The correlation between antioxidant, anti-inflammatory activity and the composition of free and bound phenolics in Chinese olive cultivars

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Research Article

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Abstract

As the food of homologous of medicine and food in China, the Chinese olive (*Canarium album* L.) is a natural phenolic antioxidant and anti-inflammatory agent. However, the disparity of anti-inflammatory activity in different cultivars and existing forms may be due to peculiar phenolic compounds that are not yet well-explored in the literature. In this study, 44 phenolic compounds were found in three species of free phenolics (FP) and bound phenolics (BP) and 17 phenolic compounds including syringic acid, caffeic acid, esculetin and so on were first reported in Chinese olive. Among cultivars, 'Tan xiang' and 'Na zhong' showed the best antioxidant and inhibition (LPS-induced NO and TNF-α production) activities in FP and BP separately. Furthermore, clustering correlation analysis showed that gallic acid, catechin, syringic acid and nobiletin contribute similarly, galloyl-bis-HHDP-glucose, epigallocatechin, dihydrokaempferol, genistein, galloylquinic acid, 4-hydroxycinnamic acid, and methyl ellagic acid pentoside may cause discrepancies to inflammatory effects in FP and BP. Collectively, our study uncovered the relationship between the constitution of phenolic compounds and bioactivities in cultivars of Chinese olives.

Introduction

As ubiquitous secondary metabolites in plants and have important beneficial effects on humans[1], phenolic compounds(PC) have more than 8,000 structures and are known to be widespread in the form of free phenolics (FP: phenolics that present in free forms within the plant cell vacuole) and bound phenolics (BP: phenolics that are linked with the insoluble macromolecule substrates of the cell wall in plant food matrix through chemically covalent bonds, hydrogen bonding, and hydrophobic interactions) [2]. However, the composition and content of FP and BP in plants are not identical, giving rise to variations in their pharmacological effects. This divergence further highlights the importance and value of studying these compounds [3].

Chinese olive (*Canarium album* L.) is a fusiform drupe fruit belonging to the *Burseraceae* family. It originated in southeastern China and is rich in varieties, especially in Guangdong Province, with more than 63 species. As one of 110 foods of homologous of medicine and food in China, Chinese olive has a long history of edible and medicinal use, which is different from the European olive (*Olea europaea* L.) used for oil extraction[4]. Traditional Chinese medicine calls it Qing guo and also believes that it can treat pharyngitis, faucitis, stomatitis, hepatitis and toxicosis, and interestingly, people in Guangdong Province add it to the soup to cure pharyngitis. Even though researchers revealed the free phenolic content is rich [5] and some common polyphenols in Chinese olive [6, 7], there is a lack of studies comparing the full individual phenolic profile of species, especially in BP.

Polyphenols can clear the contribution of inflammatory signaling by modifying the expression of several pro-inflammatory genes (lipoxygenase, nitric oxide synthases cyclooxygenase) [8]and exerting their antioxidant effects (interrupting the reactive oxygen species-inflammation cycle)[9]. Furthermore, the influence on anti-inflammatory activity may vary with changes in the composition of phenolic compounds, even the content remains the same[10]. However, the responsible factors for this

phenomenon and comparing the bioactivity of phenolic compounds combined with different forms are not yet well-explored in the literature, which is not conducive to the selection of functional products in cultivars.

This study aimed to identify and quantify the FP and BP in the composition of three commercially important Chinese olive varieties grown in China. The antioxidant activity was evaluated by three antioxidant methods and the anti-inflammatory capacities of FP and BP in lipopolysaccharide (LPS) induced oxidative macrophage injury were assessed using RAW264.7 cells.

Materials and methods

The materials and methods section are presented as Supplementary Information.

Results and Discussion

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) in Chinese olive species

The TPC and TFC for the fractions of 3 species of Chinese olive is shown in Table.1. Significant differences ($p < 0.05$) were found between the FP and BP of the three species. The study showed 'Tan xiang' had the highest TPC of FP at 70.94 ± 10.59 mg GAE/g DW, followed by 'Na zhong' and 'Xiang zhong'. However, the TPC of BP in 'Na zhong' is also the most in species, followed by 'Tan xiang', and the last is 'Xiang zhong'. The BP contributed to 9.47–15.65% of TPC and have no significant differences in all species. In FP of Chinese olive, TFC accounts for 59.18-61.00% of all species while having no significant differences. In all samples, the highest and lowest proportion were found in 'Na zhong' and 'Tan xiang', respectively. In the case of BP, significant differences in TFC content were found between all samples, where the variety 'Xiang zhong' showed the highest values and the highest percentage in TFC ratio TPC (89.89%).

