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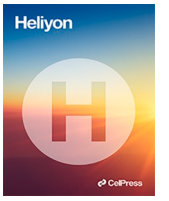

# Antimicrobial ingredients of *Zanthoxylum motuoense* and potential in fresh pork meat preservation

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ABSTRACT

Zanthoxylum motuoense (Tibetan prickly ash, MTHJ), different from the Chinese prickly ash species, is distributed only in the Tibet. Now the chemical characterization and antibacterial activity of MTHJ extracts were analyzed for the first time. As a result, Schinifoline **(12)**, γ-Fagarine **(8)**, (*2E,7E,9E*)-6 S-Hydroxy-*N*-(2-methylpropyl)-11-oxo-2, 7, 9-Dodecatrienamide **(6)**, and Neoechinulin A **(17)** were found to be the major different factors by untarget LC-MS metabolomics together with quantitative analysis on target. These four compounds were also the major antibacterial constituents. Then, the antimicrobial activity of MTHJ fractions was evaluated with colony forming units (CFU), fluorescence microscopy imaging, SEM and investigating the potential food preservation. Nutritional composition, colour and sensory evaluation of extract-treated samples were evaluated along storage time. The results suggested the MTHJ may be used for meat products preservation, and the scores were significantly higher for its unique flavor, which offered a promising choice for food safety, preservation and reducing foodborne illness.

## 1. Introduction

Tibetan prickly ash (*Zanthoxylum motuoense*, MTHJ) has been widely used as a pungent seasoning in the southeastern area of Tibet in cooking due to its unique aroma, taste, high medicinal and nutritional[1]. It is alsoconsidered as traditional food and be widely used in marinated yak meat, stir-fries and cold salads in the daily diet[2,3]. In addition to culinary applications, Tibetan prickly ash has also been used as functional food to clear away heat, antidotes, anthelmintics, and treatments of gastrointestinal problems[2]. For a long time, Tibetan prickly ash provenances were been almost isolated from the outside world[4]. Currently, there is very little documentation of the Tibetan prickly ash[5,6] used in food preservation, antimicrobial and bioactive compounds.

Deterioration and weightlessness of meat products during storage have always brought many problems for their commercialization [7] and economic value [8], microbial contamination and loss of nutrients are important factors for quality deterioration [9,10]. The

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**Abbreviations**

| | |
|---|---|
| CFU | colony-forming unit assays |
| MBC | minimum bactericidal concentration |
| MFC | minimum fungicidal concentration |
| MIC | minimum inhibitory concentration |
| MPP | mass profiler professional |
| MTHJ (Tibetan prickly ash) | Zanthoxylum motuoense Huang |
| PBS | phosphate buffered solution |
| PCA | principal component analysis |
| TIC | total ion chromatograms |
| ROS | reactive oxygen species |
| TSB | tryptic soy broth. |

bigger worry is that the food products contaminated with drug-resistant bacteria presents a serious threat to global public health[11,12], requiring the discovery and development of new classes of antimicrobial agents [13]. The other focus of attention is that the growing consumers demand for safe and naturally processed meats[14]. The use of chemical additives and synthetic preservatives, have become controversial because they may lead to the potential health damage[15–17]. However, there are many natural preservatives have been widely used in meat products [18], but due to their volatility, instability [19], and easy degradation, their biological activities often decreases [9,20]. Developing non-polluting, low-toxic and low-residue functional additions (such as prickly ash extraction) in food industry can reduce the concentration of some preservatives or eliminate the use of synthetic preservatives, and reduce the rates of antibiotic resistance and the spread of resistant bacteria [21].

The objective of this work was to found potential antimicrobial and food preservation of MTHJ extracts of different solvents and fractions with different polarity for antimicrobial and food preservation through untarget LC-MS metabolomics and targeted quantitative analysis. In order to better analyze the main difference of MTHJ extracts and fractions, we not merely estimated its antibacterial activity and mechanisms but also explored the anti-food spoilage activity. Overall, this research can provide a good reference for the rational application of Tibetan prickly ash on chilled pork and reducing foodborne diseases caused by pathogens.

## 2. Methodology

### 2.1. General experimental procedures

Using agilent UHPLC/6545 QTOF-DAD-MS/MS spectrometer to measure electron spray ionization (ESI) spectra. Column chromatography (CC) used $C_{18}$ columns (2.1 mm × 50 mm, 1.8 μm, Agilent Technologies) and BEH HILIC column (2.1 × 50 mm, 1.7 μm, Waters). MS-grade methanol, acetonitrile, and formic acid were purchased from Merck (USA). Bacteria Biofilms were analyzed under an inverted fluorescence microscopy (LeiCa, DM 2000) and stained by thiazoyl blue tetrazolium bromide (MTT). Ampicillin sodium,

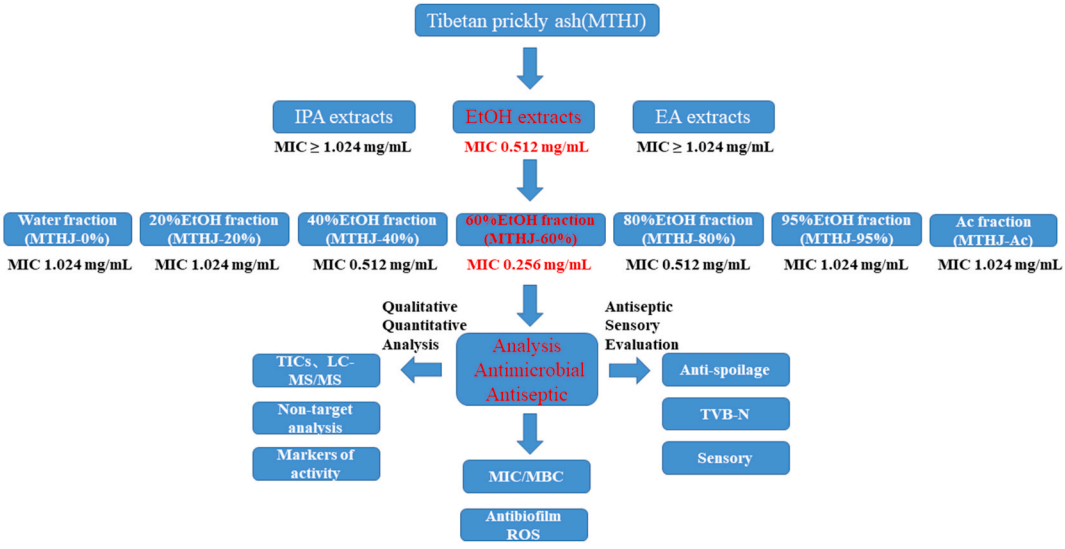


**Fig. 1.** Flow chart indicating the extraction of Tibetan prickly ash (MTHJ, *Z. motuoense*).

cefoxitin, fluconazole, vancomycin, berberine chloride, 5-(4, 6-dichlorotriazinyl) aminofluorescein (5-DTAF), crystal violet, and MTT were got from Macklin Reagents (Shanghai, China). The standard compounds (purity ≥98 %) are purchased from Mai De Sheng (Cheng Du, China). Fresh pork purchased in Haoxiansheng Supermarket in KunMing.

