while *C. albicans* was sensitive to amphotericin B. Both *S. aureus* and *Sarcina lutea* showed the highest sensitivity to *N. aba* rhizome extract as shown in Table 2.

Minimum inhibitory concentrations of *N. alba* rhizome extract and isolated compounds

The MIC of rhizome extract was equivalent to 0.25 mg/mL for standard *S. aureus* and above 2 mg/mL with standard strains of *B. subtilis, E. coli, P. aeruginosa and C. albicans* (Table 3 to 5).

Standard *S. aureus* was chosen for next studies in addition to the four clinical strains. *N. alba* extract showed high activity against Standard *S. aureus* and clinical isolates except Staph (3) isolate. Methyl gallate showed the highest activity against all tested microorganisms compared to ethyl gallate and pentagalloyl glucose.

The effect of *N. alba* extract and pure compounds on biofilm formations was studied. The minimum concentration that causes inhibition of growth was used. Methyl gallate caused a significant reduction in biofilm formation (84.9%) followed by the rhizome extract (78.8%) ($P<0.01$). Both standard strain and the clinical isolate Staph (2) were the most affected by the extract and methyl gallate followed by isolate Staph (3). The degree of inhibition was not correlated with the MIC of extract or the pure component on different tested microorganisms. Pentagalloyl glucose showed the least effect on biofilm formation. Methyl gallate showed the best antimicrobial and anti-biofilm activity in agreement with previous reports (Kang et al., 2008). This activity was attributed to its structure; a lipophilic alkyl chain at one end is connected via an ester linkage to the galloyl group bearing the polar hydroxyl groups at the other end. This amphiphilic property makes the cell membrane of *S. aureus* one of the most likely target sites of the action of alkyl gallate (Shibata et al., 2005). The antibacterial effect of alkyl gallate is due to, both, membrane disruption and affecting cell division by anti-FtsZ activity (Król et al.,

Table 4. Minimum Inhibitory Concentrations (MIC) of *N. alba* rhizome AEE and its main constituents against *S. aureus* standard strain and clinical isolates.

Microorganism	MIC (mg/mL)			
	***N. alba* AEE**	**Methyl gallate**	**Ethyl gallate**	**Pentagalloyl glucose**
Staph. aureus (ATCC 6538)	0.25	0.1	>0.1	>0.1
Staph 1	0.5	0.1	>0. 1	>0. 1
Staph 2	0.25	0.1	>0.1	>0. 1
Staph 3	2	0.1	>0.1	>0. 1
Staph 4	0.5	0.1	>0. 1	>0. 1

Table 5. Percentage reduction in biofilm formation of *Staphylococcus aureus* by *N. alba* rhizome AEE and its main constituents at MIC.

Microorganism	Percentage reduction in biofilm formation (%)			
	***N. alba* AEE**	**Methyl gallate**	**Ethyl gallate**	**Pentagalloyl glucose**
S. aureus (ATCC 6538)	78.84	84.9	62.8	19.28
Staph 1	24.40	51.67	44.49	48.32
Staph 2	77.41	85.6	73.49	10
Staph 3	70.00	80	9.3	43.35
Staph 4	4.80	66	12.72	8.78

2015). Their antibacterial mode of action was also suggested to be as surface-active agents affecting membrane integrity and hence affect biofilm formation (Takai et al., 2011).

1, 2, 3, 4, 6-penta-*O*-galloyl-β-D-glucose (β-PGG) is a prototypical gallotannin and the central compound in the biosynthetic pathway of hydrolysable tannin. β-PGG has five ester bonds formed between carboxylic groups of gallic acids and aliphatic hydroxyl groups of the glucose core. It is present in a number of medicinal herbals such as *Rhus chinensis* Mill and *Paeonia suffruticosa* and showing several biological activities (Zhang et al., 2009). In our study, PGG showed lower activity compared with methyl and ethyl gallate.

Radical scavenging activity

Methyl gallate showed the best radical scavenging activity with IC_{50} 3±0.36 µg/mL followed by ethyl gallate 4.7±0.23 µg/mL while IC_{50} of pentagalloyl glucose was 12±0.54 µg/mL compared with vitamin C 12±3.5 µg/mL. Antioxidant activity of methyl and ethyl gallate was proved by Wang et al. (2014) and Kalaivani et al. (2011), respectively. While moderate activity of ethyl gallate was previously reported by Kalaivani et al. (2011)

Other report showed that PGG showed an EC_{50} of scavenging 1, 1-diphenyl-2-picrylhydrazil (DPPH) free radical at about 1 µg/mL (1.1 µM) in test tubes, which was more potent than vitamin E (Abdelwahed et al., 2007).

Cytotoxic activity

In term of antiproliferative/cytotoxic IC_{50} values, Methyl gallate showed the highest activity against HepG2- cell line with IC_{50}= 9.61±0.3 µg/ml, while ethyl gallate and pentagalloyl glucose values were 41.9±0.23 and 41.2±0.41 µg/ml, respectively compared with Doxorubicin 0.56 µg/ml, standard cytotoxic.

PGG cytotoxic activity against hepatocellular carcinoma was comparable to ethyl gallate with IC_{50} 41.2 and 41.9 µg/ml, respectively. This was in agreement with Oh et al. (2001) who isolated PGG from the root of *Paeonia suffruticosa* and tested its *in vitro* effect on human hepatocellular carcinoma SK-HEP-1 cells. Up to 50 µM PGG inhibited the growth of SK-HEP-1 cells in a dose-dependent fashion and 30 µM PGG significantly induced G1 arrest.

Conclusion

This study is the first report for the identification and characterization of phenolic constituents in *N. alba* rhizomes and evaluation of its anti-biofilm and cytotoxic activities. *N. alba* rhizomes revealed a promising anti-biofilm activity with suggested contribution in antibacterial therapy.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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