Table.1 Fractions of phenolic compounds in Chinese olive species.

	Na zhong	Tan xiang	Xiang zhong
TPC of FP (mg GAE/g DW)	53.28 ± 1.13^{ab}	70.94 ± 7.49^{a}	51.44 ± 5.51^{b}
TFC of FP (mg RE/g DW)	32.50 ± 3.06 ^{a}	41.98 ± 3.86 ^{a}	30.80 ± 4.31 ^{a}
TPC of BP (mg GAE/g DW)	8.34 ± 0.83 ^{a}	6.72 ± 0.18 ^{a}	6.23 ± 0.67 ^{a}
TFC of BP (mg RE/g DW)	3.00 ± 0.27 ^{c}	3.80 ± 0.19 ^{b}	5.60 ± 0.13 ^{a}

Different lowercase letters in the same column indicate significant differences($p < 0.05$).

Generally, the TPC and TFC of FP in our study which is far fewer than those reported by Guo [11] and Liu[5] but similar to reported by Chang[12]. To our knowledge, this is the first time that bound phenolic content has been described in *Canarium album* L.

Identification and quantification of individual extractable phenolic compounds in Chinese olive species

Forty-four phenolic compounds were detected and 29 of which were confirmed by standards(Supplement Table 4) and the other peaks were tentatively identified by exact masses, MS/MS spectra and molecular formulas with previous results in the literature, the total ion chromatograms were presented in Fig. 1. Divided all the phenolic compounds into 7 groups to different polyphenol classes (hydroxybenzoic acids, hydroxycinnamic acids, isoflavones, flavones, flavonols, flavanols, other polyphenols and derivatives) and MS and MS/MS mass spectrometry information was shown in Supplement Table 1.

Figure 1 Total ion chromatograms of Chinese olive on UPLC-MS (A)Free phenolics (B)Bound phenolics

As shown in Supplement Table 2, 13 types of phenolic acids, 14 types of flavonoids and 11 other polyphenols were found in FP. The content of phenolic acids is abundant in the FP (64.94%-78.69% of the TPC). Quinic acid is the highest phenolic acid in Chinese olive (FP: 1229.69-1381.07mg/100g DW). Gallic acid is the third most abundant in FP (9.62-13.41mg/100g DW). The peak of ID 2 produced MS^2 fragment ions at 191.05504 m/z and 169.01312 m/z, suggesting that this is galloylquinic acid. Ulteriorly, the loss of 162 mass unit (331.06583-169.01259,329.08701-167.03391) could be considered hexoside, so the peak of ID 3 and ID 5 were tentatively identified as gallic acid hexoside and vanillic acid hexoside. Moreover, chlorogenic acid was found only in FP and significant differences were shown between the three species. In terms of flavones, flavones, flavanols and derivatives content, ‘Tan xiang’ showed significantly higher levels ($p < 0.05$) compared to other species, with values approximately twice as high as the rest.

Besides phenolic acids and flavonoids, there are also 11 non-flavonoid polyphenols present in Chinese olive that are considered vital to human health. It can be characterized that ellagic acid and its 9 derivatives, all of those can be quantified by the ellagic acid and corilagin analytical standard. The peak of ID 29 was identified as corilagin (Galloyl-HHDP-glucose) with a deprotonated ion at m/z 633.07343 $[M-H]^-$and had MS/MS fragments at m/z 300.99860 after the loss of an HHDP-group. A similar result was obtained when the peak of ID 30 showed the molecular ion peak at m/z 935.07928$[M-H]^-$. Furthermore, the loss of 15 mass unit (315.01434–300.99850), 176 mass unit(491.04654-315.01434), and 132 mass unit(447.05658–315.01450) could be considered methyl, glucuronoside, and pentoside respectively, so that the peak of ID 33,35 and 37 can be identified as ellagic acid pentoside, methyl-ellagic acid glucuronoside and methyl ellagic acid pentoside[13, 14]. The peak of ID 28(m/z 951.07397) and 32 (m/z 951.07397) were identified as geraniin isomer and geraniin due to their different retention times.