### 2.2. Plant material

Air-dried MTHJ seed (No. Luo 20,181,209) was collected in December 2018 in MoTuo city, Tibet and checked with http://www.theplantlist.org. The botanical identification was performed by Zhao-Jie Wang from Yunnan Provincial Center for Research & Development of Natural Products. All specimens dealt with in this study are housed in the Key Laboratory of Medicinal Chemistry for Natural Resource, Yunnan University. Flow chart shown in Fig. 1.

### 2.3. Pathogenic microorganisms

*Staphylococcus aureus* (ATCC 25923) was received from Northwest A&F University. *Canidida albicans* (ATCC 14053) was received from the Second People's Hospital of Yunnan Province. MRSA 3 (170,208,345, resistant) were isolated strains received from the First People's Hospital of Zunyi City. Trypticase soy broth (HKM, Guangzhou, China). The strains were cultured on tryptic soy agar (TSA), and a single colony was selected from the TSA plate for inoculation in tryptic soy broth (TSB) at 37 °C with shaking at 180 rpm for 24 h to obtain a bacterial suspension in logarithmic phase prior to the experiment.

### 2.4. Preparation of MTHJ extracts

Dried and ground herbs of MTHJ (20 g) and extracted with 95 % (v/v) aqueous ethanol, propan-2-ol, and ethyl acetate (200 mL × 3, 24 h each) under ultrasonic conditions, and the solvent was evaporated in vacuum. Crude extract (1 g) was subjected by food grade D101 macroporous resin column (4.8 × 68 cm i. d.), eluted with step gradient of mixed solutions (ethanol/water 0:100, 20:80, 40:60, 60:40, 80:20, 95:5 v/v, and acetone, each 1 L) to generate seven fractions (MTHJ-0% to MTHJ-Ac, respectively). Finally, all fractions were prepared for further LC-MS/MS analysis, antimicrobial and food preservation tests. Dissolve the standard substances in mass spectrometry grade methanol and dilute it to a range of concentrations for qualitative and quantitative analysis of sample.

### 2.5. Identification of compounds from MTHJ by UHPLC-QTOF-DAD-MS/MS

Agilent 1290 UHPLC combined with 6545 Q-TOF-DAD/MS system (Agilent Technologies, Palo Alto, CA, USA) was used for the qualitative and quantitative analysis of bioactive compounds in MTHJ extracts. All samples have passed 0.22 μm PTFE membrane filtration before injection and separation was performed by a C18 column at 35 °C with a flow rate of 0.15 mL/min. The other conditions were recorded in Supporting Information.

### 2.6. Untarget LC-MS metabolomics analysis

Untarget LC-MS metabolomics was performed using a liquid chromatography-high-resolution mass spectrum (LC-HRMS) system (Agilent UHPLC/6545 QTOF-MS/MS) coupled with Profinder B.08.00, Mass Profiler Professional, and SIMCA 14.1 software. Sample separation was performed as described previously. The scan range of the ESI-MS was $m/z$ 20–2000. The ESI voltage was 3500 V in positive ion mode. A nebulizing gas of 35 (psig) and a drying gas (8 L/min) were applied for ionization, using nitrogen in both cases. Non-targeted screening workflow of Profinder and Mass Profiler Professional software (B.08.00) was adopted with a medium peak detection sensitivity to export peak area and identify all the compounds. Next, PCA analysis was performed using the PCA method from the SIMCA 14.1, and the divergence compounds of different fractions were shown as Loadings Scatter Plot.

### 2.7. Evaluation of antimicrobial activity in vitro

Microbial cultures were grown in TSB or on standard TSA plates. According to CLSI guidelines (CLSI, 2019)[22], broth microdilution assays were carried out to evaluate the antimicrobial activity of different fractions and compounds by the determination of MIC, MBC and MFC.

As described previously, 100 μL aliquots of bacterial or fungal cultures (~$10^8$ CFU) were seeded into 96-well polystyrene microtiter plates (Corning, NY, USA) at 37 °C for 24 h, to allow biofilm formation. Non-adhered cells were removed with a pipette and plates washed three times using 200 μL PBS. Then, existing biofilms were incubated at 37 °C in mixed solvents with ½ MIC, 1 MIC, 2 MIC, and 4 MIC concentrations of fractions and TSB, and then incubated for a further 24 h. Each treatment included six parallel wells. Biofilms incubated with TSB only were used as blank controls, fluconazole or ampicillin sodium salt as positive control. Evaluation of biofilm mass was carried out with crystal violet and thiazoyl blue tetrazolium bromide[23].

For fluorescence microscopy qualitative observations, bacterial or fungal cultures (~$10^8$ CFU) were grown on glass coverslips at 37 °C for 24 h in 24-well plates supplemented with 1 mL of TSB to allow biofilm formation. Coverslips were washed to remove unattached cells and treated with 2 mL PBS. Biofilms were incubated at 37 °C in mixed solvents with ½ MIC, 1 MIC, 2 MIC, and 4 MIC concentrations of fractions and TSB. After incubation, the medium was removed, and fluorescent dye (5-DTAF) was used after washed three times using PBS. The labeled biofilms were then washed with sterile water to remove free dye.

### 2.8. Evaluation anti-food spoilage activity in food industry

The MTHJ-60 % fraction was tested for anti-food spoilage activities and food preservations at concentration (1, 10 mg/mL). The pork was cut into 3 cm × 4 cm pieces and divided in three groups. Each group had three parallel samples. Two groups were treated with 1 and 10 mg/mL MTHJ-60 % respectively, and one group was treated with PBS served as a control group. After 1 h, take out the sample, dry the surface at 50 °C, and store in a 4 °C refrigerator. Repeat the above operation once a day. The total number of bacterial colonies was determined according to Chinese standard GB 4789.2–2016. After serial dilutions, ten-fold dilutions of the aliquots were inoculated onto TSA plates and incubated at 37 °C for 24 h. The results are expressed as CFU/g fresh pork, and parallel determination of each sample was performed three times.

