SUPPLEMENTARY MATERIAL

Communication

Phytochemical Composition and Enzyme Inhibition Studies of *Buxus papillosa* C.K. Schneid.

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Received: date; Accepted: date; Published: date

1. *Phytochemical composition*

1.1. HPLC-PDA polyphenolic quantification

HPLC analyses were performed on a Waters liquid chromatograph equipped with a model 600 solvent pump and a 2996 photodiode array detector and Empower v.2 Software (Waters Spa, Milford, MA, USA) was used for acquisition of data. A C18 reversed-phase packing column (Prodigy ODS (3), 4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA, USA) was used for the separation and the column temperature was set at 30 ± 1°C using a Jetstream2 Plus column oven. The UV/Vis acquisition wavelength was set in the range of 200–500 nm. The quantitative analyses were achieved at the maximum wavelength for each compound. The injection volume was 20 μL. The mobile phase was directly on-line degassed by using Biotech DEGASi, mod. Compact (LabService, Anzola dell'Emilia, Italy). Gradient elution was performed using the mobile phase water-acetonitrile (93:7, v/v, 3% acetic acid) as reported in the literature (16-18). All the prepared sample solutions were centrifuged and the supernatant was injected into the stock solutions of phenolics were made at a concentration of 1 mg/mL in a final volume of 10 ml of methanol. Working solutions of mixed standards at the concentrations of 10, 25, 50, 75, 100, 150 and 200 μg/mL were made by dilution of stock solution in volumetric flasks with the mobile phase. Then the standards were injected into the HPLC-PDA system. Working solutions of mixed standards at the concentrations of 0.25, 0.5, 1, 2.5, 5, 10, and 20 μg/mL were made by dilution of stock solution in volumetric flasks with the mobile phase. Then the standards were injected into the HPLC-PDA system. Each solid sample was weighted and solubilized in mobile phase in 1:1 (*w:v*) ratio. In this case, the obtained concentrations (μg/mL) correspond to the total amount (μg/mg). After solubilization, the samples were centrifuged at 12000 *x g* and filtered through a micro-filter before HPLC injection. Standard phenolic compounds were applied for identifying and quantifying individual phenolic components in the samples as μg/g dry extract [1, 2].

1.2. UHPLC-MS secondary metabolites identification

Secondary metabolites were evaluated by RP-UHPLC-MS. UHPLC of Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source was used. Column specifications were as: Agilent Zorbax Eclipse XDB-C18, narrow-bore 2.1×150 mm, 3.5 μm (P/N: 930990-902). Column and auto-sampler temperatures were maintained at 25 °C and 4 °C, respectively. The flow rate was 0.5 mL/min. Mobile phases used were: A - 0.1% formic acid in water, B - 0.1% formic acid in acetonitrile. The injection volume was 1.0 μL. Run time was 25 min and post-run time was 5 min. Full scan MS analysis was done over a range of m/z 100–1000 using electrospray ion source in negative mode. Nitrogen was supplied as nebulizing and drying gas at flow rates of 25 and 600 L/hour, respectively. The drying gas temperature was 350 °C. The fragmentation voltage was optimized to 125 V. Analysis was performed with a capillary voltage of 3500 V. Data was processed with Agilent Mass Hunter Qualitative Analysis B.05.00 (Method: Metabolomics-2017- 00004.m). Identification of compounds was done from Search Database: METLIN_AM_PCDL-Ne 170502.cdb, with parameters as: Match tolerance: 5 ppm, Positive Ions: +H, +Na, $+NH_4$, Negative Ions: H [3].

2. *Enzyme assays*

2.1. α-Amylase inhibition assay

Sample solution (1 mg/mL; 25 μL) was mixed with the α-amylase solution (ex-porcine pancreas, EC 3.2.1.1, Sigma) (50 μL) in phosphate buffer (pH 6.9 with 6 mM sodium chloride) in a 96-well microplate and incubated for 10 min at 37◦C. After pre-incubation, the reaction was initiated with the addition of starch solution (50 μL, 0.05%). Similarly, a blank was prepared by adding a sample solution to all reaction reagents without enzyme (α-amylase) solution. The reaction mixture was incubated 10

min at 37◦C. The reaction was then stopped with the addition of HCl (25 µL, 1 M). This was followed by the addition of the iodine-potassium iodide solution (100 µL). The sample and blank absorbance were read at 630 nm. The absorbance of the blank was subtracted from that of the sample and the α-amylase inhibitory activity was expressed as acarbose equivalents (mmol ACE/g extract) [4].