As shown in Supplement Table 3, 14 types of phenolic acids, 8 types of flavonoids, and 8 other polyphenols were found in BP. The specie of ‘Tan xiang’ showed significantly higher levels compared to

other species at the content of hydroxybenzoic acids and derivatives. But in the specie of ‘Na Zhong’, the amount of hydroxycinnamic, isoflavones, and other polyphenols and their derivatives present are outstanding. Furthermore, caffeic acid, esculetin, ferulic acid, genistein, amentoflavone, and dihydrokaempferol only be found in bound phenolics.

To our knowledge, gallic acid hexoside, vanillic acid hexoside, syringic acid, caffeic acid, esculetin, 4-hydroxycinnamic acid, isoferulic acid, astragalin, nobiletin, gallocatechin, procyanidin B1, galloyl-bis-HHDP-glucose, paeonol, ellagic acid pentoside, methyl-ellagic acid glucuronoside, methyl ellagic acid pentoside, and syringaldehyde were first reported in Chinese olive through our study. In this study, 15 and 6 phenolic compounds were only found in free and bound phenolics separately, suggesting that they may have the potential to modulate multiple health benefits in the gut microbiome and intestinal immune response for human health[3].

Antioxidant Capacity of Chinese olive

Past results showed the antioxidant activity of phenolic compounds was connected with TPC significantly. Therefore, this study measured the antioxidant activities of Chinese olive by DPPH(1,1-diphenyl-2-picrylhydrazyl), ABTS(2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate)), and ORAC assays in different species (Fig. 2). For DPPH radical scavenging activity, ‘Tan xiang’ and ‘Na zhong’ have the best antioxidant activity in FP and BP but no significant differences were found in FP and BP, respectively.Similarly, the results using the ABTS study, ‘Tan xiang’ also has the best antioxidant activity in FP but followed by ‘Na zhong’ and ‘Tan xiang’. Significant differences were observed between ‘Xiang zhong’ and other species. ORAC results showed that for FP of three species, ‘Tan xiang’ also has the best antioxidant activity and significant differences with other species. The antioxidant ability of ABTS assay and ORAC assay showed a similar trend to the DPPH assay in BP and ‘Na zhong’ was found similar significant differences with ‘Xiang zhong’ in both studies (ABTS assay and ORAC assay).

Anti-inflammation Capacity of Chinese olive

Cell viability was carried out to determine whether the extracts had cytotoxic effects on RAW264.7 cell and to further evaluate their anti-inflammation capacity (Fig. 3A-B). The concentration in 50μg/mL was designated after cell cytotoxicity assay (range from 25–200μg/mL).

We used NO assay and ELISA KIT(TNF-α, IL-6) to further explore the anti-inflammation capacity of phenolics [15] (Fig. 3C-E). All intervene groups of species significantly decreased NO production and cytokine secretion. BP experimental group was also lower than FP experimental group. In FP experimental groups of TNF-α and IL-6 assays, there was no significant difference between ‘Na zhong’ and ‘Tan xiang’, but both had extremely significant differences ($p < 0.01$) from the ‘Xiang zhong’. In the case of BP experimental groups, there was no significant difference in TNF-α assay, ‘Na zhong’ and ‘Tan xiang’ were significantly lower than ‘Xiang zhong’ in the production of IL-6. Strikingly, our data demonstrate that the ability of BP to inhibit cytokine production (TNF-α, IL-6) was greater than FP in ‘Na zhong’ and had significant differences($p < 0.01$) separately.

Figure 3 Anti-inflammatory effect of Chinese olive extracts on RAW264.7 cells. (A) Effects of FP on cell viability. (B) Effects of BP on cell viability. (C) NO production. (D)TNF-α production. (E)IL-6 production. Different lowercase letters in the same column indicate significant differences($p < 0.05$) between Chinese olive species.