The determination of total volatile base nitrogen (TVB-N) shall refer to the microdiffusion method of Chinese Standard GB 5009.228–2016. Results were expressed as mg/100 g of fresh pork and each of the sample was parallelly determined three times. The judgment criteria were as follows: TVB-N value $<$ 10 mg/100 g was fresh meat; 10 mg/100 g $<$ TVB-N value $<$ 20 mg/100 g was aged meat; 20 mg/100 g $<$ TVB-N value was spoiled meat.

Sensory evaluation (scoring test) shall be conducted according to the sensory index of Chinese meat hygiene standard GB 2707–2016. The physical properties like smell, color, elasticity and physical state were assessed by sensory evaluation. The judgment criteria of freshness: sensory score $\geq$90 was fresh meat; 90 $>$ sensory score $\geq$65 was aged meat; sensory score $<$ 65 was spoiled meat.

### 2.9. Evaluation of the nutritional components of pork

Evaluation of the nutritional components of pork including amino acid, fatty acids, lauric acid, and peptides content were also performed. LC–MS targeted metabolomics analysis of pork, significantly different small molecules or peptides of MTHJ extract-treated-group were tested. MS/MS data analyses have been performed as described of non-target metabolomics analysis. Sample separation was performed using $C_{18}$ column and HILIC column.

### 2.10. Statistical analysis

All experiments were performed at least three times and the results are presented as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism 8.0 statistical software (GraphPad, La Jolla, CA, USA). Differences between groups were analyzed by ANOVA and Student's t-test. A *P*-value of less than 0.05 was considered statistically significant.

## 3. Results and discussion

### 3.1. Phytochemical investigation of the MTHJ extracts

The total ion chromatograms (TICs) of MTHJ extracts in +ESI modes are shown in Fig. 2. Extracts of different solvent from the same species have a clear difference, MTHJ fractions of different eluants also show large differences in TICs. A total of 21 compounds were identified and quantified in MTHJ-60 % of the best antimicrobial activity using QTOF-MS/MS (shown Fig. 2, Table 1 and Table 2). Aligning all peaks and export them for principal component analysis (PCA). The figure includes two principal components (pc 1 and pc 2). As shown in the score plots (Fig. 3), the fraction MTHJ-60 % with the best antibacterial activity showed great differences compared with other fractions. In Fig. 3C, we marked and found the biomarkers include Schinifoline **(12)**, γ-Fagarine **(8)**, (*2E,7E,9E*)-6 S-

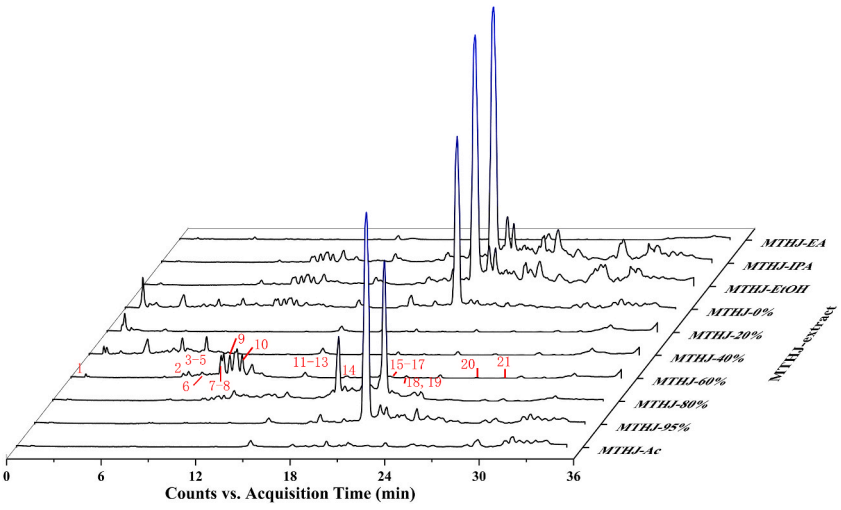


**Fig. 2.** The total ion chromatograms (TICs) of MTHJ extracts and fractions.

**Table 1**
Chemical profiles of MTHJ extracts under UPLC-Q-TOF/MS investigation.

| Peak | RT[a](time) | ESI mode | *m/z* | MS/MS Fragment ions | Molecular Formula | Compounds | Reference |
|---|---|---|---|---|---|---|---|
| 1 | 0.71 | $[M+H]^+$ | 203.0339 | 203.0304, 175.1104, 159.0439,131.0464, | C11H6O4 | Xanthotoxol | standard |
| 2 | 10.23 | $[M+H]^+$ | 247.0965 | 213.1628,161.0951,121.0644, 81.0681 | C14H14O4 | Nodakenetin | standard |
| 3 | 10.24 | $[M+H]^+$ | 229.0859 | 214.0607, 197.0582, 187.0743, 157.0692 | C14H12O3 | Xanthyletin | standard |
| 4 | 10.61 | $[M+H]^+$ | 264.1958 | 145.0646, 119.0861, 98.9737, 84.9589 | C16H25NO2 | Alpha-Hydroxy-Sanshool | standard |
| 5 | 10.86 | $[M+H]^+$ | 282.2064 | 167.1275, 133.0986, 57.0694 | C16H27NO3 | (*2E,7E,9E*)-10,11-Dihydroxy-*N*-(2-methylprooyl)-2,7,9-dodecatrienamide | standard |
| 6 | 11.63 | $[M+H]^+$ | 278.1751 | 214.1221, 196.1116, 156.0797, 83.0485 | C16H23NO3 | (*2E,7E,9E*)-*N*-(2-Hydroxy-2-methylpropyl)-6,11-dioxo-2,7,9-dodecatrienamide | standard |
| 7 | 12.46 | $[M+H]^+$ | 260.0917 | 245.0680,227.0582, 162.9844, 132.9728 | C14H13NO4 | Skimmianine | standard |
| 8 | 12.79 | $[M+H]^+$ | 230.0812 | 215.0562,198.0126, 186.0529, 113.9397 | C13H11NO3 | $\gamma$-Fagarine | standard |
| 9 | 13.80 | $[M+H]^+$ | 231.1016 | 175.0361, 147.0442, 91.0546 | C14H14O3 | Demethylsuberosin | standard |
| 10 | 14.32 | $[M+H]^+$ | 245.1172 | 219.0647,215.0685,191.0703 | C15H16O3 | Suberosin | standard |
| 11 | 18.24 | $[M+H]^+$ | 280.1907 | 222.1489, 205.0858, 117.0679 | C16H25NO3 | (*2E,7E,9E*)-6 S-Hydroxy-*N*-(2-methylpropyl)-11-oxo-2,7,9-dodecatrienamide | standard |
| 12 | 18.47 | $[M+H]^+$ | 258.1852 | 187.0915, 174.0839, 159.0614, 132.0535 | C17H23NO | Schinifoline | standard |
| 13 | 18.95 | $[M+H]^+$ | 288.1131 | 280.1902, 246.1846, 184.1328 | C18H13N3O | Rutaecarpine | standard |
| 14 | 19.76 | $[M+H]^+$ | 217.0495 | 202.0233, 174.0284, 118.0392, 90.0451 | C12H8O4 | 8-Methoxypsoralen | standard |
| 15 | 21.13 | $[M+H]^+$ | 248.2073 | – | – | – | unknown |
| 16 | 21.18 | $[M+H]^+$ | 389.1595 | 348.1224,258.1840,157.0162 | C21H24O7 | (+)-Syringaresinol | standard |
| 17 | 21.96 | $[M+H]^+$ | 324.1707 | 669.3167, 256.1087 | C19H21N3O2 | Neoechinulin A | standard |
| 18 | 22.42 | $[M+H]^+$ | 290.2115 | 274.2166,262.1803,210.1483 | C18H27NO2 | Galma- Hydroxy-Sanshool | standard |
| 19 | 22.62 | $[M+H]^+$ | 348.1236 | 332.0563, 318.0405, 304.0614, 290.0459 | C21H18NO4+ | Nitidine | standard |
| 20 | 26.41 | $[M+H]^+$ | 309.1333 | 235.0571, 219.0624, 205.0470, 191.0676 | C16H20O6 | Toddalolactone | standard |
| 21 | 29.60 | $[M+H]^+$ | 296.222 | 278.1737,205.0841,95.0476 | C17H29NO3 | (*2E,6E,8E*)-10-Methoxyl-11-hydroxy-*n*-(2-methylpropyl)-2,6,8-dodecatrienamide | standard |

