HPLC-DAD finger printing, antioxidant, cholinesterase, and α-glucosidase inhibitory potentials of a novel plant Olax nana

Muhammad Ovais1,2, Muhammad Ayaz3*, Ali Talha Khalil4, Sayed Afzal Shah5, Muhammad Saeed Jan3, Abida Raza6, Muhammad Shahid7 and Zabta Khan Shinwari1,8

Abstract

Background: The medicinal importance of a novel plant Olax nana Wall. ex Benth. (family: Olacaceae) was revealed for the first time via HPLC-DAD finger printing, qualitative phytochemical analysis, antioxidant, cholinesterase, and α-glucosidase inhibitory assays.

Methods: The crude methanolic extract of O. nana (ON-Cr) was subjected to qualitative phytochemical analysis and HPLC-DAD finger printing. The antioxidant potential of ON-Cr was assessed via 1,1-diphenyl,2-picrylhydrazyl (DPPH), 2,2-azinobis[3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) and hydrogen peroxide (H2O2) free radical scavenging assays. Furthermore, acetylcholinesterase (AChE) & butyrylcholinesterase (BChE) inhibitory activities were performed using Ellman’s assay, while α-glucosidase inhibitory assay was carried out using a standard protocol.

Results: The qualitative phytochemical analysis of ON-Cr revealed the presence of secondary metabolites like alkaloids, flavonoids, tannins, sterols, saponins and terpenoids. The HPLC-DAD finger printing revealed the presence of 40 potential compounds in ON-Cr. Considerable anti-radical activities was revealed by ON-Cr in the DPPH, ABTS and H2O2 free radical scavenging assays with IC50 values of 71.46, 72.55 and 92.33 μg/mL, respectively. Furthermore, ON-Cr showed potent AChE and BChE inhibitory potentials as indicated by their IC50 values of 33.2 and 55.36 μg/mL, respectively. In the α-glucosidase inhibition assay, ON-Cr exhibit ed moderate inhibitory propensity with an IC50 value of 639.89 μg/mL.

Conclusions: This study investigated Olax nana for the first time for detailed qualitative phytochemical tests, HPLC-DAD finger printing analysis, antioxidant, anticholinesterase and α-glucosidase inhibition assays. The antioxidant and cholinesterase inhibitory results were considerable and can provide scientific basis for further studies on the neuroprotective and anti-Alzheimer’s potentials of this plant. ON-Cr may further be subjected to fractionation and polarity guided fractionation to narrow down the search for isolation of bioactive compounds.

Keywords: Olax nana, Phytochemical analysis, HPLC-dad, Acetylcholinesterase (AChE), Butyrylcholinesterase (BChE), DPPH, H2O2, ABTS, α-glucosidase inhibitory assays

* Correspondence: ayazuop@gmail.com
3Department of Pharmacy, University of Malakand, Khyber Pakhtunkhwa (KPK), Chakdara 18000, Pakistan

© The Author(s). 2018 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
**Background**

Humans have used medicinal plants as a remedy against various diseases since time immemorial [1]. The presence of various bioactive components in different parts of plants make them an important resource for the treatment of various diseases. Furthermore, the side effects associated with some of the synthetic medications and antibiotic resistance demands intermittent research for alternative solutions [2, 3]. In addition, to their direct therapeutic use, medicinal plants have been explored to fabricate various nanoscaled materials for potential biomedical applications [4–9]. Natural products based alternative therapies are still practiced in many countries and globally approximately 80% of population trust on herbal medicine as a primary source of therapeutic remedies [10]. In the last decade, a revival has been observed in the use of medicinal plants and herbal medicines. It is forecasted that the global market for herbal medicine will expand over time due to the preference of consumers for natural medicines [11]. Moreover, by the year 2017, the worldwide herbal supplements and remedies market is forecasted to reach $107 billion [12].

The genus *Olax* belongs to family *Olacaceae* [13], which contains several medicinally important plants [14]. Among the *Olacaceae* species, *Olax subschorpioidea*, is reported to have applications in the management of analgesic, cancer, yellow fever, inflammatory diseases, mental illness, Alzheimer’s disease, parasitic, microbial infections and hepatological disorders [14–23]. *Olax scandens* revealed the presence of highly useful phytochemicals like sitosterol, octacosanol, oleic acid, oleuronic acid and β-sitosterol [24]. Several studies confirmed the pharmacological importance of *O. scandens* in bacterial infections, cancer, headache and psoriasis [25–27]. Moreover, the aqueous extract of *Olax zeylanica* was found effective against skin diseases and exhibits photoprotective activity, whereas, *Olax disstiflora* have mosquito repellent properties [28, 29]. The phytochemical analysis of *Olax mannii* Oliv revealed the presence of three new flavonoid triglycosides including kaempferol 3-O-[(β-D-glucopyranosyl-(1–2)-α-L-arabinofuranoside)-7-O-α-L-rhamnopyranoside], kaempferol 3-O-[(β-D-arabinopyranosyl-(1–4)-α-L-rhamnopyranoside)-7-O-α-L-rhamnopyranoside], kaempferol 3-O-[(α-D-apiofuranosyl-(1–2)-α-L-arabinofuranoside)-7-O-α-L-rhamnopyranoside], kaempferol 3-O-[(α-D-apiofuranosyl-(1–2)-α-L-arabinofuranoside)-7-O-α-L-rhamnopyranoside], kaempferol 3-O-α-L-rhamnopyranoside and fourteen already known flavonoids glycosides which are potential anti-cancer and anti-inflammatory agents [30].

Free radicals are implicated in the progression of several disorders like, cancer, ischemic heart diseases, neurodegenerative diseases, diabetics, reperfusion injuries, arthritis and atherosclerosis [31]. Free radicals from various sources like toxins, environmental pollutants and deep fried foods cause abnormal genes expression and proteins synthesis which initiate degenerative reactions in the body [32]. In the living systems, generation of free radicals occur via oxidation process, which are nullified to non-radical forms in the human body by natural antioxidants. Hence, apart from our own immune system which acts against these free radicals, natural antioxidants are of prime importance to counter them [33]. Synthetic antioxidants like tertiary butyl hydro quinone, propyl gallate, butylated hydroxytoluene and butylated hydroxy anisole are associated with adverse effects to human health. Hence, natural antioxidants form plants are found to be best alternative for synthetic antioxidants [33]. Many published studies validate the potential antioxidant potential of medicinal plants crude extracts and of isolated pure compounds [34, 35]. Flavonoids and phenolics are considered as potent antioxidants due to the presence of hydroxyl groups and conjugated ring structures, which via hydrogenation or complexation scavenge free radicals [36].

