Supplementary Online Material of Journal of Insects as Food and Feed (https://doi.org/10.3920/JIFF2022.0148)

Dietary *Periplaneta americana* extract improved the growth performance, immune, and antioxidative status, and crowding stress responses in Nile tilapia

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Supplementary Materials and methods

Identification of active components in *Periplaneta americana* extract

The active components of PAE were analysed by LC-MS method. For the identification of choline and L-carnitine, 0.5 ml of the PAE, 0.3 ml 1% formic acid-acetonitrile solution, and 100 mg steel beads were put into a 2 ml Eppendorf tube (EP tube), which was placed in a high-throughput tissue grinding machine to grind them at 50 Hz for 60s. After centrifugation at 12,000 rpm and 4℃ for 20 min, 20 µl of the supernatant were removed to 2 ml centrifuge tube, mixed with 10 µl of internal standard solution and 750 µl of 1% formate-acetonitrl solution, following vortex oscillation for 30s and centrifugation at 12000 rpm 4℃ for 10 min. Then, the supernatant of 500 µl was filtered through 0.22 µm membrane to obtain filtrate for analysis. Chromatographic separations were conducted with a ACQUITY UPLC® BEH HILIC column (2.1×100 mm, 1.7 µm, American Waters company) with flow rate of 0.4 ml/min and injection volume of 5 µl at 40 °C. The mobile phase was composed of (A) water (containing 0.1% formic acid and 10 Mm ammonium formate) and (B) acetonitrile. The following gradient procedures were used: 0~1 min, 20% A; 1~2 min, 20~30% A; 2~2.5 min, 30% A; 2.5~3 min, 30~50% A; 3~3.5 min, 50% A; 3.5~4 min, 50~20% A; 4~6 min, 20% A. A Q Exactive hybrid Q-Orbitrap mass spectrometer with a heated ESI source (SCIEX, AB4000) was used to record HRMS data. The ESI source parameters were shown as follows: Ion source temperature, 500℃; Ion source voltage, 5000 V; Colash gas, 6 psi; Air curtain air, 30 psi; atomized and auxiliary gas, 50 psi. Choline and L-carnitine were identified by their retention time and relative ion-flow intensity. Choline and L-carnitine were quantified by their peak area.

For identification of protocatechuic acid, 500 µl of PAE were first soaked in 2 ml of aqueous NaOH (4 M). The solution was hydrolysed for 2h at 40 °C in a gas bath. The pH was adjusted to 2 with 4 M aqueous HCl. The hexane layer was removed by shaking the mixture with 2 ml of hexane for 20 min at room temperature. The aqueous layer was extracted with ethyl acetate (22 ml), and the mixed extract was concentrated to near dry at 35℃ on a rotary evaporator. Before analysis, the residue was dissolved in 200 µl of 50% methanol/water and transferred to insert-equipped vials. Then, the sample were analysed using an UPLC-Orbitrap-MS system, composed of HPLC (Vanquish, UPLC, Thermo, USA) and high-resolution mass spectrometry (Q Exactive, Thermo, USA). The UPLC parameters were set as follows, column, Waters HSS T3 (50*2.1 mm, 1.8 µm); mobile phase (A) ultrapure water (containing 0.1% acetic acid) (B) acetonitrile (containing 0.1% acetic acid); flow rate, 0.3 ml/min; column temperature, 40 °C; injection volume, 2 µl. The following gradient procedures were used: 0~2 min, 90%A : 10% B; 2~6 min, 90%A : 10% B；6~8 min, 40%A : 60% B; 8~12 min，90%A : 10% B. Finally, A Q Exactive hybrid Q–Orbitrap mass spectrometer with a heated ESI source (Thermo Fisher Scientific) was used to record HRMS data. The ESI source parameters were shown as follows: sheath gas, 40 arb; aux gas, 10 arb; sweep gas, 0 arb; spray voltage, -2.8 kV; aux gas heater temperature, 350 ℃; capillary temperature, 320 ℃. Data were collected and processed on the Q-Exactive with Xcalibur 4.1 and TraceFinder™4.1 Clinical (Thermo Scientific), respectively.