[a] RT:Retention time.

Hydroxy-*N*-(2-methylpropyl)-11-oxo-2,7,9-dodecatrienamide **(6)**, and Neoechinulin A **(17).** The identified penylpropanoids can be grouped two types: coumarins and lignans. According the literature and standard subatances, coumarins of breaking behavior alway generating the corresponding high-abundance fragment ions under the collision-induced dissoxiation: $[M + H–CO_2]^+$, $[M + H–CO–CO]^+$, $[M + H–CO]^+$, $[M + H–CH_3]^+$, and $[M + H–2CH_3]^+$ [1]. For instance, the main ions found on the mass spectra of the 8-Methoxypsoralen (**14**) was seen at *m/z* 217 $[M+H]^+$ and *m/z* 202 $[M + H–CH_3]^+$. In addition, there are five types alkaloids identified, including amines, quinolines, protoberberine, indoles, and amides. Such as $\gamma$-Fagarine **(8)** belongs to quinolines, the $MS^2$ spectrum shows methoxy substituents fragmentations producing $[M + H–CH_3]^+$ and $[M + H–OCH_3]^+$ ions, at *m/z* 215.0562 and 200.0322. Besides, the $MS^2$ spectrum of the Nitidine **(19)** shows methoxy substituents fragmentations producing $[M - CH_4]^+$ and $[M\text{-}2CH_3]^+$ ions at *m/z* 332.0563 and 318.0405. According to other fragment ions and literature reports, it can be determined that it is $\gamma$-Fagarine **(8)** and Nitidine **(19)**[24].

### 3.2. *In vitro antimicrobial activity of MTHJ*

The antibacterial activity of ten MTHJ fractions (three solvent extracts and seven fractions) were carried out to determine the MIC. The antibacterial activity of fractions was assessed against potentially pathogenic microorganisms including bacterial, multidrug-resistant bacterial, and fungal strain. Table 3 shows that MTHJ fractions had the inhibition effect on *S. aureus*, MRSA and *C. albicans*, and as good as positive control (ampicillin sodium salt, fluconazole, and berberine), especially MRSA and *C. albicans*. When the concentration of MTHJ-60 % was higher than 1.024 mg/mL, MTHJ-60 % (pH 7.2) had potential to eliminate Gram-positive bacteria and fungus. According to the results, the MIC or MFC against *S. aureus*, MRSA, and *C. albicans* was in the range of 0.256–1.024 mg/mL. MTHJ fractions, especially MTHJ-60 % showed a better broad-spectrum antimicrobial activity than those of other three positive controls. Despite the chemical composition of extracts using three solvents and seven fractions are significant differences (Fig. 3 A and B). In total, 21 major compounds (standard compounds) had varying *anti*-MRSA and *anti-C. albicans* activities

**Table 2**
Content of the major conpounds in different solvent extracts of MTHJ and different components of EtOH extract segmented by food grade D101 macroporous resin by UHPLC-ESI-HRMS/MS (μg/g of dry extract).