Alzheimer’s disease (AD) is a common neurodegenerative disorder characterized by behavioral turbulence, cognitive dysfunction and imperfection in the routine activities. Its prevalence is high among individuals of age above sixty years [37]. Currently only five drugs have been approved for clinical use, among which four are cholinesterase inhibitors [38]. The cholinesterase’s including acetyl cholinesterase (ACHE) and butyrylcholinesterase (BChE) catalytically metabolize acetylcholine (ACh) in the synaptic cleft [39]. Acetylcholine is an important neurotransmitter involved in the transmission of impulses across the synapse and is vital in the acquisition and storage of memory. The level of ACh has been found to be depleted in AD [40]. Among the options is the use of inhibitors of AChE and BChE which will restore the activity of ACh at the synapse. Among the clinically approved cholinesterase inhibitors, two are from natural products including galantamine and rivastigmine. Consequently, natural products are under consideration for the development of more useful cholinesterase inhibitors [41].

Diabetes is a major health problem which is associated with high blood glucose level in the body. The α-glucosidase enzyme plays a key role in the control of glucose level inside the body as they are related to postprandial glucose excursion in a person suffering from diabetes [42, 43]. α-Glucosidase breakdown carbohydrates into glucose and therefore increases the glucose level inside the body. Henceforth, α-glucosidase inhibition (AGI’s) is considered as a popular strategy for controlling post prandial glucose level. Many plants have been researched for natural AGI’s like *Punica granatum*, *Pine bark* and *Andrographis paniculata* [44].

To the best of our information there is no single report on the phytochemical investigation and pharmacognostic features (anticholinesterase, antioxidant and α-glucosidase inhibitory potential) of *O. nana*. Therefore, the present research was undertaken to investigate and scientifically
validate the use of *O. nana* aqueous methanolic extract against ailments such as diabetes and neurogenerative
diseases.

**Methods**

**Plant collection and extraction**

*O. nana* whole plant was collected from Swat district of Khyber Pakhtunkhwa, Pakistan in June 2016 (Fig. 1). The plant was authenticated by botanical taxonomist Syed Afzal Shah (PhD Candidate) and Dr. Mushtaq (plant taxonomist) at the Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. A sample was deposited at MOSEAL Laboratory, Department of Biotechnology, Quaid-i-Azam University with a voucher number MOSEAL-344. The plant was gently rinsed with running distilled water followed by shade drying for 10 days. Subsequently, the dried leaves were ground via cutter mill to obtain a fine powdered material that weigh ~1 kg. In a 1 L of 70% aqueous methanol, 300 g of the powdered plant was soaked for 5 days in a shaking incubator (37 °C), with frequent sonication (60 Hz for 5 mints every day) using a sonicator (Elmasonic E 60 H - Cousins UK). The extraction step was repeated thrice, with the addition of extract to the original one and filtered via muslin cloth, ultimately followed by final filtration through Whatman® qualitative filter paper, Grade 1. Furthermore, in a rotary evaporator (EYELA N-1300S-W 115 V, Tokyo Rikakikai Co., LTD), the crude methanolic extract (ON-Cr) was concentrated, ultimately resulting in a ~20 g mass of dark brownish semisolid [45].

**Chemical and drugs**

For antioxidant assays, 1,1-diphenyl, 2-picrylhydrazyl (DPPH) and 2, 2-azinobis [3-ethylbenzthiazoline]-6-sulfo-
nic acid (ABTS) (CAS 1898–66-4 and CAS 30931–67-0 Sigma Aldrich, USA,K2S2O4 (Riedel-de Haen Germany) were purchased from authorized dealers in Pakistan. For anticholinesterase and α-glucosidase studies, AChE from *Electric eel* (type-VI-S, CAS 9000–81-1), BChE from equine serum lyophilized (CAS 9001–08-5) and α-

**Phytochemical analysis**

**Preliminary phytochemical tests**

For the identification of various secondary metabolites including alkaloids, flavonoids, glycosides, saponins, ste-
rols, tannins, terpenoids and anthraquinones, qualitative phytochemical tests were performed following standard protocols as reported previously [46].

**Determination of alkaloids (Dragendorff’s method)**

The crude methanolic extract (0.2 g) was taken in a conical flask and sulphuric acid (2%) was added. The conical flask was placed on a hot water bath for 2 min and then cooled. The sample was then filtered and treated with Dragendorff’s reagent in a test tube. The orange red color precipitate in the test tube showed the presence of alkaloids.
**Test for flavonoids**
In a conical flask, dried powdered material was boiled with 10 mL of distilled water in a hot water bath for about 5 min and then filtered. After cooling the mixture, few drops of sodium hydroxide solution (20%) was added to 1 mL of the cooled filtrate, which changed the color to yellow. Further addition of 2% HCl changed the yellow color of mixture to colorless.

**Test for tannins**
To 2 g of sample, 20 mL of distilled water was added and boiled for about 5 min in a hot water bath. The boiled solution was filtered immediately and then cooled at room temperature. A 1 mL of filtrate was taken and added to 5 mL of distilled water. Then 10% of ferric chloride (2–3 drops) was added. The appearance of bluish-black precipitate or brownish-green color showed the presence of tannins.

**Test for anthraquinones**
A 2 g powdered plant sample was taken and macerated with ether. The solution was shaken and checked, the appearance of red, violet or pink color in the aqueous layer indicated the presence of anthraquinone.