2.2. Tyrosinase inhibition assay

Sample solution (1 mg/mL; 25 µL) was mixed with tyrosinase solution (40 µL, Sigma) and phosphate buffer (100 µL, pH 6.8) in a 96-well microplate and incubated for 15 min at 25◦C. The reaction was then initiated with the addition of L-DOPA (40 µL, Sigma). Similarly, a blank was prepared by adding a sample solution to all reaction reagents without enzyme (tyrosinase) solution. The sample and blank absorbance were read at 492 nm after a 10 min incubation at 25◦C. The absorbance of the blank was subtracted from that of the sample and the tyrosinase inhibitory activity was expressed as kojic acid equivalents (mg KAE/g extract) [2, 5].

2.3. Lipoxygenase inhibition assay

For the determination of lipoxygenase activity, a total volume of 200 µL assay volume was used [6]. To each well of 96- well UV plate, 150ul of 100Mm, PH 8.0 potassium phosphate buffer, 10µl of test compound and 20 µL of LOX enzyme was added. The contents were mixed and pre-incubated for 5 minutes. Pre-read was measured at 234nm. Then 20µl of a substrate (linoleic acid) was added to initiate the reaction in each well. It was incubated for 10 minutes and change in absorbance was measured while taking after-read at 234nm. All reactions were carried out in triplicates. Quercetin was used as a positive control. The percentage inhibition was calculated by the formula:

Inhibition (%) = (Abs of cont – Abs of test comp/ Abs of cont) × 100

IC_{50} values for active compounds were determined by using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc.).

3. Statistical analysis

All the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The differences between the different extracts were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's significant difference post hoc test and value $p < 0.05$ was considered significant. This treatment was carried out using SPSS v. 14.0 program. Graph Pad Prism software (San Diego, CA, USA, Version 6.03) was used to calculate IC_{50} values.

FIGURES

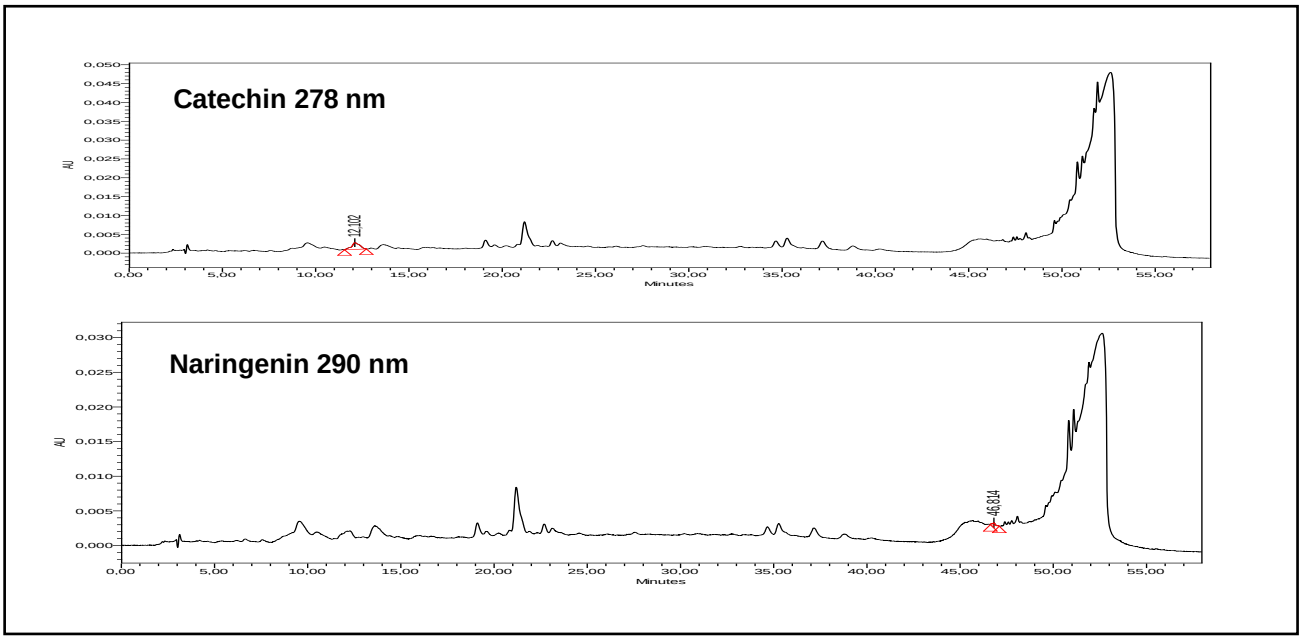

Figure S1. HPLC chromatograms of phenolics quantified in *B. papillosa* aerial-MeOH extract.

