Comparison of phytochemical composition and anti-hyperglycemic activity of Liubao tea from different fermentation years

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Using response surface methodologies, two Liubao tea (LBT) extracts, LBT-5 and LBT-7, were optimized for different fermentation durations. Phytochemical composition analysis assessed the chemical composition and distribution of tea pigments. Their inhibitory effects on glucose-metabolic enzymes were analysed. Furthermore, an insulin-resistant (IR) cell model was constructed to study their regulation of glucose metabolism and underlying mechanism. LBT-7, aged longer, exhibited higher polyphenols and pigments. Assessments on glucose-metabolic enzymes and IR regulation confirmed LBT's capability to enhance glucose consumption, modulate cytokines and activate the IRS-PI3K-Akt-GLUT4 pathway. Notably, LBT-7 demonstrated a stronger effect due to its higher tea pigment.

Keywords: Anti-hyperglycemic activity, enzyme inhibition, Liubao tea, phytochemical composition, PI3K-Akt pathway.

TEA consumption has been a long-standing concept associated with health benefits in China, attributing to the presence of bioactive substances, such as polysaccharides, proteins and polyphenols, which exhibit significant effects in antioxidation, hypoglycemia, hypolipidemia and hypotension[1]. Notably, different processing methods yield teas with distinct compositions. Among six major types, dark tea (DT) is the only one involving microbial fermentation during its manufacturing process, imparting unique flavour and functionality[2].

DT is a distinct fermented tea through solid-state fermentation, including variations like Pu-erh, Qingzhuan and Liubao tea (LBT)[3]. Due to its specific processing, DT presents marked differences in polyphenol and theanine compositions. Research reveals that LBT contains about 10% polyphenols and 4.45% total flavonoids[3], offering potential benefits in preventing CCl_4-induced liver injury in mice due to its antioxidant capacity[4]. However, studies on LBT's anti-hyperglycemic effects are currently limited.

Diabetes, characterized by prolonged high blood glucose levels, poses risks of cardiovascular, kidney and eye diseases[5]. Type 2 diabetes (T2DM) constitutes over 90% of diabetes cases, arising from impaired pancreatic beta-cell function, insulin deficiency and insulin-resistance (IR) in target organs[6]. DT has shown promise in reducing blood glucose levels by mitigating IR and influencing key glucose-related enzyme metabolism[3,7]. Specifically, LBT has been found to activate the PI3K-Akt-PPARs-GLUT2 signalling cascade, addressing metabolic disorders and IR[8]. Nevertheless, the impact of different years on LBT composition and its anti-hyperglycemic activity remains underexplored.

In this study, we investigated LBT extracts from different fermentation years and optimized their extraction process through single-factor and orthogonal experiments, analysed composition differences, and evaluated *in vitro* anti-hyperglycemic activity, laying the groundwork for LBT's rational development.

Materials and methods

Chemicals and reagents

LBTs (storage for 5 and 7 years, namely LBT-5 and LBT-7) were purchased from Guangxi Wuzhou Shengyuan Tea Co., Ltd (Wuzhou, China). LO2 human hepatocytes were purchased from Chinese Academy of Sciences (Shanghai, China). High-glucose (4.5 g L^{-1}) DMEM complete medium was purchased from HyClone Co. (Los Angeles, USA). CCK-8 assay kit was purchased from Solarbio (Beijing, China). Insulin receptor substrate (IRS), phosphorylated insulin receptor substrate (p-IRS), phosphatidylinositol 3-kinase p85 (PI3K p85), phosphorylated protein kinase B (p-Akt), protein kinase B (Akt) and glucose transporter 4 (GLUT4) antibodies were purchased from Abcam Co. (California, USA). All other chemicals and solvents were of analytical grade.

Optimizing the extraction of LBT aqueous extracts

The optimal extraction method of LBT extracts was performed according to the previous report[8]. Briefly, under the conditions of a fixed liquid–solid ratio of 10 : 1, an extraction temperature of 90°C, an extraction time of 2 h,

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and a centrifugation time of 15 min, the effects of liquid–solid ratio (5 : 1, 10 : 1, 15 : 1, 20 : 1 and 25 : 1), extraction temperature (60°C, 70°C, 80°C, 90°C and 100°C), and extraction time (1, 1.5, 2, 2.5 and 3 h) on the extraction rate of aqueous extracts were studied.