**Test for Saponins**
The powdered plant material (0.2 g) along with 10 mL of distilled water was taken in a conical flask and boiled in a water bath for about 10 min. The hot mixture was then filtered and allowed to cool. A 1 mL of filtrate along with 2 mL of distilled water was taken in a test tube and vigorously shaken for 2 min. The formation of froth indicates the presence of saponins in the filtrate. Olive oil was then added drop wise to the froth and after shaking the mixture, a formation of emulsion was observed.

**Test for glycosides**
To a test tube containing a mixture of concentrated sulphuric acid (1 mL), aqueous plant extract (5 mL) and glacial acetic acid (2 mL), a drop of ferric chloride was added. The concentrated sulphuric acid remained beneath the mixture. The appearance of brown ring indicated the presence of cardiac glycoside.

**Test for Terpenoids (Salkowski’s test)**
For the identification of Terpenoids, powdered plant material was dissolved in 5 mL of aqueous solution and boiled in a water bath and then allowed to cool. The solution is filtered, and 2 mL of chloroform was added to the filtrate in a test tube; followed by addition of 3 mL of concentrated sulphuric acid. The appearance of reddish brown interface indicated the presence of terpenoid in the sample.

**High performance liquid chromatography-Diaode Array detection (HPLC-DAD) analysis**

**Samples preparation**
The extract sample for HPLC was prepared by mixing 20 mL of hydromethanolic mixture (1:1 v/v) with 1 g of ON-Cr. The resultant mixture was heated in a water bath at 70 °C for 1 h with subsequent cooling. Thereafter, the sample was centrifuged for 10 min at 4000 rpm. Finally, 2 mL of test sample was filtered through a Whatman filter paper into HPLC vials.

**HPLC–DAD procedure**
An Agilent 1260 infinity high-performance liquid chromatography (HPLC) system provided with ultraviolet array detector (UVAD), quaternary pump, auto-sampler and degasser was used. Separation was done using an Agilent Zorbax Eclipse XDB-C18 column. A gradient system consisting of solvent B (methanol: acetic acid; deionized water, 100: 20: 180, v/v) and solvent C (methanol: acetic acid: deionized water, 900: 20: 80, v/v) was used. The gradient program was started with 100% B at 0 min, 85% B at 5 min, 50% B at 20 min, 30% B at 25 min, and 100% C for 30–40 min [47]. Using this sequence, elution occurred after 25 min. For analysis of compounds, the ultraviolet array detector (UVAD) was set at 280 nm and the spectra were recorded from 190 to 500 nm. Two standard compounds, rutin and pyrogallol were detected in the HPLC analysis.

**Antioxidant assays**

1,1-diphenyl, 2-picrylhydrazyl (DPPH) free radical scavenging assay ON-Cr was tested for DPPH free radical scavenging assay as previously reported [35, 48]. Briefly, 0.004% DPPH reagent solution was prepared and added to increasing concentration of ON-Cr extract (125, 250, 500 and 1000 μg/mL) with subsequent incubation for 30 min. The absorption of reaction mixture was measured spectrophotometrically (UV–3000 O.R.I. Germany) at 517 nm. Ascorbic acid was used as positive control. The percent scavenging activity of samples was calculated using the following equation:

$$
\text{% scavenging activity} = \frac{\text{absorbance of control} - \text{absorbance of plant extract}}{\text{absorbance of control}} \times 100
$$

The experiment was performed in triplicate. The IC50 values were calculated using GraphPad Prism® (version 4.0, Sandiego, CA, USA).

2, 2-Azinobis [3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) free radical scavenging assay For further evaluation of Olax nana antioxidant potentials, ABTS free radical scavenging assay was performed [41, 49]. The principle of this assay is based on the test sample ability to scavenge...
ABTS free radical cations, ultimately leads to reduced absorbance at 734 nm. After mixing, K₂S₂O₈ (2.45 mM) and ABTS (7 mM), the solution was kept in dark for 12–16 h to form ABTS free radical cations. A 300 μL of ON-Cr test sample (1000 μg/mL-31.25 μg/mL) was gently mixed with ABTS solution (3.0 mL) in a cuvette. The absorbance of the mixture was measured after every 1 min, for a period of 6 min continuously on a UV-VIS spectrophotometer (UV-3000 O.R.I. Germany). As a positive control, ascorbic acid was used. The assay was performed in triplicate and calculation of percent inhibition was done using the following equation:

\[
\%\text{scavenging effect} = \left( \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \right) \times 100
\]

The percent inhibition and EC₅₀ value (extract concentration required for 50% reduction of ABTS radicals) were then calculated for expression of antioxidant potential.

**Hydrogen peroxide (H₂O₂) free radicals scavenging assay** As per our previously reported method, the H₂O₂ scavenging activity of ON-Cr was elucidated [50]. A solution of H₂O₂ (2 mM) was prepared in phosphate buffer (50 mM, pH 7.4). In a 0.3 mL of phosphate buffer solution (50 mM), 0.1 mL of test sample was added, which was followed by addition of 0.6 mL of H₂O₂ and then vortexed. The absorbance of solution was measured at 230 nm after a period of 10 min (UV-3000 O.R.I. Germany), in comparison to blank. The following equation was used for the calculation of H₂O₂ free radical scavenging activity:

\[
\text{Hydrogen peroxide scavenging activity} = \left( \frac{1 - \text{sample absorbance}}{\text{absorbance of control}} \right) \times 100
\]

**Anticholinesterase assays**

The Ellman’s assay was performed to assess the AChE and BChE enzymes inhibition potentials of ON-Cr [51]. The principle of anticholinesterase assay is based on hydrolysis of acetylthiocholine iodide and butyrylthiocholine iodide by respective enzymes, leading to the formation of 5-thio-2-nitrobenzoate anion, which complexes with DTNB to form a yellow color compound, after evaluation by UV-VIS spectrophotometer (UV-3000 O.R.I. Germany).

**Preparation of solutions** Phosphate buffer solution (0.1 M and pH: 8.0) was prepared as previously reported [48]. For adjusting the pH, potassium hydroxide was used. The final concentrations of 0.03 U/mL and 0.01 U/mL were obtained for AChE (518 U/mg solid) and BChE (7–16 U/mg) after respective dilution in the freshly prepared buffer pH 8.0. Using distilled water, DTNB (0.0002273 M), acetylthiocholine iodide and butyrylthiocholine iodide (0.0005 M) solutions were prepared and stored in a refrigerator (8 °C). The respective dilutions were also prepared for galantamine (positive control) in methanol.