| Compounds/No | MTHJ-EA | MTHJ-EtOH | MTHJ-IPA | MTHJ-0% | MTHJ-20 % | MTHJ-40 % | MTHJ-60 % | MTHJ-80 % | MTHJ-95 % | MTHJ-Ac |
|---|---|---|---|---|---|---|---|---|---|---|
| **Xanthotoxol (1)** | 0.16 ± 0.06 | 0.12 ± 0.04 | 0.14 ± 0.02 | – | 0.18 | – | 0.20 | 0.11 | 0.14 | – |
| **Nodakenetin (2)**[a] | 105.77 ± 3.3 | 125.26 ± 8.4 | 104.1 ± 4.2 | 0.18 | – | 16.64 | 281.11 | 1.8 | – | – |
| **2-Chloroacetanilide (3)**[a] | 2.76 ± 1.6 | 3.77 ± 0.4 | 3.45 ± 0.3 | – | – | 5.28 | 9.2 | – | – | – |
| **Alpha-Hydroxy-Sanshool (4)** | 9953.36 ± 66 | 9021 ± 52 | 9358 ± 59 | 4.37 | 47.53 | 28127.7 | 3147 | 63.84 | 9.33 | – |
| **(*2E,7E,9E*)-10,11-Dihydroxy-*N*-(2-methylprooyl)-2,7,9-dodecatrienamide (5)**[d] | 149.51 ± 23.7 | 134.13 ± 42.38 | 88.31 ± 6.5 | – | – | 248.30 | 61.0 | – | – | – |
| **(*2E,7E,9E*)-*N*-(2-Hydroxy-2-methylpropyl)-6,11-dioxo-2,7,9-dodecatrienamide (6)** | 8327.31 ± 45.1 | 1410.33 ± 99.3 | 7086.45 ± 69.2 | 6.32 | – | 4124.99 | 4694.74 | 105.58 | 28.29 | – |
| **Skimmianine (7)**[c] | 0.20 ± 0.03 | – | 0.22 ± 0.01 | 0.45 | 0.15 | 0.18 | 0.53 | – | – | – |
| **γ-Fagarine (8)** | 0.22 ± 0.06 | 0.55 ± 0.08 | 0.28 ± 0.03 | 0.18 | – | – | 0.36 | – | – | – |
| **Demethylsuberosin (9)**[a] | 3.10 ± 0.3 | – | 2.19 ± 0.3 | – | – | – | 2.2 | – | – | – |
| **Suberosin (10)** | 2.39 ± 0.42 | – | 1.28 ± 0.87 | – | – | – | 4.87 | – | – | – |
| **(*2E,7E,9E*)-6*S*-Hydroxy-*N*-(2-methylpropyl)-11-oxo-2,7,9-dodecatrienamide (11)** | 4641.95 ± 112.6 | 3509.62 ± 88.3 | 2932.48 ± 159.3 | – | – | – | 27414.15 | 3775.94 | 46.41 | 6.56 |
| **Schinifoline (12)** | 3.43 ± 0.13 | 5.73 ± 0.74 | 3.47 ± 1.16 | 0.5 | 0.49 | 1.37 | 6.52 | – | 0.40 | – |
| **Rutaecarpine (13)**[e] | 437.92 ± 8.9 | 55.54 ± 12 | 360.68 ± 20.6 | 37.0 | 11.79 | 3.95 | 5.79 | 38.57 | 172.93 | 46.39 |
| **8-Methoxypsoralen (14)**[a] | 17.48 ± 0.83 | 2.56 ± 0.94 | 14.13 ± 0.78 | – | – | – | – | 0.87 | 5.68 | 2.76 |
| **(+)-Syringaresinol (16)**[a] | 3,13 ± 1.1 | – | 1.04 ± 0.5 | – | – | – | – | 0.40 | 0.84 | 0.40 |
| **Neoechinulin A (17)**[e] | – | 0.61 ± 0.36 | 0.44 ± 0.22 | – | – | – | 0.2 | – | – | – |
| **Galma- Hydroxy-Sanshool (18)**[b] | 4750.38 ± 32.64 | 459.68 ± 93.33 | 4019.28 ± 68.1 | – | – | – | – | 2217.41 | 178.48 | – |
| **Nitidine (19)** | 27.73 ± 1.1 | 2.81 ± 0.9 | 21.53 ± 0.59 | – | – | – | – | 1.20 | – | 5.82 |
| **Toddalolactone (20)**[a] | 1.87 ± 0.05 | 5.24 ± 0.01 | 1.41 ± 0.3 | – | – | – | – | – | 1.15 | – |
| **(*2E,6E,8E*)-10-Methoxyl-11-hydroxy-*N*-(2-methylpropyl)-2,6,8-dodecatrienamide (21)**[d] | 111.12 ± 6.56 | 9.98 ± 0.82 | 82.91 ± 6.91 | – | – | – | – | – | 23.32 | – |

-: means that the compound was not present in the extract. a: This compound was semi-quantified by xanthotoxol. b: This compound was semi-quantified by alpha-hydroxy-sanshool. c: This compound was semi-quantified by γ-fagarine. d: This compound was semi-quantified by (*2E,7E,9E*)-6 S-hydroxy-*N*-(2-methylpropyl)-11-oxo-2,7,9- dodecatrienamide. e: This compound was semi-quantified by nitidine.

(shown in Table 3), MICs of Schinifoline **(12)**, γ-Fagarine **(8)**, (*2E,7E,9E*)-6 S-Hydroxy-*N*-(2-methylpropyl)-11-oxo-2,7,9-dodecatrienamide **(11)**, and Neoechinulin A **(17)** were 20–128 μg/mL. Of concern is that the Schinifoline **(12)** was the characteristic component of different MTHJ extracts and fractions (Table 2 and Fig. 3C), which was the major compound in MTHJ-60 % (the fraction of the best antimicrobial activity). This study shows that the method of combining analysis and antibacterial activity can effectively obtain active compounds of *Z. motuoense*.

Amides and alkaloids possess a natural *anti*-biofilm activity mainly due to the electrostatic interaction between their cationic amino groups and anionic extracellular polymeric substance (EPS), thus resulting in changes in membrane permeability and damage of its barrier properties. Coincidentally, amides and alkaloids are the main characteristic constituents of the MTHJ-60 %. To investigate the affinity between MTHJ-60 % and bacterial biofilm, preformed *C. albicans* biofilm was incubated with MTHJ extract, then washed and collected for fluorescence intensity quantification or qualitative analysis. As shown in Fig. 4, bioslime film incubated with MTHJ extract showed a strong fluorescence intensity demonstrating that MTHJ-60 % can anchor to bacteria bioslime film and significantly decreased biofilm biomass (Fig. 4 A, B, C). Therefore, disruption of biofilm would reduce the risk of generating antimicrobial