Chemical composition analysis

The polysaccharide content was determined by the phenol–sulphuric acid method[9]. The polyphenol content was determined by the Folin–Ciocalteu method (Chinese national standard GBT8313-2018). The protein content was determined by the Bradford method[10]. The contents of theaflavins (TFs), thearubigins (TRs) and theabrownins (TBs) were determined by extraction method[3]. Typical tea pigments, including theobromine, gallic acid, gallocatechin (GC), caffeine, epigallocatechin (EGC), catechin, epicatechin (EC), caffeic acid, epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), p-coumaric acid, ferulic acid, epicatechin gallate (ECG), theaflavin-3-gallate (TF-3-G), theaflavin-3′-gallate (TF-3′-G), and theaflavin 3,3′-digallate (TF-3,3′-diG), were determined by high-performance liquid chromatography (HPLC)[11].

α-Glucosidase and α-amylase activity inhibition

The enzyme activity inhibition of α-glucosidase and α-amylase was analysed according to the method described by Zhu *et al.*[12].

Inhibition of advanced glycation end products (AGEs) formation and sucrase activity

The inhibition of AGEs formation and sucrase activity was evaluated according to reports by Liu *et al.*[13] and Minai-Tehrani *et al.*[14] respectively.

Cell test evaluation

All the cell model studies are conducted with three independent culture conditions with triplicates each time.

Cell culture: LO2 hepatocytes were cultured in high-glucose DMEM medium supplemented with 10% FBS and 100 U mL^{-1} penicillin-streptomycin solution at 37°C, 5% CO_2 and 95% humidity.

Cell viability: The IR cell model was established following the method described by Zhu *et al.*[3]. Briefly, LO2 cells in the logarithmic growth phase were digested, and the cell density was adjusted to 2×10^4 mL^{-1}. Then, 100 µL of cell suspension was seeded per well in a 96-well plate. Following cell adhesion, all groups except the control were treated with 10^{-7} M recombinant human insulin (RHI) (Solarbio, Beijing, China) for 36 h to establish an IR cell model. Subsequently, the culture medium was discarded, and cells were exposed to various concentrations of the samples (50, 100, 200, 400, 800, 1000 µg mL^{-1}) for an additional 24-h incubation period. The cell viability was assessed using the CCK-8 kit according to the manufacturer's instructions.

Glucose consumption and IR-related cytokine levels: The experiment consisted of four groups: control, model, LBT-5 and LBT-7. Control and model groups were treated with PBS. The control group was cultured in a high-glucose DMEM medium without RHI for 48 h. Moreover, model, LBT-5 and LBT-7 groups were cultured in high-glucose DMEM medium containing 10% RHI for 48 h. After 48 h of induction modelling, the medium was replaced. The sample groups (100 µg mL^{-1}) were incubated in high-glucose DMEM complete medium containing 10% RHI for 36 h, while the model and control groups were incubated in their respective original medium for 36 h. Before the end of the incubation, the levels of glucose, insulin-like growth factor 1 (IGF-1), superoxide dismutase (SOD), tumour necrosis factor-α (TNF-α), interleukin 6 (IL-6) and C-reactive protein (CRP) in the cell-free supernatant were quantified using commercially available kits.

Glucose metabolism-related enzyme activity: The measurement of key enzymes involved in glucose metabolism, including glucose 6-phosphatase (G-6-Pase), glycogen synthetase (GS) and phosphoenolpyruvate carboxykinase (PEPCK) was performed as per the guidelines outlined in the instruction manual of the respective ELISA kit.

RT-qPCR and western blotting: RT-qPCR and western blotting assays were conducted following the procedures outlined in a previous study[3]. The primer sequences used for RT-qPCR are provided in Supplementary Table 1.

Statistical analysis

Data processing was conducted using GraphPad Prism 9.0 software. A *P*-value less than 0.05 indicates a significant difference.

Results

Optimal extraction

Single-factor experiments (Figure 1 *a–c*) clearly demonstrated the significant impact of varying extraction temperature, liquid-to-solid ratio, and extraction time on the efficiency of LBT water extraction. Based on these findings, the optimal conditions for LBT extract were determined as follows: 90°C for extraction temperature, a liquid–solid ratio of 20 : 1 and an extraction time of 2.5 h.