**Spectroscopic analysis** In a cuvette containing 205 μL of ON-Cr extract, 5 μL of enzyme solution was added, followed by addition of 5 μL DTNB reagent. In a water bath, the resulting solution was incubated for 15 min at 30 °C, with addition of substrate solution (5 μL) subsequently. Furthermore, using a UV-VIS spectrophotometer (UV-3000 O.R.I. Germany) the absorbance was calculated at 412 nm. As a standard inhibitor of cholinesterase, 10 μg/mL of galantamine was used as positive control, while the components other than ON-Cr extract were considered as negative control. At 30 °C for 4 min of reaction time, the absorbance values at specific intervals were noted. The assays were conducted in triplicate, while the change in absorption rate with time gave calculations for the % enzyme activity and % enzyme inhibition by galantamine and ON-Cr sample.

**α-glucosidase inhibitory assay**

The protocol of McCue et al., was adopted for the assessment of ON-Cr α-glucosidase inhibitory potential [52]. An enzyme solution was prepared by mixing 120 μL of phosphate buffer (0.1 M, pH 6.9) with α-glucosidase enzyme (20 μL of 0.5 unit/mL). The solution of substrate having p-nitrophenyl-α-D-glucopyranoside was prepared in phosphate buffer (0.1 M, pH 6.9). The ON-Cr test sample was prepared in a concentration range of 31.25–1000 μg/mL, followed by mixing with enzyme solution and incubation for 15 min at 37 °C. Furthermore, to the mixture of enzyme and test sample, substrate solution (20 μL) was added and incubated under the same conditions. The reaction was completed by the addition of sodium carbonate solution (80 μL of 0.2 M). Using a UV-VIS spectrophotometer (UV-3000 O.R.I. Germany), the absorbance of samples was recorded at 405 nm. As a positive control, acarbose was used while the sample devoid of α-glucosidase was used as a blank. The experiment was performed in triplicate, while the following equation was used for the calculation of percent inhibition:

\[
\%\text{inhibition} = \left( \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \right) \times 100
\]

The concentration of ON-Cr extract which inhibited substrate hydrolysis via AChE and BChE enzymes by 50% (IC₅₀ value) was calculated from the dose response curve [53]. The kinetics of enzyme activity was measured in the presence of increasing concentrations of extract.
Statistical analysis
All the assays were performed three times, while values were represented as ± S.E.M. One-way ANOVA followed by Bonferroni’s multiple comparison post hoc test was used for the analysis of antioxidant activity and enzyme inhibition assays. At 95% confidence interval, a P value of <0.05 was considered as statistically significant. Lineweaver-Burk plots (1/v versus 1/[s]) where v is reaction velocity and [s] is substrate concentration were plotted from assays using a range of plant extract concentrations. The \( V_{\text{max}} \) and \( K_m \) values were determined using Michaelis-Menten kinetics.

Results

Phytochemical analysis
The preliminary phytochemical analysis of ON-Cr revealed the presence of various secondary metabolites like alkaloids, flavonoids, tannins, sterols, saponins and terpenoids. Table 1 highlights the observational results of all the phytochemical tests. In the HPLC-DAD analysis of ON-Cr, several components were separated in different ratios but gallic acid derivative (25.57%), hydroxybenzoic acid derivative (17.81) and rutin (5.35%) were compounds present in higher concentrations as summarized in Fig. 2 and Table 2. The highest concentrated signals were observed at retention times of 6.9, 5.8, 5.2, 6.3 min which were 25.57, 17.81, 14.41 and 8.19% respectively.

Antioxidant assays
In the DPPH free radical scavenging assay, ON-Cr at the concentrations of 31.25, 62.50, 500 and 1000 \( \mu \)g/mL demonstrated % inhibition not significantly different when compared to the positive control (ascorbic acid) \( (P > 0.05) \) at the same concentration. As shown in Fig. 3, the IC\(_{50}\) value of ON-Cr was 71.46 \( \mu \)g/mL, while that of ascorbic acid was 31.31 \( \mu \)g/mL. Interestingly, in ABTS free radicals scavenging assay, ON-Cr only at the concentration of 62.05 \( \mu \)g/mL demonstrated significantly different % inhibition in comparison to that of ascorbic acid \( (P < 0.001 \text{ at } 95\% \text{ confidence interval}) \). While at the concentrations of 31.25, 125, 250, 500 and 1000 \( \mu \)g/mL, the % inhibition values were not significantly different when compared to ascorbic acid at similar concentrations \( (P > 0.05 \text{ at } 95\% \text{ confidence interval}) \). As shown in Fig. 4, the IC\(_{50}\) value of ON-Cr was 72.55 \( \mu \)g/mL, while that of ascorbic acid (positive control) was 32.37 \( \mu \)g/mL. In the H\(_2\)O\(_2\) anti-radicals assay, ON-Cr at the concentrations of 31.25, 62.50 \( \mu \)g/mL demonstrated % inhibition significantly different in comparison to that of ascorbic acid \( (P < 0.001 \text{ at } 95\% \text{ confidence interval}) \). While at the concentrations of 125, 250, 500 and 1000 \( \mu \)g/mL, the % inhibition values were not significantly different when compared to ascorbic acid at similar concentrations \( (P > 0.05 \text{ at } 95\% \text{ confidence interval}) \).