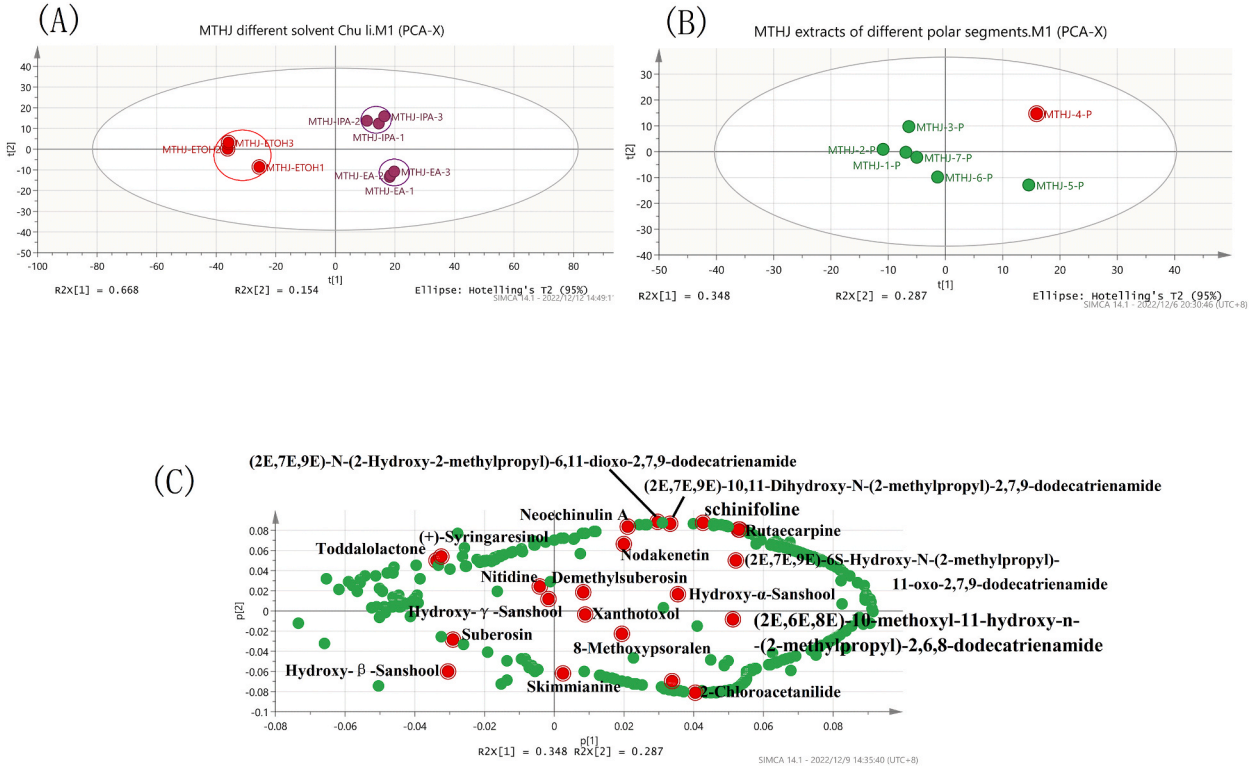


**Fig. 3.** Main different metabolites among MTHJ extracts. Score plot of different solvent extracts (A) and different fractions (B). (C) Loading plot. Each point represents the mean of three extracts duplication.

**Table 3**
Antimicrobial activity of MTHJ extracts/fractions.

| fractions/compounds | C. albicans ATCC 14053 | | MRSA 3 | | *S. aureus* ATCC 25923 | |
|---|---|---|---|---|---|---|
| | MIC mg/ml | MFC mg/ml | MIC mg/ml | MBC mg/ml | MIC mg/ml | MBC mg/ml |
| MTHJ-EtOH | 0.512 | 1.024 | 0.512 | 1.024 | 0.512 | > 1.024 |
| MTHJ-IPA | 1.024 | > 1.024 | > 1.024 | > 1.024 | > 1.024 | > 1.024 |
| MTHJ-EA | 1.024 | > 1.024 | 1.024 | > 1.024 | > 1.024 | > 1.024 |
| MTHJ-0% | 0.512 | > 1.024 | > 1.024 | > 1.024 | > 1.024 | > 1.024 |
| MTHJ-20 % | 0.512 | > 1.024 | 0.512 | 1.024 | 1.024 | > 1.024 |
| MTHJ-40 % | 0.512 | 1.024 | 0.512 | 1.024 | 1.024 | 1.024 |
| MTHJ-60 % | 0.256 | 0.512 | 0.512 | 0.512 | 0.512 | 1.024 |
| MTHJ-80 % | 0.512 | 0.512 | 1.024 | > 1.024 | 1.024 | > 1.024 |
| MTHJ-95 % | 0.512 | > 1.024 | > 1.024 | > 1.024 | > 1.024 | > 1.024 |
| MTHJ-Ac | > 1.024 | > 1.024 | > 1.024 | > 1.024 | > 1.024 | > 1.024 |
| Fluconazolea | 0.128 | > 0.256 | – | – | – | – |
| Vancomycina | – | – | 0.004 | 0.008 | 0.001 | 0.008 |
| Ampicillina | – | – | 0.512 | 1.024 | 0.002 | 0.008 |
| Berberinea | > 1.024 | > 1.024 | 0.512 | 1.024 | 0.512 | 1.024 |
| Schinifolineb | 0.02 | 0.04 | 0.02 | 0.02 | 0.02 | 0.04 |
| γ-Fagarineb | 0.128 | 0.256 | 0.128 | 0.256 | 0.128 | 0.128 |
| Neoechinulin A | 0.064 | 0.128 | 0.128 | 0.128 | 0.064 | 0.256 |

- mean MIC was significantly greater than 2.048 mg/mL.
a positive controls of antimicrobial use.
b activity compounds in MTHJ extracts.

resistance.

*S. aureus* and MRSA are the main pathogens causing serious foodborne disease in human or livestock, and biofilms are most frequently formed in food-related environments[25]. MTHJ-60 % showed effect against MRSA bioslime films after 24 h treatment in contrast with blank control (Fig. 4 B, D), which means that MTHJ extracts as flavoring agent could be used as natural preservatives in the food industry to reduce bacterial growth, especially resistant bacteria.