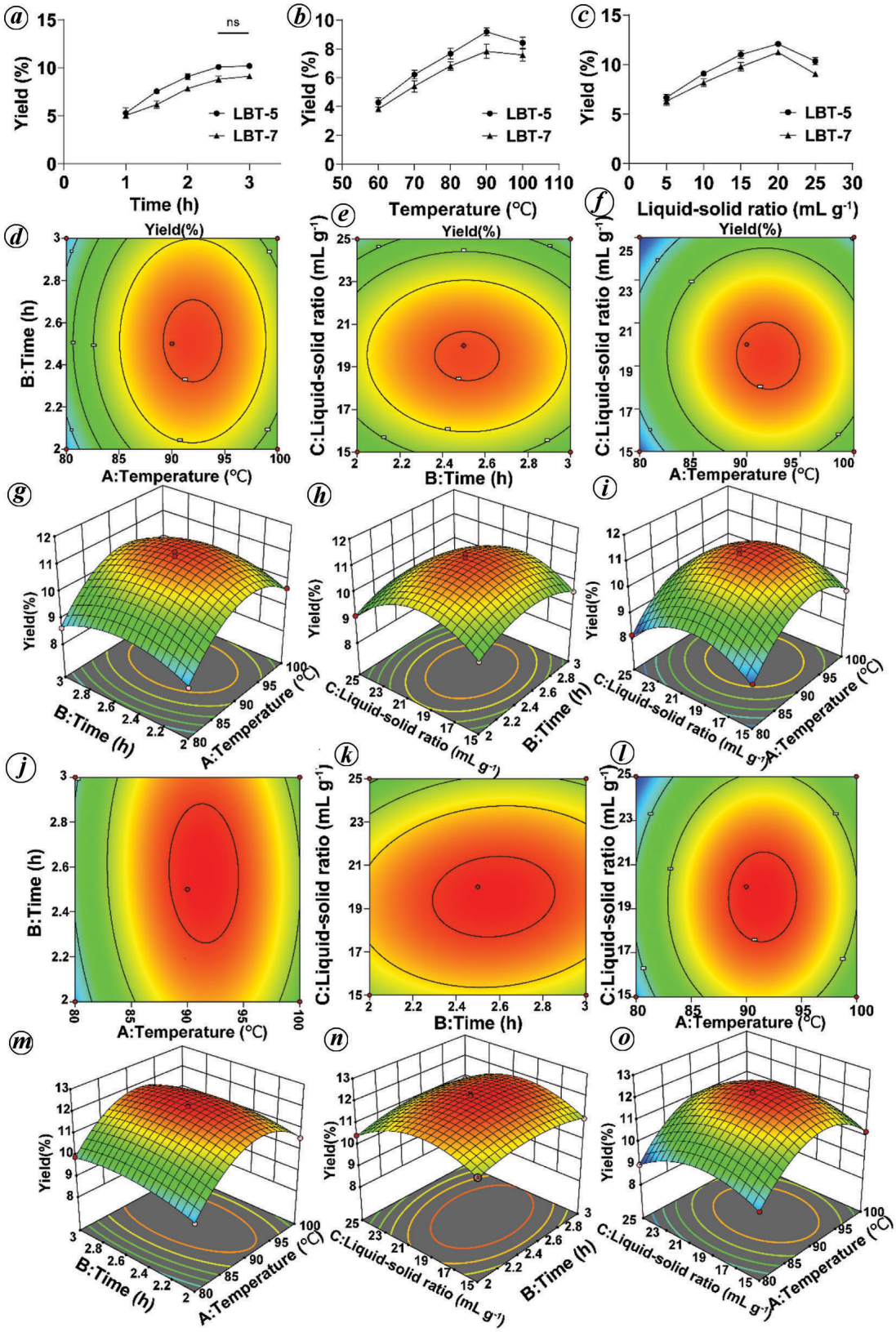

Figure 1. Single factor showing (***a***) the effects of extraction time, (***b***) extraction temperature, (***c***) liquid-to-solid ratio conditions on LBT-5 and LBT-7 extract yields, (***d–f***) response surface analysis showing the impact of the pairwise interaction of three factors on the yields of LBT-5 and LBT-7 extracts, including contour plots, (***g–i***) response surface plots of LBT-5 (*D*, *G*) $Y = f(B, A)$, (*E*, *H*) $Y = f(C, B)$, and (*F*, *I*) $Y = f(C, A)$, as well as (*j–l*) contour plots and (***m–o***) response surface plots of LBT-7 (*J*, *M*) $Y = f(B, A)$, (*K*, *N*) $Y = f(C, B)$ and (*L*, *O*) $Y = f(C, A)$.