Table 1 Different phytochemical tests for aqueous methanolic extract of Olax nana

| S. No | Phytochemical tests     | Observations                                      | Results |
|-------|-------------------------|---------------------------------------------------|---------|
| 1     | Alkaloids               | ppt.                                              | +       |
| 2     | Flavonoids              | Formation of yellow color which changed to colorless on acid addition | +       |
| 3     | Glycosides              | Reddish ppt. is not formed                        | -       |
| 4     | Tannins                 | Formation of bluish-black color                   | +       |
| 5     | Sterols                 | Green to pink color was absent                    | +       |
| 6     | Saponins                | Frothing bubbles are formed                       | +       |
| 7     | Anthraquinones          | Red, violet or pink color are not formed in aqueous layer | -       |
| 8     | Terpenoids              | Appearance of reddish brown color                 | +       |

Fig. 2 HPLC-DAD chromatogram of methanolic extract of Olax nana
interval). As shown in Fig. 5, the IC$_{50}$ value of ON-Cr was 92.33 μg/mL, while that of ascorbic acid was 22.01 μg/mL.

**Anticholinesterase assays**

In the acetylcholinesterase inhibitory activity, the IC$_{50}$ value for ON-Cr was 33.2 μg/mL, while that of

| Peak | RT (min) | Peak height (mAU) | Peak area % | Proposed identity of compound* | HPLC-DAD λmax (nm) | References |
|------|----------|------------------|-------------|--------------------------------|---------------------|------------|
| 1    | 2.5      | 13.29053         | 0.320       | Ascorbic Acid                  | 244                 | Mladu et al., 2012 |
| 2    | 5.2      | 1168.32886       | 14.41       | Gallic acid derivative         | 273, 279, 288       | Aaby et al., 2007 |
| 3    | 5.3      | 504.3362         | 3.19        | Gallic acid derivative         | 280                 | Mladu et al., 2012 |
| 4    | 5.8      | 456.6558         | 17.81       | Hydroxybenzoic acid derivative | 280                 | Santos et al., 2014 |
| 5    | 6.3      | 151.39925        | 8.20        | Hydroxybenzoic acid derivative | 274                 | Santos et al., 2014 |
| 6    | 6.9      | 163.31013        | 25.57       | Gallic acid derivative         | 271, 278, 287       | Aaby et al., 2007 |
| 7    | 11.1     | 8.41879          | 0.33        | Kaempferol-7-O-glucoside       | 254                 | Ibrahim et al., 2015 |
| 8    | 11.6     | 26.59156         | 0.70        | p-Coumaric acid derivative     | 313                 | Santos et al., 2014 |
| 9    | 13.2     | 35.75774         | 1.36        | Isovitexin-4-O-glucoside       | 254                 | Ibrahim et al., 2015 |
| 10   | 14.3     | 18.4162          | 0.57        | Caffeic acid                  | 242; sh 298; 328    | Carazzone et al., 2013 |
| 11   | 14.9     | 6.63179          | 0.17        | Gallic acid derivative         | 280                 | Santos et al., 2014 |
| 12   | 15.5     | 19.11495         | 0.46        | Hydroxybenzoic acid derivative | 278                 | Santos et al., 2014 |
| 13   | 15.7     | 19.45154         | 0.43        | Hydroxybenzoic acid derivative | 278                 | Santos et al., 2014 |
| 14   | 16.9     | 92.68282         | 2.47        | p-Hydroxybenzoic acid         | 256                 | Santos et al., 2014 |
| 15   | 17.0     | 15.39486         | 0.23        | Caffeoylmalic acid             | 327, 300, 268       | Santos et al., 2014 |
| 16   | 17.5     | 26.84283         | 0.60        | bis-HHDP-glucose              | 232                 | Aaby et al., 2007 |
| 17   | 18.1     | 14.13607         | 0.21        | Quercetin-3-O-triglucoside     | 256; 340            | Lin et al., 2011 |
| 18   | 18.8     | 77.88322         | 1.70        | Galloyl-HHDP-glucose           | 232                 | Aaby et al., 2007 |
| 19   | 18.9     | 102.11224        | 2.93        | Apigenin-7-O-rutinoside        | 254                 | Ibrahim et al., 2015 |
| 20   | 19.9     | 19.88177         | 0.46        | P-coumaric acid derivative     | 228; 316            | Santos et al., 2014 |
| 21   | 20.7     | 57.97879         | 2.78        | Vanillic acid                 | 260; 292            | Santos et al., 2014 |
| 22   | 21.4     | 10.30173         | 0.39        | Caffeic acid                  | 238; 298 sh; 323    | Santos et al., 2014 |
| 23   | 22.3     | 89.48333         | 5.35        | Rutin                         | 155.3661            | Reference Standard |
| 24   | 23.2     | 4.74129          | 0.10        | Syringic acid                 | 274                 | Santos et al., 2014 |
| 25   | 23.5     | 2.65536          | 0.04        | Proanthocyanidin trimer       | 284                 | Aaby et al., 2007 |
| 26   | 24.1     | 54.95284         | 1.32        | Quercetin-di-glucoside        | 256; sh 268; 350    | Llorach et al., 2008 |
| 27   | 24.7     | 38.34863         | 0.87        | Quercetin glycoside           | 256; sh 266; 354    | Llorach et al., 2008 |
| 28   | 25.4     | 1.90931          | 0.04        | Quercetin glycoside           | 256; sh 266; 354    | Llorach et al., 2008 |
| 29   | 25.9     | 11.029           | 0.26        | Caffeoylmalic acid            | 244; sh 298; 328    | Llorach et al., 2008 |
| 30   | 26.6     | 17.19059         | 0.49        | P-Coumaric acid               | 228; 310            | Santos et al., 2014 |
| 31   | 27.953   | 5.04913          | 0.09        | Pyrogallol                    | 244; sh 298; 328    | Reference Standard |
| 32   | 27.7     | 89.48333         | 2.58        | Cichoric acid                 | 244; sh 298; 328    | Carazzone et al., 2013 |
| 33   | 29.2     | 8.83803          | 0.22        | Caffeic acid derivative       | 242; sh 298; 322    | Santos et al., 2014 |
| 34   | 31.3     | 120.93526        | 2.08        | Quercetin-3-(caffeoylglucoside)-7-glucoside | 252; sh 268; 332 | Santos et al., 2014 |
| 35   | 32.7     | 14.63641         | 0.26        | Quercetin-3-galactoside       | 256; 268 sh; 356    | Santos et al., 2014 |
| 36   | 33       | 14.05107         | 0.24        | Quercetin-3-O-glucoside       | 256; 268 sh; 356    | Santos et al., 2014 |
| 37   | 34       | 3.52791          | 0.06        | Rosmarinic acid               | 330; 290 sh        | Santos et al., 2014 |
| 38   | 36.3     | 2.60122          | 0.08        | Quercetin-3-feruloylsophoroside | 256; sh 268; 334 | Lin et al., 2011 |
| 39   | 39.6     | 2.02323          | 0.06        | Rosmarinic acid derivative    | 290; 328           | Santos et al., 2014 |
| 40   | 40.5     | 2.04721          | 0.04        | cyanidin-3-glucoside          | 278 sh; 232         | Aaby et al., 2007 |