Multivariate data analysis

The (dis)similarities among the different cultivars were well visualized by the Partial least squares discriminant analysis (PLS-DA) score plot and clustering heatmap (Fig. 4A), that concerning the most influential variables which together explain 90.60% of the total variance separately and show how the samples are grouped in terms of their cultivars. The indicating decent stability and predictive power of the model were established by an R^2 (0.966) and Q^2 (0.841). A higher variable importance in projection (VIP) indicates a higher contribution of the explanatory variables and higher differential correlation. VIP > 1 was used as the screening criteria in this study to obtain isoquercitrin, kaempferol 3-O-glucoside, vanillic acid hexoside, methyl-ellagic acid glucuronoside, galloyl-bis-HHDP-glucose, genistin, hyperoside, epicatechin, procyanidin B1, procyanidin B2, dihydrokaempferol, gallocatechin, chlorogenic acid, genistein, ellagic acid glucuronoside, galloylquinic acid, gallic acid hexoside, and esculetin which may potentially be the main factors causing the difference in cultivars among the free and bound phenolics of three species, serving as indicative markers to a certain extent.

The correlation between the phenolic compounds and the bioactivity was determined using the Spearmans correlation (Fig. 4B-C). Obviously, ellagic acid and derivatives showed a strong positive correlation with bioactivity due to the presence of many phenolic hydroxyl groups in their structures [16] [17]. In FP, 4-hydroxybenzoic acid, hyperoside, 4-hydroxycinnamic acid, isoflavones, flavonols, and derivatives and paeonol showed significant positive correlation in the antioxidant and anti-inflammatory activity respectively. Our study revealed that all of those phenolic compounds in ‘Tan xiang’ exhibited significantly higher concentrations compared to other cultivars by antioxidant assays. In BP, six phenolic compounds showed a significant positive correlation as the potential anti-inflammatory active components, suggesting their potential anti-inflammatory properties [18, 19]. When focusing on the activity of inhabit in FP and increase in BP, study found gallic acid, catechin, syringic acid and nobiletin have the same trend of cytokine production but the results were opposite in corilagin and methyl ellagic acid pentoside. Combined with the results of anti-inflammation capacity, elucidated that the anti-inflammatory capacity of BP are superior to that of FP at the same dose. This result suggested the anti-inflammatory of free and bound phenolics may correlated with other factors. On the one hand, bioactivities of phenolic compounds may be influenced by their forms. Galloyl-bis-HHDP-glucose, epigallocatechin and dihydrokaempferol and genistein were only found in FP and BP respectively, they showed a significant correlation with inflammation. On the other hand, the interaction between different forms of phenolic compounds cannot be neglected. Likewise, galloylquinic acid, 4-hydroxycinnamic acid, and methyl ellagic acid pentoside significantly inhibited the production of IL-6, not bound phenolics.

Conclusion

This study identified 44 phenolic compounds in Chinese olives and 18 phenolic compounds are the main factors causing differences in cultivars. The advantages of different cultivars were cleared, such as ‘Tan xiang’ in FP showed the highest TPC and antioxidant activity, and ‘Na zhong’ in BP showed the best inhibition in NO and TNF-α production. Furthermore, the bioactivity of phenolics in Chinese olive may be influenced by those special phenolics that only exist in some form or are synergistic with unclear phenolics. In conclusion, this study provides evidence of the relevant differences in the characterization of phenolic compound content and profile in different cultivars and proposes phenolic compounds that may be associated with inflammation reduction.

Abbreviations

FP: free phenolics; BP: bound phenolics; PC: phenolic compounds; LPS: lipopolysaccharide; TPC: total phenolic content; TFC: total flavonoid content; UPLC-Q-Exactive Orbitrap-MS: UPLC-MS; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate); ORAC: Oxygen Radical Absorbance Capacity; PLS-DA: Partial least squares discriminant analysis; VIP: variable importance in projection.

Declarations

Author Contributions Fangqing He: Methodology, Investigation, Writing. **Yixuan Du:** Formal analysis. **Zhuangguang Pan:** Data curation. **Huize Zeng:** Software. **Haolin Luo:** Visualization. **Junyi Wang:** Resources. **Yuanming Sun:** Supervision, Conceptualization. **Meiying Li:** Funding acquisition, review & editing.

Data availability No data was used for the research described in the article.