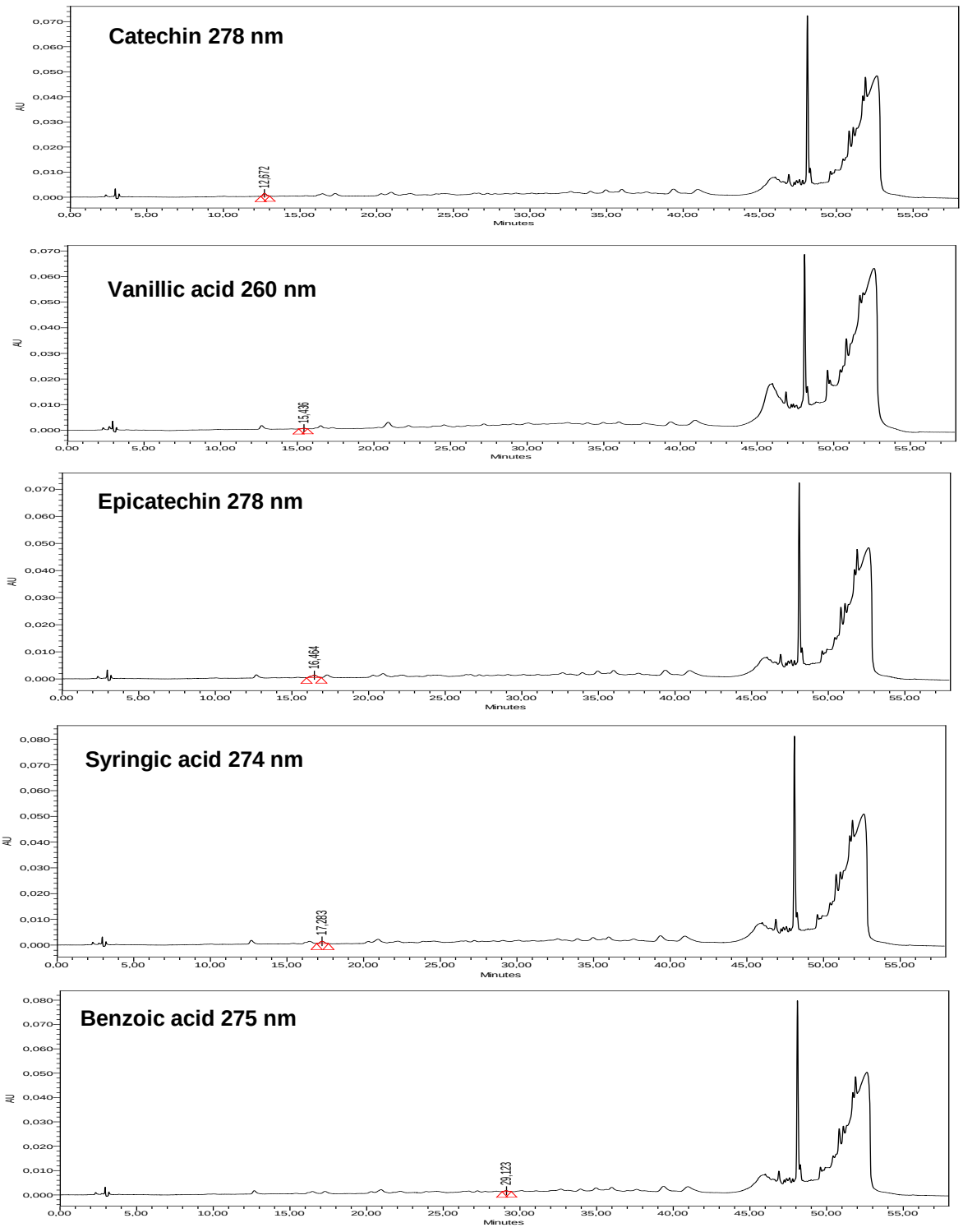

Figure S2. HPLC chromatograms of phenolics quantified in *B. papillosa* aerial-DCM extract.