*The compounds were identified by comparing absorption spectra of the sample with the standard compounds or from the values reported in the literature.
galantamine was 19.26 μg/mL (Fig. 6). Furthermore, in butyrylcholinesterase inhibitory activity, ON-Cr demonstrated potential IC\textsubscript{50} value of 55.36 μg/mL, while that of galantamine was 24.99 μg/mL (Fig. 7). The strong inhibitory propensity of ON-Cr was further corroborated from the Linewaer-Burk plots (Fig. 8) in which ON-Cr prospectively inhibited the enzymatic activities of both AChE and BChE, the inhibitory effect was comparable to that of the positive control, galantamine. The K\textsubscript{m} value of the substrates, ON-Cr and galantamine for AChE were 20.94 and 79.03 μg/min, respectively. Similarly, for BChE, the K\textsubscript{m} values of ON-Cr and galantamine were noted as 24.32 and 75.35 μg/min, respectively.

### α-glucosidase inhibitory assay

The percent inhibition of α-glucosidase via ON-Cr was found to be dose-dependent. The IC\textsubscript{50} value of ON-Cr was 639.89 μg/mL, while that of acarbose used as positive control was 61.19 μg/mL. The percent inhibition of α-glucosidase of ON-Cr in a concentration range of 31.25–1000 μg/mL is shown in Table 3. The Linewaer-Burk plots for the inhibition of α-glucosidase by ON-Cr and galantamine is shown in Fig. 9. The dissociation constant (K\textsubscript{m}) and V\textsubscript{max} values of ON-Cr for α-glucosidase were 31.11 μg and 51.01 μg/min, respectively, which were comparable to that of positive control, acarbose (30.19 μg and 76.17 μg/min), thus showing potent inhibitory proclivity against α-glucosidase.

### Discussion

In the present study, detailed phytochemical investigation and pharmacogonostic activity of Olax nana aqueous methanolic extract has been reported for the first time. Presence of various secondary metabolites like alkaloids, flavonoids, tannins, sterols, saponins and terpenoids have been found in the preliminary phytochemical analysis. Interestingly, in the HPLC-DAD results the highest concentrated signals were of gallic acid derivatives, hydroxybenzoic acid derivatives and rutin. Previously, Olax scandens has been reported for the presence of various essential phytochemicals like oleanolic acid, octacosanol, aleanolic acid and β-sitosterol etc. [54].
Furthermore, chemical investigation of *Olax mannii* Oliv also reported to contain useful flavonoid glycoside and derivatives [30].

Free radicals are generated during metabolic processes in the body and are implicated in a variety of disorders including neurodegenerative disorders, coronary heart disease, cancer, diabetes and immune-suppression [55, 56]. The hydrogen peroxide and singlet oxygen are included in the list of non-free radicals, while nitric oxide, lipid peroxyl, superoxide and hydroxyl are common free radicals [57]. Naturally, the lethal effect of free radicals is nullified in our body via various defense mechanisms, including the protective antioxidant system and chain breaking antioxidants generation [58]. Furthermore, extensive tissue injury occurs when free radical generation rate surpasses the limit of natural scavenging mechanisms. Hence, many diseases including neurogenerative disorders can be cured with the help of drugs having free radical scavenging ability. Plants
are a valuable resource of antioxidants which have been reported to protect from free radicals induced damage [59]. Significant anti-radical activities was revealed by ON-Cr in the DPPH, ABTS and H2O2 free radical scavenging assays with IC50 values of 71.46, 72.55 and 92.33 μg/mL, respectively.

In the treatment of neurological disorders, potential inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes are of prime importance. These enzymes are involved in the catabolism of acetylcholine (ACh), hence have applications in the treatment of Alzheimer’s disease (AD) [60], along with many other neurological disorders [61]. AD is connected with memory impairment, change of behavior and cognitive dysfunction [62]. A decline in the amount of ACh via malfunctioning of various biochemical pathways ultimately leads to AD [63]. AChE along with BChE result in the termination of signal transmission carried out in the synapse via ACh. Hence, by inhibiting these key metabolizing enzymes, we can treat AD and many other neurological disorders [64]. Unfortunately, the available drugs are associated with hepatotoxicity along with many adverse side effects and show effectiveness only in mild type of AD [65]. Hence, it's of prime importance to explore novel remedies for AD, which is effective and have less side effects propensities. Plants are widely explored for potential therapeutic compounds effective in the treatment of neurological disorders [66]. Many research groups including ours have also explored the potential of medicinal plants as valuable source for the treatment of neurodegenerative disorders especially AD [35, 67]. In the current study, very potent acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition potential was demonstrated by ON-Cr. Interestingly, in both acetylcholinesterase and butyrylcholinesterase inhibitory activities of ON-Cr, all the concentrations (31.25, 62.50, 125, 250, 500 and 1000 μg/mL) demonstrated % inhibition values not significantly different in comparison to galantamine (standard drug) at the same concentration (P > 0.05 at 95% confidence interval). Previously, Olax Subscorpioidea is also reported to possess neuroprotective, anxiolytic and anti-depressant properties [18, 21]. Significant results noticed in the antioxidant and anticholinesterase assays of ON-Cr may be attributed to the predominant presence of Gallic acid derivatives among the identified compounds (Table 2), which is a common poly phenol and strong antioxidant having promising neuroprotective potentials [68, 69]. The plant extracts also contain ascorbic acid which is a strong antioxidant. Quercetin and its derivative s are extensively studied as neuroprotective and antioxidant agents [70–72]. ON-Cr contains several quercetin derivatives which may be responsible for the antioxidant and cholinesterase inhibitory potentials of extracts samples. Pyrogallol, another poly phenolic compound was identified via comparison with the standards. This compound is a potent auto-oxidant and stimulates the activation of indigenous antioxidant system [73]. Rutin has a well established antioxidant and neuroprotective potentials [74, 75]. The current antioxidant and anti-cholinesterase potentials of ON-Cr can be attributed to the presence of these compounds. Yet further studies