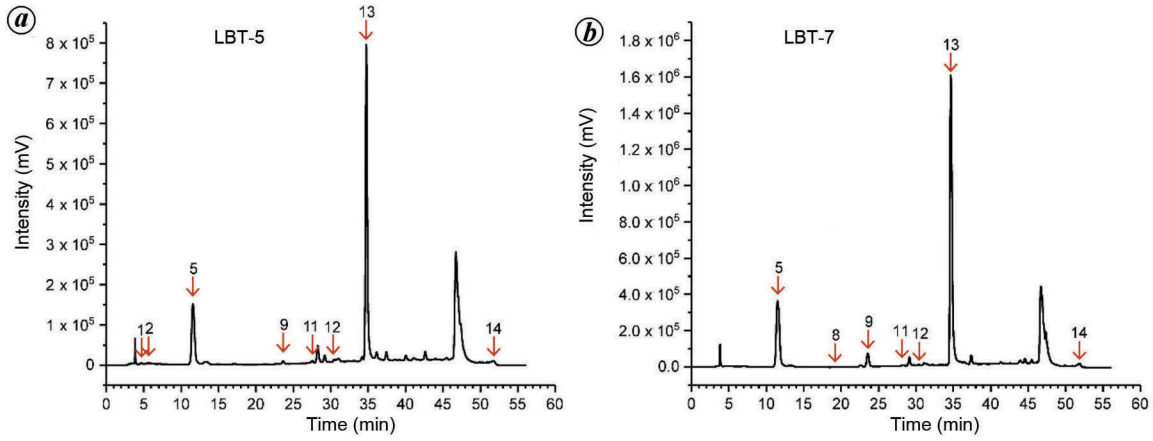

Figure 2. HPLC chromatograms at 280 nm showing the distribution of different typical tea pigments in LBT-5 and LBT-7 extracts at different retention times. (***a***) LBT-5, (***b***) LBT-7. (1) Theobromine (4.940 min); (2) Gallic acid (5.593 min); (3) GC (7.783 min); (4) Caffeine (10.390 min); (5) EGC (11.299 min); (6) Catechin (14.451 min); (7) EC (18.103 min); (8) Caffeic acid (18.890 min); (9) EGCG (23.142 min); (10) GCG (26.364 min); (11) *p*-Coumaric acid (27.716 min); (12) Ferulic acid (30.543 min); (13) ECG (34.527 min); (14) TF-3-G (52.6637 min).

Table 1. Main component content in Liubao tea (LBT)-5 and LBT-7 extracts

Category	LBT-5	LBT-7
Theaflavin (%)	0.09 ± 0.01	0.13 ± 0.01*
Thearubigin (%)	0.86 ± 0.02	1.72 ± 0.03*
Theabrownin (%)	4.19 ± 0.03	8.20 ± 0.05*
Polysaccharide (%)	4.41 ± 1.14	3.62 ± 0.48*
Protein (%)	15.87 ± 0.30	13.89 ± 0.32*
Polyphenols (%)	15.51 ± 1.40	22.41 ± 0.35*
Theobromine (mg g^{-1})	0.85 ± 0.03	ND
Gallic acid (mg g^{-1})	0.86 ± 0.04	ND
Gallocatechin (mg g^{-1})	ND	ND
Caffeine (mg g^{-1})	ND	ND
Epigallocatechin (EGC, mg g^{-1})	11.61 ± 0.46	32.78 ± 1.36*
Catechin (mg g^{-1})	ND	ND
Epicatechin (mg g^{-1})	ND	ND
Caffeic acid (mg g^{-1})	ND	0.61 ± 0.03
Epigallocatechin gallate (mg g^{-1})	1.19 ± 0.11	9.58 ± 0.77*
Gallocatechin gallate (mg g^{-1})	ND	ND
p-Coumaric acid (mg g^{-1})	0.64 ± 0.01	1.16 ± 0.07*
Ferulic acid (mg g^{-1})	1.27 ± 0.08	0.71 ± 0.02*
ECG (mg g^{-1})	53.30 ± 1.4	181.69 ± 2.79*
TF-3-G (mg g^{-1})	0.77 ± 0.03	2.89 ± 0.11*
TF-3′-G (mg g^{-1})	ND	ND
TF-3,3′-diG (mg g^{-1})	ND	ND

*Significant difference from the LBT-5 group, $P < 0.05$. ND, Not detected.

Using data from the single-factor experiments, we meticulously designed a response surface optimization experiment for LBT water extraction (Supplementary Tables 1 and 2). For LBT-5, the extraction rate ranged from 8.94% to 12.16%, whereas for LBT-7, it ranged from 8.64% to 11.25%. Analysis of variance (Supplementary Tables 3 and 4) highlighted the significant influence of extraction temperature on LBT-7's extraction rate, and both extraction temperature and liquid–solid ratio on LBT-5's extraction rate. The *P* values for both models were <0.01, indicating their significance. The lack-of-fit term had a *P* value exceeding 0.05, confirming the model's utility. All signal-to-noise ratios exceeded 4, affirming its effectiveness for experimental design.

Regarding the interaction between factors (Figure 1 *d*–*o*), a strong interaction was observed between extraction time and the liquid–solid ratio for LBT-5, while a stronger interaction was noted between extraction temperature and liquid–solid ratio for LBT-7. Optimal conditions for LBT-5 were 91.44°C for extraction temperature, 2.57 h for extraction time, and a liquid–solid ratio of 19.57 : 1, resulting in an extract yield of 12.16%. For LBT-7, the optimal conditions were 92.01°C for extraction temperature, 2.51 h for extraction time and a solid–liquid ratio of 19.48 : 1, yielding an extract yield of 11.11%. Verification experiments supported these predicted yields, with actual values of 12.14% ± 0.04% for LBT-5 and 11.07% ± 0.05% for LBT-7.