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Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Figures

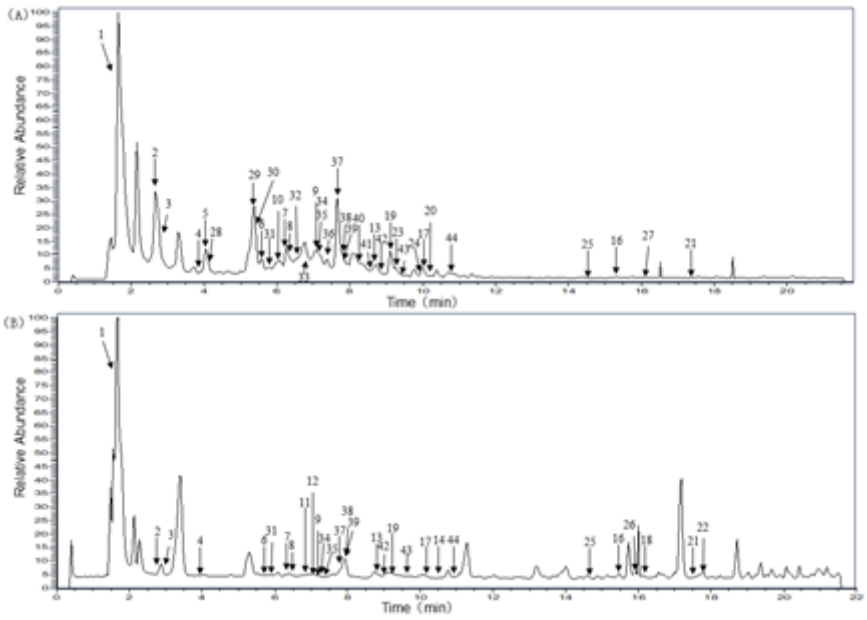

Figure 1

Total ion chromatograms of Chinese olive on UPLC-MS (A)Free phenolics (B)Bound phenolics

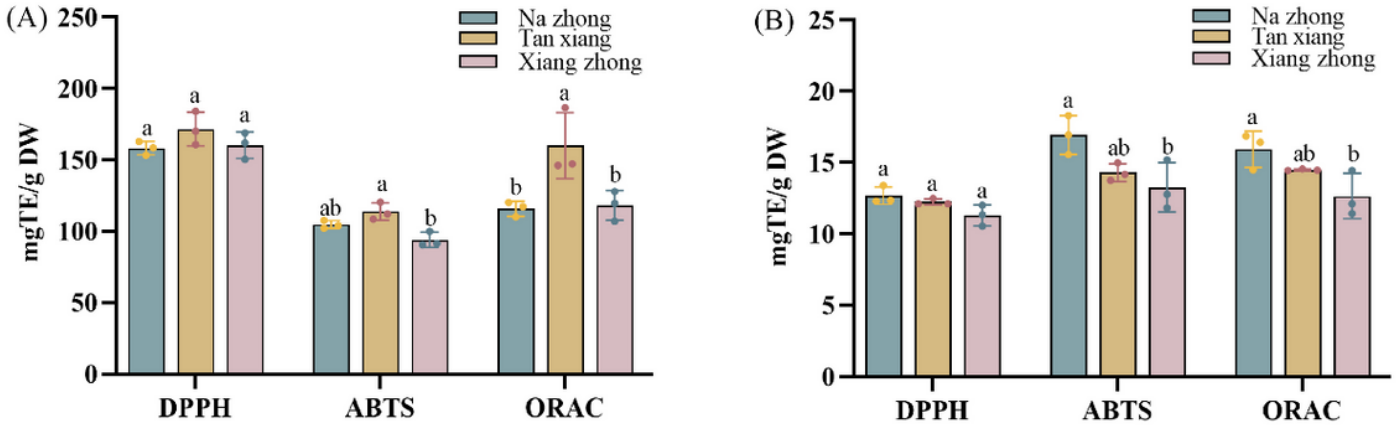

Figure 2

(A)The antioxidant capacity of free phenolics in Chinese olive species. (B)The antioxidant capacity of bound phenolics in Chinese olive species. Values represent mean ±standard deviation(n=3). Different lowercase letters in the same column indicate significant differences(p<0.05) between Chinese olive species.