### Table 3 α-Glucosidase inhibitory activity of Olax nana

| Concentration (μg/mL) | % inhibition of extract | IC50 (μg/mL) | Acarbose IC50 (μg/mL) |
|-----------------------|-------------------------|-------------|-----------------------|
| 1000                  | 55.24±0.33              | 639.89      | 77.76±0.17            |
| 500                   | 47.35±0.49              | 73.99±0.08  | 61.19                 |
| 250                   | 41.45±0.23              | 64.97±0.17  | 55.65±0.43            |
| 125                   | 37.18±0.09              | 55.65±0.43  | 49.50±0.45            |
| 62.5                  | 33.08±0.22              | 49.50±0.45  | 44.21±0.26            |
| 31.25                 | 29.79±1.12              | 44.21±0.26  |                       |

![Fig. 9 Lineweaver-Burk plots representing the reciprocal of initial α-glucosidase velocity versus the reciprocal of substrate concentration in the presence of different concentrations of ON-Cr (a) and the positive control, galantamine (b)](image-url)
regarding the isolation and testing of the plant samples will validate its potential use as a neuroprotective agent in folk medicine.

In the early stage of diabetes mellitus type 2, postprandial hyperglycemia occurs due to impaired secretion of insulin after meal. It is believed that free radicals may cause hyperglycemia and may result in complications, like retinopathy, nephropathy, and memory impairment [76]. The dietary carbohydrates in our meal are digested by a group of enzymes known as glucosidases. Acarbose is one of the key inhibitor of glucosidase, which ultimately downregulate the process of carbohydrate digestion and delays its absorption into the blood. Hence, potential inhibitors of glucosidases can ultimately prevent type 2 diabetes mellitus development via lowering of glucose after meal [77]. Plants have been reported as a great source of these type of inhibitors [78, 79]. Our results showed interesting inhibition of α-glucosidase via ON-Cr. The dissociation constant (Km) and Vmax values as reported by ON-Cr and acarbose were comparable to each other (Fig. 9). To the best of our knowledge, this is the first report of α-glucosidase inhibition via any Olax specie.

Conclusions
This present research is an exclusive report on the phytochemical composition, antioxidant, cholinesterase and α-glucosidase inhibitory potentials of Olax nana methanolic extract. The results of enzyme inhibitions and anti-radical activities were highly significant. These results indicate that Olax nana, is highly enriched with potential antioxidant compounds which can be exploited as potential therapy for the treatment of many disorders. In light of the potential biomedical application as demonstrated by the above results, further studies on the bioassay-guided isolation of potential active compounds and their therapeutic evaluation is recommended.

Acknowledgements
The authors are grateful to the Department of Pharmacy, University of Malakand, Khyber Pakhtoonkhwa, Pakistan, for providing laboratory facilities to conduct experimental work.

Funding
This research has received no specific grant from any funding agency in the public, commercial, or not for-profit sectors.

Availability of data and materials
The data presented in this manuscript belong to research work of Muhammad Ovais and has not been deposited in any repository yet. However, the materials are available to the researchers upon request.