Chemical composition analysis

Table 1 displayed key findings on constituents in LBT-5 and LBT-7 extracts. LBT-5 showed higher tea polysaccharide and protein levels, yet lower tea polyphenols and characteristic tea pigments compared to LBT-7. Interestingly, TB content was prominent in LBT-5 and LBT-7. HPLC was employed for a deeper understanding of phenolic composition in LBT-7 and LBT-5 (Figure 2 and Table 1), revealing higher ECG (181.69 mg g^{-1}) and EGC (32.78 mg g^{-1}) contents in LBT-7, whereas LBT-5 showed ECG at 53.3 mg g^{-1} and EGC at 11.61 mg g^{-1}.

Inhibitory impact on enzyme activity in glucose metabolism

The glucose metabolism-related enzyme activity experiment validated the concentration-dependent anti-hyperglycemic

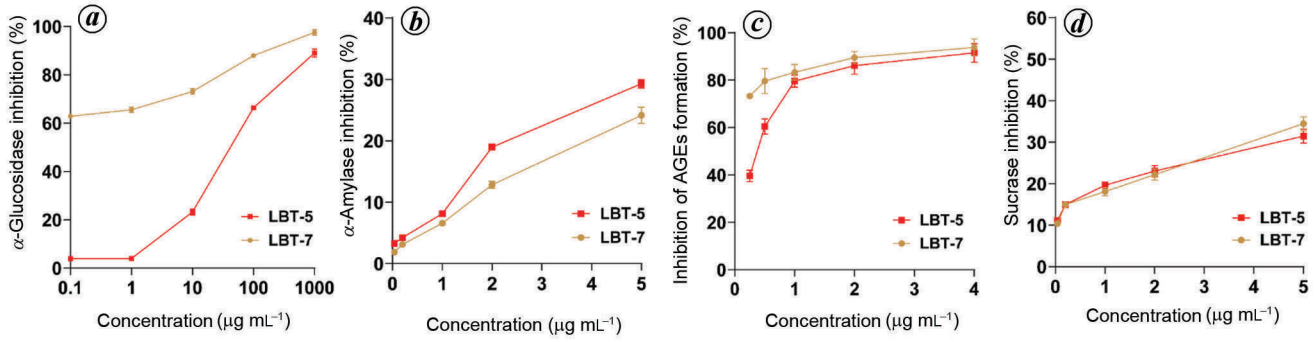

Figure 3. Inhibitory effects of LBT-5 and LBT-7 extracts on the activities of some glucose metabolism enzymes, including (***a***) α-glucosidase, (***b***) α-amylase, (***c***) formation of AGEs and (***d***) sucrase.

effect of the two LBT extracts. As depicted in Figure 3, LBT-7 exhibited robust inhibition of α-glucosidase and AGEs formation, while LBT-5 showed enhanced α-amylase inhibition, with no significant sucrase difference between the two.

Cellular anti-hyperglycemic evaluation

The LO2 liver cell experiment confirmed LBT's anti-hyperglycemic effect on IR. Cellular viability assays (Figure 4 *a* and *b*) showed enhanced cell proliferation by both tea concentrations (50–1000 μg mL^{-1}), peaking at 100 μg mL^{-1}. Glucose consumption assessments (Figure 4 *c*) indicated LBT-7's higher potency. Figure 4 *d*–*h* demonstrated that LBT-5 and LBT-7 stimulated IGF-1 and SOD secretion while inhibiting TNF-α, IL-6 and CRP. Experimental findings (Figure 4 *i*–*k*) indicated LBT's influence on these enzymes, underscoring its anti-hyperglycemic potential.

The underlying mechanism of LBT's action was further explored. The results (Figure 5 *a*–*d*) revealed enhanced mRNA expression levels of PI3K, Akt, IRS-1 and GLUT-4 due to LBT extracts, with more pronounced effects observed in LBT-7. Western blot experiments (Figure 5 *e*–*k*) demonstrated LBT's capability to boost IRS, PI3K, pAkt, and GLUT-4 protein expressions, with LBT-7 showing greater efficacy.