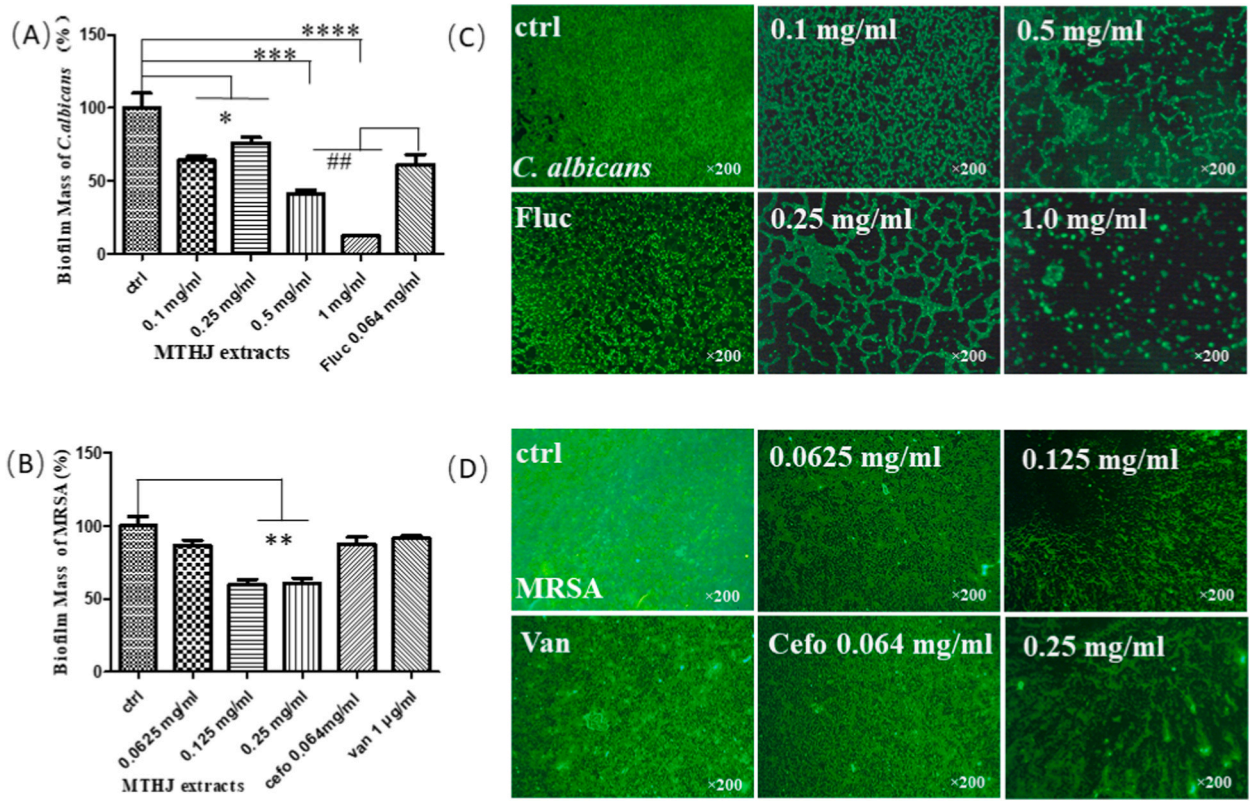


**Fig. 4.** Biofilms formed by *C. albicans* and MRSA were exposed to MTHJ extracts (MTHJ-60 %). (A) The biofilm mass of *C. albicans* was determined by the crystal violet assay (OD at 595 nm). (B) The biofilm mass of MRSA was determined by the crystal violet assay (OD at 595 nm). (C) Fluorescence images of the *C. albicans* biofilm. (D) Fluorescence images of the MRSA biofilm. Values are mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 versus controls (ctrl); ##P < 0.01 versus fluconazole (fluc) or vancomycin (van). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### 3.3. Anti-food spoilage activity in food industry

Pork is perishable due to oxidation and microbial spoilage[26]. However, the fresh pork contains large numbers of bacteria and provide the nutrients needed for the microorganisms to sustain themselves, survive, and grow (lg CFU/g ~4, in Fig. 5 A, 0-day). The microorganism factors are highly influential for meat flavor, but foodborne pathogens, such as *S. aureus* and *C. albicans* should be inhibited selectively. A total number of colonies in pork are shown in Fig. 5, during the storage time, the sum total of bacterial count of all the samples in each group showed a trend of total biomass gain. The number of colonies in the MTHJ 60%-treated-group (MTHJ-1 mg/mL, MTHJ-10 mg/mL) was significantly less than controls from 3 to 10 days, and with a concentration-dependent manner. The corresponding characterized sensory score of MTHJ 60%-10 mg/mL treated-group are greater than 90 within 10 days, which was counted as a satisfactory flavor and quality in meat and marinated meat. The sensory score of MTHJ 60%-1 mg/mL treated-group are greater than 80 within 3 days, and greater than 75 within 10 days, which was counted as aged meat. PBS treated-group (ctrl) was counted as spoiled meat (sensory score ≤60) from 3 to 10-days (Fig. 5 B, C). Pork quality of smell, color, elasticity and physical state becomes worse (Fig. 5 D), low water content, loose muscular tissue, and juiciness scores (red arrow) of meats decreased significantly. The TVB-N value of ctrl group significantly increased, from 0.03 to 32.38 mg/100 g, which was identified as the smell of rotting flesh. However, The TVB-N values of MTHJ-1 mg/mL and MTHJ-10 mg/mL group were less than 10 mg/100 g, these tissues do dehydrate and muscle fibre do form a wrinkled structure. MTHJ 60 % has been applied to inhibit the growth of resistant pathogens in pork and decrease the risk of contracting foodborne illness, confirming ability to resist foodborne drug-resistant bacteria and potential in food preservation.

### 3.4. Different nutritional components of pork

Deterioration of pork can cause the breakdown, destruction, and loss of its main components such as protein and fat, seriously reducing its nutritional value. As we can see, there were significant differences in amino acids, peptides, and lipids among different groups. The contents of *N*-palmitoyl phenylalanine, Val Pro Arg, Val Leu Pro, Pro Ala Tyr, Ala Thr Ile, Phe Thr Lys, and Arg Thr Trp in MTHJ-treated pork were significantly higher than those in the fresh and PBS-treated group (shown in Fig. 6). Values of *N*-palmitoyl