Authors’ contributions
MO and MA carried out experimental work, data collection, evaluation, literature search and wrote the manuscript. ZKS supervised research work, helped in study design and drafted the final version of the manuscript. AKT, SAS, MSJ, AR and MS helped in plant collection, processing data interpretation and statistical analysis. All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interest.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References
1. Khalil AT, Khan I, Ahmad K, Khan YA, Khan J, Shinwari ZK. Antibacterial activity of honey in north-west Pakistan against select human pathogens. J Tradit Chin Med. 2014;34(1):86–9.
2. Khalil AT, Khan I, Ahmad K, Khan YA, Khan M, Khan MI. Synergistic antibacterial effect of honey and Herba Ocimi Basilici against some bacterial pathogens. J Tradit Chin Med. 2013;33(6):810–4.
3. Ahmad K, Talha Khalil a, Somaya R. antifungal, phytotoxic and hemagglutination activity of methanolic extracts of Ocimum Basilicum. J Tradit Chin Med. 2016; 36(6):394–8.
4. Khalil AT, Ovais M, Ullah I, Ali M, Shinwari ZK, Khamlisch S, Maaza M. Sageretia thea (Osbeck) mediated synthesis of zinc oxide nanoparticles and its biological applications. Nanomedicine (Lond). 2017 Aug;12(15):1767–89.
5. Khalil AT, Ovais M, Ullah I, Ali M, Shinwari ZK, Maaza M. Physical properties, biological applications and biocompatibility studies on biosynthesized single phase cubic oxide (Co3O4) nanoparticles via Sageretia Thea (Osbeck), Arab J Chem. 2017. https://doi.org/10.1016/j.arabjc.2017.07.004.
6. Khalil AT, Ovais M, Ullah I, Ali M, Shinwari ZK, Hassan D, Maaza M. Sageretia Thea (Osbeck) mediated synthesis of ZnO nanoparticles and their in vitro pharmacogastic, antioxidant and cytotoxic potential. Artif Cells Nanomed Biotechnol. 2017;1–15. https://doi.org/10.1080/21691401.2017.1345928.
7. Khalil AT, Ovais M, Ullah I, Ali M, Khan Shinwari Z, Maaza M. Biosynthesis of iron oxide (Fe2O3) nanoparticles via aqueous extracts of Sageretia Thea (Osbeck) and their pharmacogastic properties. Green Chemistry Letters and Reviews. 2017;10(4):186–201.
8. Ovais M, Khalil AT, Raza A, Khan MA, Ahmad I, Islam NU, Saravanan M, Ulbaid MF, Ali M, Shinwari ZK. Green synthesis of silver nanoparticles via plant extracts: beginning a new era in cancer theranostics. Nanomedicine. 2016;11(23):3157–77.
9. Ovais M, Raza A, Naz S, Islam NU, Khalil AT, Ali S, Khan MA, Shinwari ZK. Current state and prospects of the phytotheranostics colloidal gold nanoparticles and their applications in cancer theranostics. Appl Microbiol Biotechnol. 2017;101(9):3551–65.
10. World Health Organization. Health of indigenous peoples. Geneva, Switzerland: Factsheets N° 326; 2007 [http://www.who.int/mediacentre/ factsheets/fs326/en/].
11. Global Herbal Medicine Market Research Report [https://www.hexaresearch.com/research-report/global-herbal-medicine-market/].
57. Halliwell B, Gutteridge JM: Free radicals in biology and medicine. Oxford university press, USA, 2015.
58. Jacob RA. The integrated antioxidant system. Nutr Res. 1995;15(5):755–66.
59. Kumar S, Mishra A, Pandey AK. Antioxidant mediated protective effect of Parthenium Hysterophorus against oxidative damage using in vitro models. BMC Complement Altern Med. 2013;13(1):120.
60. Nawaz SA, Choudhary MI. New cholinesterase inhibiting bisbenzylisoquinoline alkaloids from Cocculus Pendulus. Chem Pharm Bull. 2004;52(7):802–6.
61. Kundiah N, Pai M-C, Senanarong V, Looi I, Ampil E, Park KW, Karanam AK, Christopher S. Rivastigmine: the advantages of dual inhibition of acetylcholinesterase and butyrylcholinesterase and its role in subcortical vascular dementia and Parkinson’s disease dementia. Clin Interv Aging. 2017;12:697.
62. Rathore S, Habes M, Iftikhar MA, Shacklett A, Davatzikos C. A review on neuroimaging-based classification studies and associated feature extraction methods for Alzheimer’s disease and its prodromal stages. NeuroImage. 2017;:
63. Callahan PM, Bertrand D, Bertrand S, Plagenhoef MA, Terry AV. Tiopetron sensitizes α7 containing nicotinic receptors to low levels of acetylcholine in vitro and improves memory-related task performance in young and aged animals. Neuropsychopharmacology. 2017;117:422–33.
64. Xiao S, Wang T, Ma X, Qin Y, Li X, Zhao Z, Liu X, Wang X, Xie H, Jiang Q. Efficacy and safety of a novel acetylcholinesterase inhibitor octohydroaminoacodine in mild-to-moderate Alzheimer’s disease: a phase II multicenter randomised controlled trial. Age Ageing. 2017;
65. McNeney-King A, Osman W, Edginton AN, Rao PP. Cytochrome P450 derivatives. Neurochem Int. 2006;48(4):263–74.
66. Zbarsky V, Datla KP, Parkar S, Rai DK, Aruoma OI, Dexter DT. Neuroprotective properties of the natural phenolic antioxidants curcumin and naringenin but not quercetin and fisetin in a 6-OHDA model of Parkinson’s disease. Proc Nutr Soc. 2017;1–7.
67. Ahmad S, Ullah F, Sadiq A, Ayaz M, Imran M, Ali I, Zeb A, Ullah F, Shah MR. Potential benefits of phytochemicals against Alzheimer’s disease. Clin Interv Aging. 2017;12:697.
68. Yilmaz Y, Toledo RT. Major flavonoids in grape seeds and skins: antioxidant activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. Neurochem Int. 2006;48(4):263–74.
69. Zbarsky V, Datla KP, Parkar S, Rai DK, Aruoma OI, Dexter DT. Neuroprotective properties of the natural phenolic antioxidants curcumin and naringenin but not quercetin and fisetin in a 6-OHDA model of Parkinson’s disease. Free Radic Res. 2005;39(10):1119–25.
70. Ossoola B, Kääriäinen TM, Männistö PT. The multiple faces of quercetin in neuroprotection. Expert Opin Drug Saf. 2009(4):397–409.
71. Dok-Go H, Lee KH, Kim HJ, Lee EH, Lee J, Song YS, Lee Y-H, Jin C, Lee YS, Cho J. Neuroprotective effects of antioxidative flavonoids, quercetin(+)-dihydroquercetin and quercetin 3-methyl ether, isolated from Opuntia Ficus-Indica Var. saboten. Brain Res. 2003;965(1):130–6.
72. Shao J, Wu Z, Yu G, Peng X, Li L. Allelopathic mechanism of pyrogallop to Microcystis Aeruginosa PCC7806 (cyanobacteria): from views of gene expression and antioxidant system. Chemosphere. 2009;75(7):924–8.
73. Yang J, Guo J, Yuan J. In vitro antioxidant properties of rutin. LWT-Food Science and Technology. 2008;41(6):1080–6.
74. Torngjaroenbuangam W, Ruksee N, Chantratitkul P, Pakdeesarong N, Kongbuntad W, Goustrapong P. Neuroprotective effects of quercetin, rutin and okra (Abelmoschus Esculentus Linn.) in dexamethasone-treated mice. Neurochem Int. 2011;59(5):677–85.
75. Martim A, Sanders a, Watkins DJ. diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol. 2003;17(1):24–38.
76. Liu L, Deseo MA, Morris C, Winter KM, Leach DN. Investigation of α-glucosidase inhibitory activity of wheat bran and germ. Food Chem. 2011;126(2):553–61.
77. Fu G, Li W, Huang X, Zhang R, Tian K, Hou S, Li Y. Antioxidant and alpha-glucosidase inhibitory activities of isoflavonoids from the rhizomes of Ficus tikoua. Nat Prod Res. 2017;1–7.
78. Zhao J-Q, Wang Y-M, Yang Y-L, Zeng Y, Mei L-J, Shi Y-P, Tao Y-D. Antioxidants and α-glucosidase inhibitors from “Luchiya” (young leaves and shoots of Sibiraea laevigata). Food Chem. 2017;230:117–24.