For identification of lactic acid, citric acid, and pantothenic acid, 0.5 ml of the PAE, 0.5 ml 30% aqueous methanol solution (containing 0.1% formic acid), and 100 mg steel beads were put into a 2 ml EP tube, which was placed in a high-throughput tissue grinding machine to grind them at 60 Hz for 100s. After centrifugation at 12,000 rpm 4 ℃ for 10 min, the supernatant was filtered through 0.22 μ m membrane and 50 μl of the supernatant were removed to 2 ml centrifuge tube, mixed with 200 μl 30% aqueous methanol solution (containing 0.1% formic acid). After vortex oscillation for 60s, the obtained samples were put into a test bottle for analysis. Chromatographic separations were conducted with a ACQUITY UPLC® BEH C18 column (2.1×100 mm, 1.7 μm, American Waters company) with flow rate of 0.4 ml/min and injection volume of 5 μl at 40 °C. The mobile phase consists of (A) water (Contains 0.1% formic acid) and (B) Methanol water (containing 0.1% formic acid). The following gradient procedures were used: 0~3 min, 30% B; 3~5 min，30~50% B; 5~7 min，50~90% B; 7~9 min，90% B; 9~13 min, 30% B. A Q Exactive hybrid Q–Orbitrap mass spectrometer with a heated ESI source (SCIEX, AB4000) was used to record HRMS data. The ESI source parameters were shown as follows: Ion source temperature, 500℃; Ion source voltage, -4500 V; Colash gas, 6 psi; Air curtain air, 30 psi; atomized and auxiliary gas, 50 psi. Choline and L-carnitine were identified by their retention time and relative ion-flow intensity. Choline and L-carnitine were quantified by their peak area.

Lysozyme (Kit No. A050-1-1),
Because the concentration of turbid bacterial solution is hydrolyzed by lysozyme and the transmittance rises, the lysozyme content may be determined based on the change in transmittance in a certain concentration of turbid bacterial solution.Thus, turbidimetry is used to measure the lysozyme content.

Total protein (TP) (Kit No. A045-4-2) ,
The protein converts Cu^{2+} to Cu^{+} under alkaline conditions, , and Cu^{+} produces a purple compound with BCA reagent, having a maximum absorption peak at 562nm. By measuring the absorption, the absorbance may be computed.

Albumin (ALB) (Kit No. A028-2-1)
Albumin is capable of binding to anionic dyes. Bromophenol green binds to albumin from yellow to green at pH4.0, and the depth of color is proportional to albumin content.

Complement C3 (Kit No. E032-1-1)
Using immunopbiactric assay, complement C3 antibody and complement C3 cause antigen antibody reaction to form an immune complex. The change in the turbidity is detected at 340nm, and the magnitude of the change being directly proportional to the complement C3 content.

C4 (Kit No. E033-1-1)
Using immunopbiactric assay, complement C4 antibody and complement C4 cause antigen antibody reaction to form immune complex. The change in the turbidity is detected at 340nm, and the magnitude of the change being directly proportional to the complement C4 content.

Malondialdehyde (MDA) (Kit No. A003-1-2),
Malondialdehyde (MDA) in the peroxide lipid degradation product can condense with

thiobarbituric acid (TBA) to generate a red product with a maximum absorption peak at 532nm. This method is called the TBA method because the substrate is thiobarbituric acid (Thibabituric Acid TBA)

Superoxide dismutase (SOD) (Kit No. A001-3-2),
Superoxide anion radical (O_2-) is produced through the xanthine and xanthine oxidase reaction system, which oxidizes hydroxylamine to form nitrite, which is colored purple under the action of chromogen, and its absorbance is measured by a visible light spectrophotometer. When the measured sample contains SOD, it has a specific inhibitory effect on the superoxide anion radical, reducing the nitrite formed. When the absorbance value of the measured tube is lower than the absorbance value of the measured tube, the SOD vitality in the measured sample can be calculated by the formula.

Catalase (CAT) (Kit No. A007-1-1),
The reaction of H_2O_2 decomposition by catalase (Catalase) can be rapidly suspended by adding ammonium molybdate. The remaining H_2O_2 and ammonium molybdate produces a pale yellow complex, measuring the amount of change at 405nm, which can calculate the vitality of CAT.

Glutathione (GSH) (Kit No. A006-2-1),
The prototype glutathione (GSH) can react with dithiodinobenzophenic acid (DTNB) to produce a yellow compound that can quantitatively quantify the content of the prototype glutathione (GSH) at 405nm.

Alkaline phosphatase (AKP) (A059-2-2),
Alkaline phosphatase breaks down benzene diodium phosphate, producing free phenol and phosphate. Phenols acts with 4-aminoamapiroline in alkaline solution by oxidation of potassium feriryanide to produce red quinone derivatives. The enzyme vitality can be determined according to the depth of red.