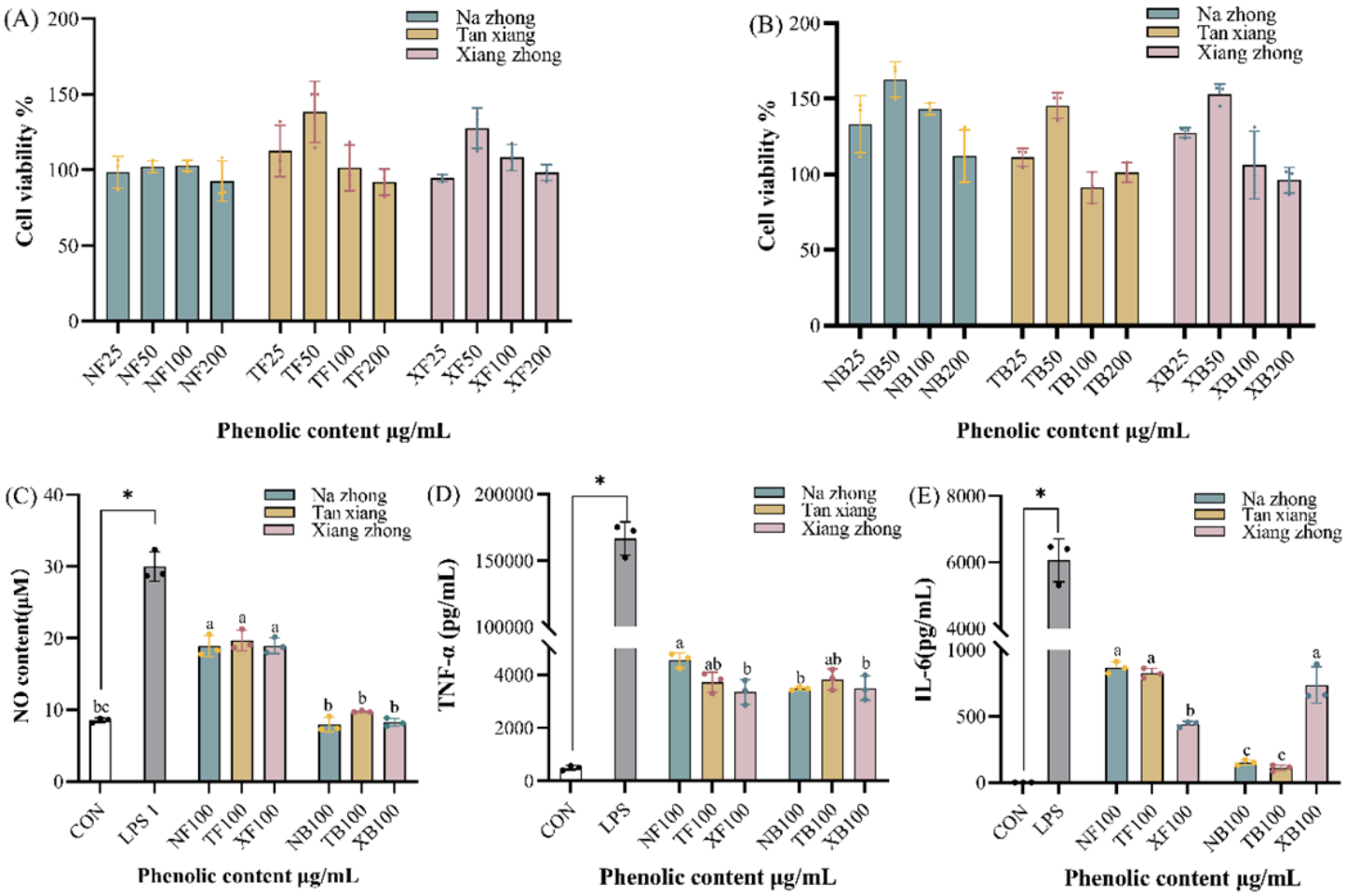

Figure 3

Anti-inflammatory effect of Chinese olive extracts on RAW264.7 cells. (A) Effects of FP on cell viability. (B) Effects of BP on cell viability. (C) NO production. (D)TNF-αproduction. (E)IL-6 production. Different lowercase letters in the same column indicate significant differences(p<0.05) between Chinese olive species.