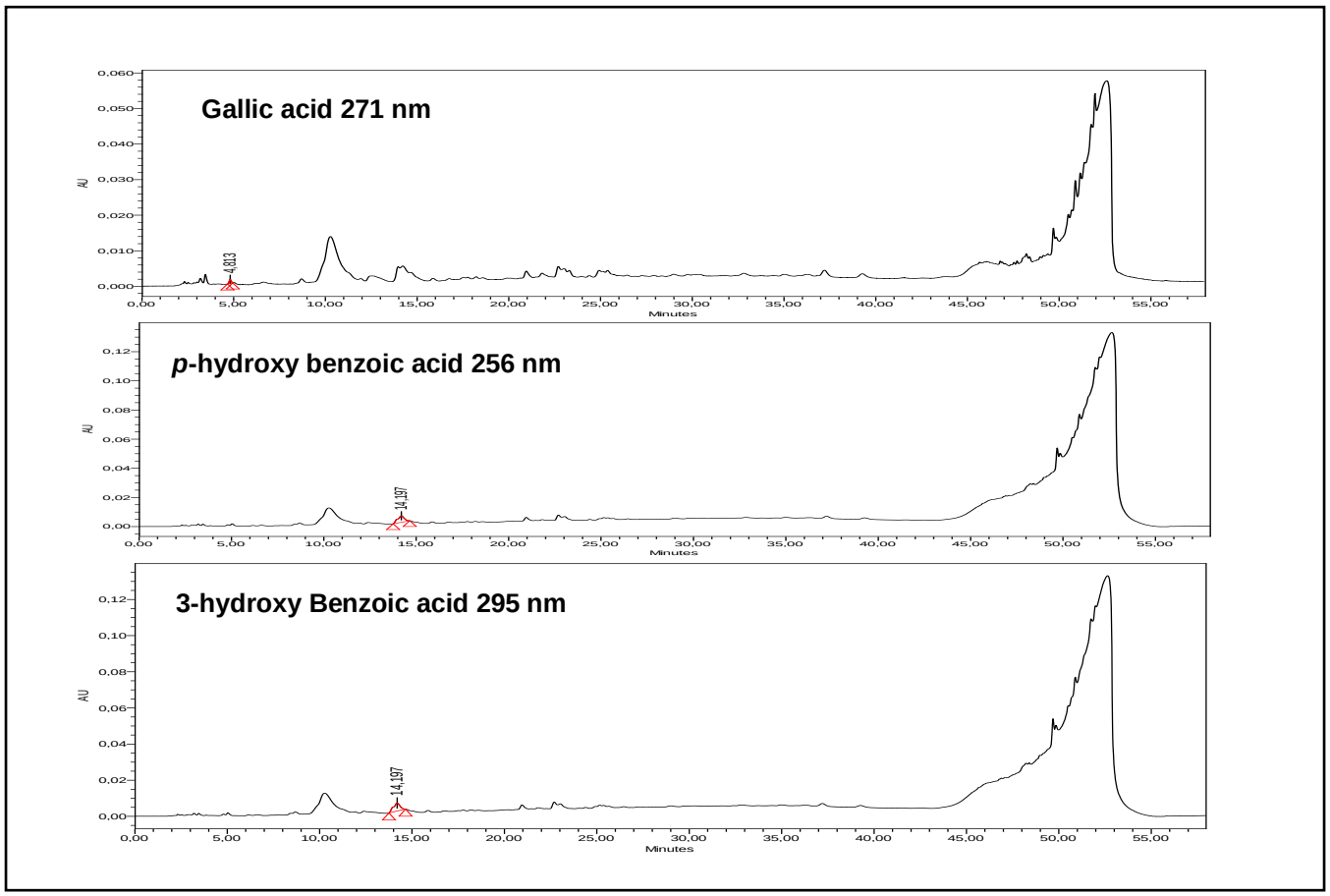

Figure S3. HPLC chromatograms of phenolics quantified in *B. papillosa* stem-MeOH extract.