Discussion

T2DM primarily arises from IR, marked by the disrupted insulin-mediated glucose handling in skeletal muscle, hepatocytes and adipocytes[5]. The complex nature of its pathogenesis makes the exact causes of this condition challenging to pinpoint. Consequently, significant research efforts have been focused on identifying dietary strategies to prevent and alleviate diabetes. This study has refined the extraction process of LBT using response surface methodology, revealing that LBTs exerted concentration-dependent inhibitory effects on enzymes involved in glucose metabolism. Importantly, these teas significantly impact the IRS–PI3K–Akt–GLUT4 signalling pathway, improving insulin sensitivity, reducing oxidative stress, and diminishing inflammation, contributing to their anti-hyperglycemic properties.

Extended storage has been shown to increase microbial activity within LBT, leading to a higher consumption of carbon and nitrogen sources[15]. Tea polysaccharides and proteins, key sources of these elements in tea, tend to decrease in concentration after prolonged storage. Interestingly, the content of TB was notably higher in LBT samples stored for longer periods, aligning with Zhu *et al.*[3]'s findings, highlighting TB's critical role in dark tea's colouration. The changes in LBT's composition over time are due to the depletion of some components and the generation of new compounds influenced by microbial actions. The microbial fermentation unique to dark tea can transform certain elements into polyphenols and flavonoids. The oxidation of tea polyphenols during extended fermentation results in the transformation of TFs and TRs into TBs[15]. Cheng *et al.*[16] noted that TB content in aged Qingzhuan tea was 15.92, 18.08 and 24.14 mg g^{-1} for 5, 10 and 15 years respectively. Additionally, there were substantial differences in ECG content between new tea and tea stored for five years[16].

Enzymatic assays related to glucose metabolism revealed that LBT-7 exhibited a robust inhibitory effect on α-glucosidase and the formation of AGEs, whereas LBT-5 enhanced the inhibitory effect on α-amylase. Liu *et al.*[17] had previously reported a potent inhibitory effect of Qingzhuan DT extract on α-glucosidase, with EGCG and ECG being the primary contributors to this effect. Similarly, Zhu *et al.*[3] reported strong glycosidase inhibition in LBT, correlating with proteins, pigments, caffeine and trace elements. It is likely that tea polyphenols and pigments make a substantial contribution to glycosidase inhibition. For instance, it has been observed that black tea extracts rich in TFs displayed potent inhibitory effects on sucrose–isomaltase activity, resulting in the delayed hydrolysis of sucrose, maltose and isomaltose[18]. Importantly, the contents of both EGCG and ECG were significantly higher in LBT-7 than in LBT-5, explaining the enhanced α-glucosidase activity in LBT-7. In conclusion, LBT-7, characterized by