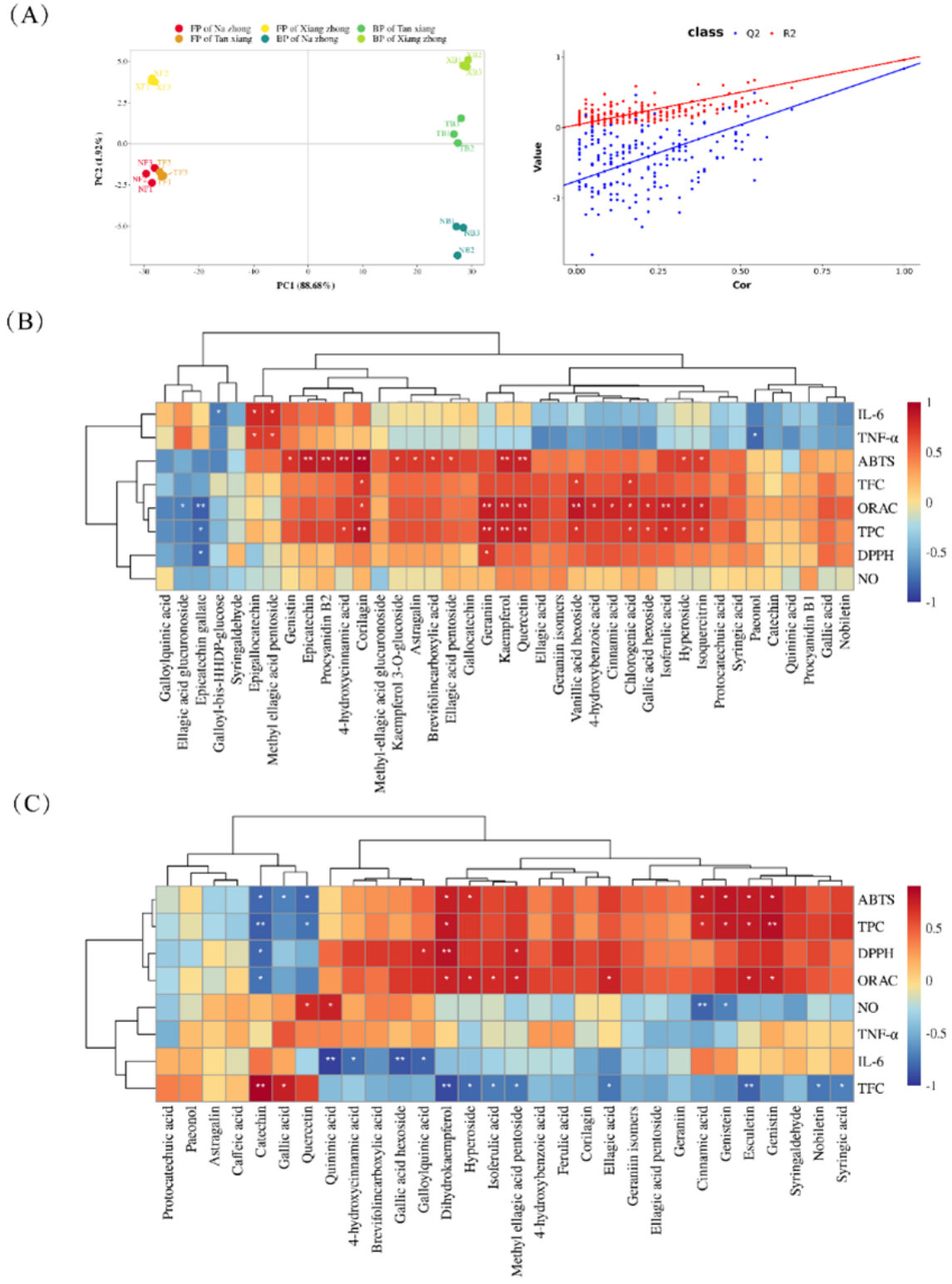

Figure 4

Multivariate analysis of the datasets of Chinese olive extracts. (A)The Partial least squares discriminant analysis (PLS-DA) score plot. NF: FP of ‘Na zhong’; TF: FP of ‘Tan xiang’; XF: FP of ‘Xiang Zhong’. NB: BP of ‘Na zhong’; TB: BP of ‘Tan xiang’; XB: BP of ‘Xiang Zhong’. (B) Clustering correlation heatmap with signs in FP. (C) Clustering correlation heatmap with signs in BP. Darker red and darker blue represent higher levels of positive and negative correlations, respectively. Significant correlations are marked with asterisks: *P<0.05, **P<0.01.

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