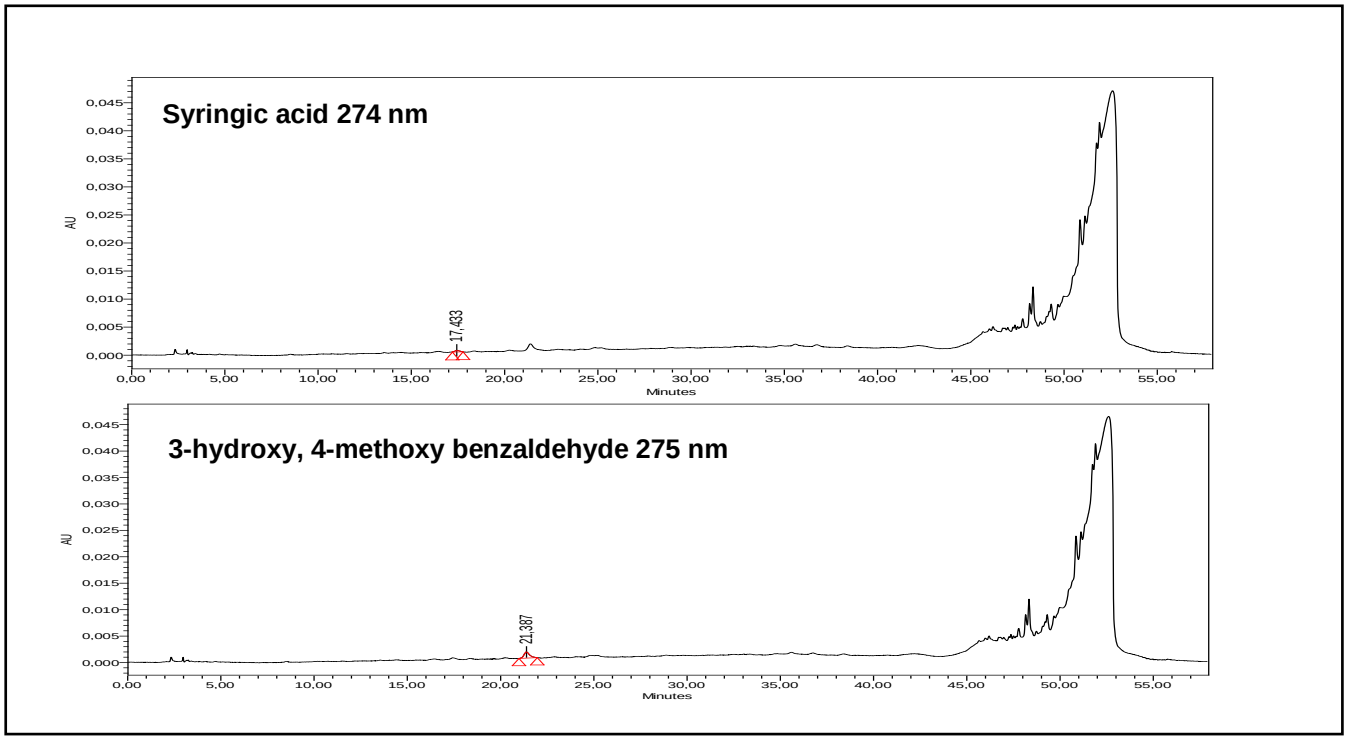

Figure S4. HPLC chromatograms of phenolics quantified in *B. papillosa* stem-DCM extract.

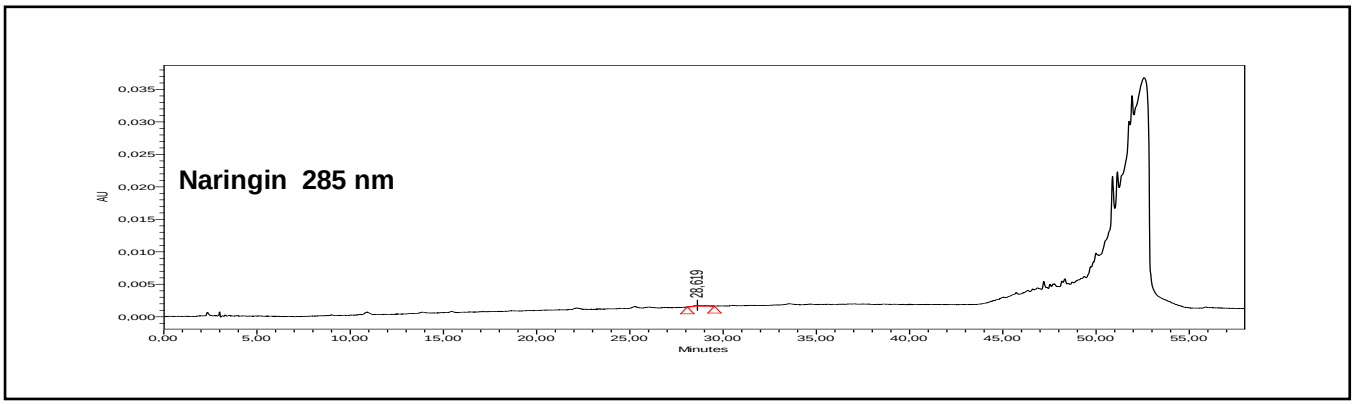

Figure S5. HPLC chromatograms of phenolics quantified in *B. papillosa* stem-hexane extract.

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