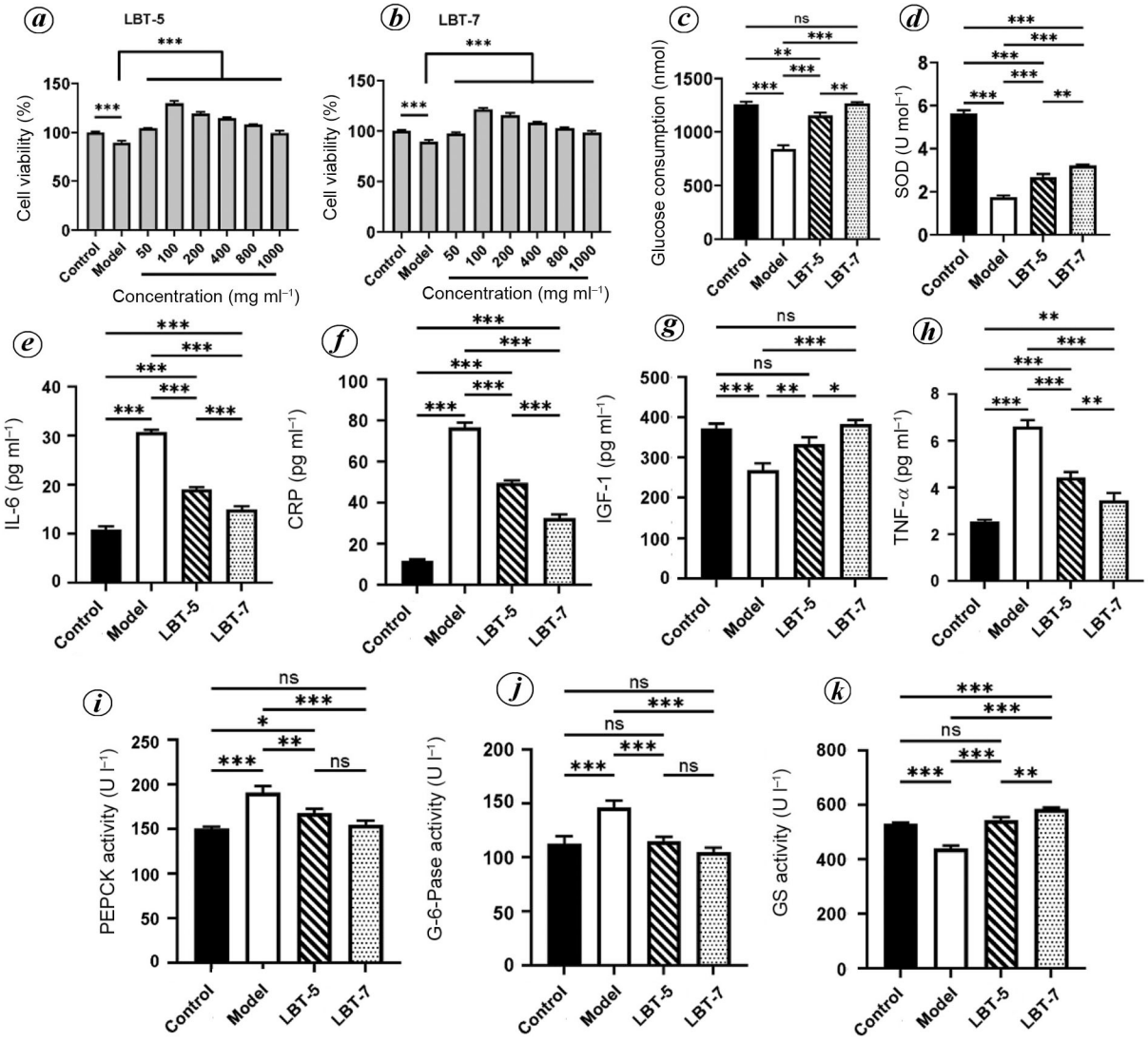

Figure 4. (***a***–***b***) Effects of LBT-5 and LBT-7 extracts on LO2 cell viability, (***c***) glucose consumption, (***d***) cytokine levels, including SOD, (***e***) IL-6, (***f***) CRP, (***g***) IGF-1 and (***h***) TNF-α, and enzyme activities related to glucose metabolism, including (***i***) PEPCK, (***j***) G-6-Pase and (***k***) GS in an insulin-resistant LO2 cells. Asterisk indicates a significant difference between two groups, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns, not significant.

extended fermentation, exhibits more pronounced regulation of glucose-metabolizing enzymes, potentially enhancing its anti-hyperglycemic activity.

Cellular experiments demonstrated that LBT-7 was more effective in increasing the mRNA expression levels of IRS-1, PI3K, Akt and GLUT-4, as well as enhancing the expression of IRS, PI3K, pAkt and GLUT-4 proteins (Figure 5). SOD, an antioxidant metalloenzyme, combats oxidative stress by catalysing the disproportionation of superoxide anion radicals[19]. CRP, an inflammatory marker, is linked to T2DM, obesity, IR and cardiovascular issues. IL-6, a pro-inflammatory cytokine, exacerbates IR[20]. TNF-α plays a role in peripheral IR[21]. These findings suggest the potential of LBT to alleviate oxidative stress, inflammation and IR, thereby reducing blood glucose levels. The significantly higher TB content in LBT-7 compared to LBT-5 potentially contributes to the anti-hyperglycemic activity of LBT-7. Crucial enzymes involved in glucose metabolism, including G-6-Pase, GS and PEPCK, promote glucose production[22]. Tea polyphenols, flavonoids, polysaccharides and TB primarily facilitate blood glucose reduction. Liu *et al.*[18] demonstrated dose-dependent glucose transport inhibition in various tea extracts correlated with catechin content, with catechins impeding glucose uptake[23]. Notably, higher polyphenol, TB, EC, EGCG and ECG levels in LBT-7 due to microbial transformation during storage elucidate its elevated anti-hyperglycemic activity compared to LBT-5 (ref. 15). The pivotal PI3K/Akt pathway plays a critical role in regulating glucose and lipid metabolism, with PI3K and AKT being essential components[24]. Previous