Aspartate aminotransferase (AST) (C010-2-1),
AST / GOT can shift the beta-ketone glutaric acid and aspartate into the amino groups and the ketone groups to generate glutamate and oxaloacetic acid. Oxaloacetate can spontaneously decarboxylate into pyruvate during the reaction. Pyruvate reacts with a 2,4 dinitrophenylhydrazine to produce a 2,4 dinitrobenzene, which appears reddish-brown in an alkaline solution. After colorization, the vitality unit of the enzyme is obtained by checking the standard curve.

Alanine aminotransferase (ALT) (C009-2-1),
At 37°C and PH7.4, glutransaminase (ALT) acts on the substrate composed of alanine and α -ketoglutaric acid,and generate pyruvate and glutamate. After 30min of the reaction (fixed time), 2,4-dinitrophenylhydrazine (DNPH) hydrochloric acid solution isadded to stop the reaction, meanwhile, DNPH reacts with ketotic acid to produce pyruvate benzene, which is reddish-brown under alkaline conditions. At 505nm, the ratio absorbance is read and the enzyme viability is calculated.

Total cholesterol (TC) (Kit NO. A111-2-1),
Cholesterol esters are broken down by cholesterol esterase to produce cholesterol, which is oxidized by cholesterol oxidase to produce hydrogen peroxide, and hydrogen peroxide reacts with phenol to form red quinones. The depth of the color of the generated quinone compounds is directly proportional to the content of cholesterol. The absorbance values of the calibration standard tube and the sample tube are determined, respectively, and the cholesterol content is calculated.

Triglycerides (TG) (Kit NO. A110-1-1)
Triglycerides are decomposed by lipase to produce glycerol, which reacts with ATP to form glycerol-3-phosphate. Then, glycerol-3-phosphate is oxidized by phosphate oxidase to produce hydrogen peroxide, and hydrogen peroxide is chromogenic with ESPMT. Colorimetric measure is conducted at 546nm, the light absorption value is proportional to the content of triglyceride. The light absorbance value of the standard tube and the sample tube are determined, respectively, to calculate the content of triglyceride in the samples.

High density lipoprotein cholesterol (HDL-C) (Kit No. A112-1-1)
High density lipoprotein cholesterol reacts with cholesterol esterase and cholesterol oxidase to produce hydrogen peroxide, which react with TOOS (N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline) to generate red and purple pigment under the catalysis of peroxidase.

Low density lipoprotein cholesterol (LDL-C) (Kit No. A113-1-1)
Low density lipoprotein cholesterol is broken down into microgranulating cholesterol by surface active agent, which reacts with cholesterol esterase and cholesterol oxidase to produce hydrogen peroxide. Hydrogen peroxide and TOOS (N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline) undergo a chromogenic reaction catalyzed by peroxidase.

Protease (Kit No. A080-2-2),
Trypsin (trypsin) catalyzes the ester chain of the substrate ethyl arginine, increasing its absorbance at 253nm. The enzyme vitality can be calculated based on the change in the absorbance.

Lipase (Kit No. A054-1-1),
The latex made of triglycerides and water has milky properties due to the absorption and scattering of the incident light by its micelles. Triglycerides in the micelles are hydrolyzed by lipase (Lipase; LPS) to divide the micelle, and thus reduce the scattering light or turbidity. The rate of reduction is related to the lipase viability.

Amylase (Kit No. C016-1-1),
α-amylase hydrolyzes starch to produce glucose, dentiose and dextrin. In cases of known and excess substrate concentration, iodine solution is added to unhydrolyzed starch to create a blue complex.

Cortisol (Kit No. H094-1-1),

This kit use the competitive method to detect the content of cortisol in the samples. Samples are added to the microplate wells pre-coated with antibodies. Then, additional biotin-labeled recognition antigen is added and incubated for 30 min at 37 ℃. Both compete with solid-phase antibodies to form immune complexes. Unbound biotin antigens are removed by washing with PBST, followed by adding avidin-HRP, and incubated for min at 37 ℃. Avidin-HRP binds to the biotin antigen. Bound HRP catalyzes TMB (tetramethylbenzidine) in blue, which is subsequently converted to yellow by acid. There is absorption peak at 450nm, and the absorbent value is negatively correlated with the concentration of antigen in the sample.

Glucose (Kit No. F006-1-1),

Glucose is broken down into gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with DHBS (3,5-Dichloro-2-hydroxybenzenesulfonic acid sodium salt) to produce red and purple pigment under the catalysis of peroxidase. The depth of color is proportional to the glucose concentration.