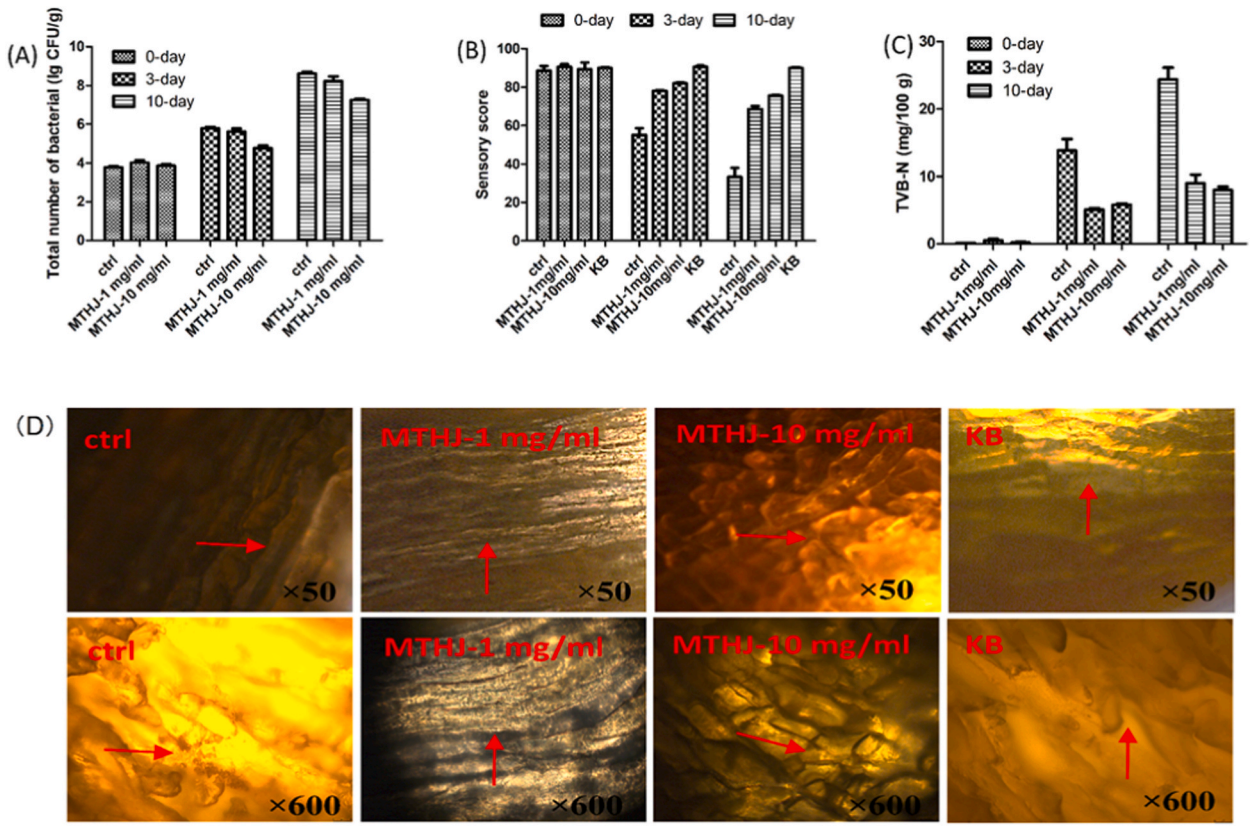


**Fig. 5.** Evaluation of anti-food spoilage activity. (A) MTHJ extracts reduced bacterial count to preserve pork. (B) Sensory score. (C) TVB-N values. (D) The microscope images scales.

phenylalanine, Val Pro Arg, Val Leu Pro, Pro Ala Tyr, Ala Thr Ile, Phe Thr Lys, and Arg Thr Trp showed a 6.5 to 20-fold increase. The nutritional components of PBS-treated pork were striking differences vs fresh pork, this result strongly implies that the effect on microorganism growth was highly significant. Lauric Acid, Gly Tyr Lys, *N*-palmitoyl glutamic acid, and Ser His Ser were reduced 6-fold. The results showed that the nutritional contents of PBS treatment group decreased.

## 4. Conclusions

Developing food preservation for meat and meat products from edible plants in the future will probably be a challenging work[27, 28]. Meat flavour in pork, quality and safety are paramount to the food industry. The obtained results in this study showed that the MTHJ-60 % as flavoring agent, exhibited strong antimicrobial ability, especially foodborne pathogen against *S. aureus*, MRSA, and *C. albicans*. Those pathogens also as the most important microbial contaminations cause meat spoilage. The results indicated that the MTHJ-60 % fraction was good to inhibit the microbial biofilm and antimicrobial much better t other fractions, which were consistent with the analysis of their Schinifoline **(12)**, $\gamma$-Fagarine **(8)**, (*2E,7E,9E*)-6 S-Hydroxy-*N*-(2-methylpropyl)-11-oxo-2,7,9-dodecatrienamide **(6)**, and Neoechinulin A **(17)**. Our results confirmed that MTHJ-60 % can potentially be a good preservative to prevent meat deterioration and corruption, and can replacing some synthetic additives which may have negative impact on human health if consumed continuouly and frequently. These results not only would account for the scientific knowledge for the traditional application of Tibetan prickly ash but also provide a raliable theoretical foundation for the further development of antimicrobial activity and the use of food preservation. There are quite a few interesting questions from theory to practical application, and We look forward to future research of the peer study that further application.

## Supporting information

MRSA was exposed to MTHJ extracts (MTHJ-60 %), and SEM observation shown in Fig. S1. The major compounds structure in different solvent extracts of MTHJ were shown in Fig. S2. The standard curve presented of standard compounds by UHPLC-DAD-MS, and were shown in Table S1. Yeilds of MTHJ fractions were shown in Table S2.

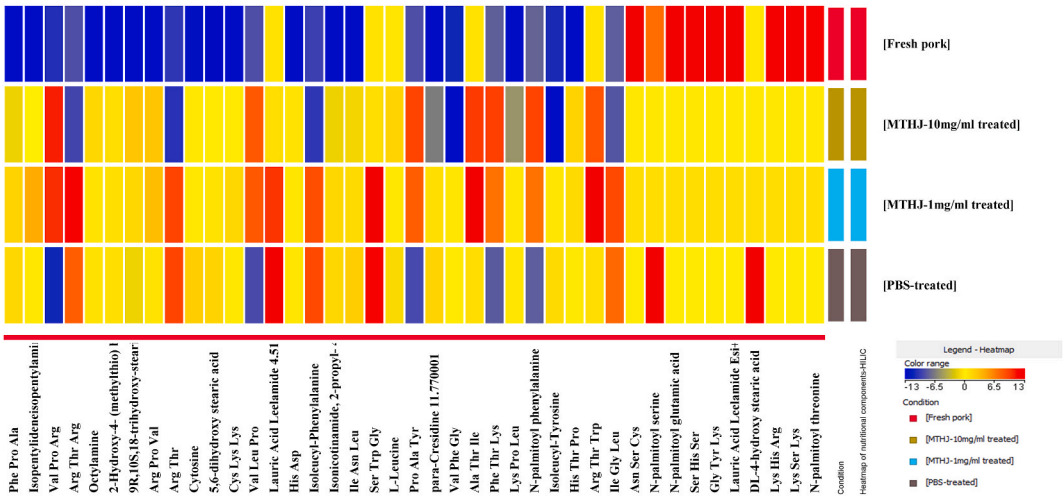


**Fig. 6.** Heatmap of nutritional components, MTHJ-treated and PBS-treated vs fresh pork.

## CRediT authorship contribution statement

**Zhao-Jie Wang:** Writing – original draft. **Huan Huang:** Data curation. **Yan-Yan Zhu:** Formal analysis. **Zhong-Shun Zhou:** Methodology. **Tie Liu:** Resources. **Xing-Chao He:** Software. **Tie-Li Zhang:** Methodology. **Xiao-Dong Luo:** Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22963.

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