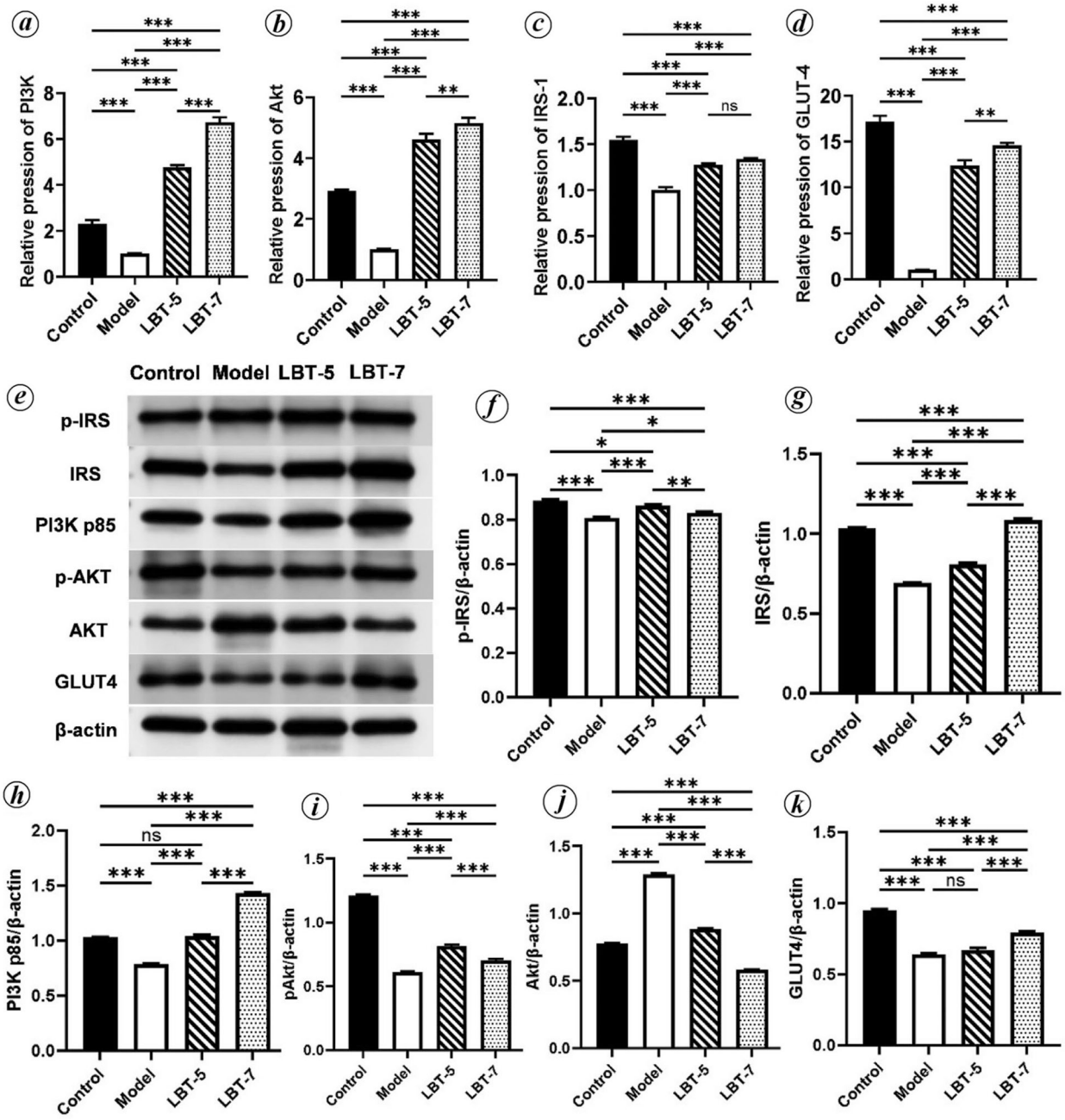

Figure 5. Effects of LBT-5 and LBT-7 extracts on mRNA expression levels of insulin-resistant LO2 cells after 36 h treatment, including (***a***) PI3K, (***b***) Akt, (***c***) IRS-1 and (***d***) GLUT4 and (***e***) protein expression levels, including (***f***) *p*-IRS, (***g***) IRS, (***h***) PI3K p85, (***i***) pAkt, (***j***) Akt and (***k***) GLUT4. Asterisk indicates a significant difference between two groups, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, ns, not significant.

studies have suggested that EC and EGC enhance GLUT4 translocation via PI3K activation, whereas EGCG impedes insulin-triggered GLUT4 translocation through the insulin signalling pathway[25]. Additionally, Zhu *et al.*[8] demonstrated LBT's involvement in the PI3K–Akt–PPARs–GLUT2 cascade signalling pathway, ameliorating metabolic irregularities and IR. Therefore, LBT extracts could exert an anti-hyperglycemic effect by activating the IRS–PI3K–Akt–GLUT4 signalling pathway.

Conclusion

The optimization of the LBT extraction procedure was achieved by implementing response surface methodology, resulting in enhanced extraction efficiency. Subsequently, the glucose metabolism-related enzyme activity experiment validated the concentration-dependent anti-hyperglycemic impact of both LBTs. Furthermore, these teas exhibited the capacity to enhance the secretion of IR-associated

cytokines through the activation of the IRS–PI3K–Akt–GLUT4 signalling pathway, thereby contributing to their anti-hyperglycemic effect.

Conflict of interest: The authors declare no conflict of interest.

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