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RESEARCH ARTICLE

Effects of Echinacea Extract on Intestinal Metabolomics in Immunosuppressive Rats

Jiali Yang[1], Yucheng Guan[1], Jiaxin Chen[1], Yalin Su[1], Shiwei Zhang[1], Khalid Mehmood[2], Muhammad Usman Saleem[3] and Dayou Shi[1]*

[1]College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China
[2]Department of Pathology, Faculty of Veterinary and Animal Sciences, The Islamia University of Bahawalpur-63100, Pakistan
[3]Department of Biosciences, Faculty of Veterinary Sciences, Bahauddin Zakariya University Multan, Pakistan
*Corresponding author: shidayou@scau.edu.cn

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A B S T R A C T

Echinacea purpurea is known as an important herbal medicine and has been frequently used for over last 300 years as a medicinal herb. This herb is well known for its effects on immune system, which can change the contents of different metabolites in animals. The mechanisms of its immunoregulation on various metabolomics are poorly known and are still unclear. Therefore, this study was executed to explore the effects of cyclophosphamide (CY) on normal control group (NC group) treated with sterile saline and treatment group exposed to *Echinacea* extract (EE) to determine the effects on immunosuppression group (IS group, induced by CY) and its association with metabolomics. Here we reported that body weight, indices of immune organs, serum biochemical and cytokine contents were affected by CY and EE had the potential to reverse adverse effects through increasing IFN-γ and TNF-α level, significantly increased quantity of total protein (TP), and albumin (ALB). The results on the Gas Chromatography-Mass Spectrometer (GC-MS) technique illustrated that amino acid and energy metabolism involved in differential metabolites between IS and NC groups with vitamin B6 metabolism, pantothenate, and coenzyme A(CoA) biosynthesis metabolic pathways. Results indicated that myo-inositol, lactic acid, pyruvate are potential marker substances between EE-IS group and IS group with a principal impact on the metabolic pathways including inositol phosphate metabolism, phosphatidylinositol signaling system, ascorbate and aldarate metabolism and pyruvate metabolism. Overall, this study identified the involvement of different metabolites pathways of EE in immunosuppression rats which provided valuable evidence of EE immunoregulation effect via metabolomics and multiple bioanalytic methods.

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INTRODUCTION

The *Echinacea* species include *Echinacea angustifolia*, *Echinacea pallida* and *Echinacea purpurea,* which are mainly cultivated in North America and Europe (Owens *et al.,* 2014). Due to medicinal properties, *Echinacea* species are frequently applied externally on wounds, burns and insect bites, toothache, and throat infections and internally to relieve pain, coughs, gastrospasms and snake bites treatments. *Echinacea* has been widely used as an herbal medicine and dietary supplement. *E. purpurea* has numerous bioactive compounds such as caffeic acid derivatives, glycoproteins, polysaccharides and alkylamides. It is believed that the glycoproteins, polysaccharides and alkylamides are the main immunomodulatory chemical compounds in roots of *E. purpurea* and play a pivotal role as antivirals, antinflammation and antimicrobial agents (Catanzaro *et al.*, 2018). Moreover, different studies have determined that it causes beneficial effects on the immune system, promotes the growth of immune organs, improves blood indexes, helps in the regulation of immune cells proliferation and enhance the cellular and humoral immune functions (Khattab *et al.*, 2019; Khalaf *et al.*, 2019). It also alters the gut microbiota and increases Bacteroides concentrations, reducing the *Lactobacillus* and *Bifidobacterium* abundance. Moreover, recent studies related to immunomodulatory effects of *E. purpurea* have shown that endophytic bacteria are the key factors in macrophage-stimulating activity (Todd *et al.*, 2015).

Immunosuppression may cause immune system disorders and increase the risks of infections associated with various reasons such as environment, drugs, or other treatments. Several methods have been used to establish immunosuppressive models, including radiation injury, thymectomy, gene knockout and immunosuppressive agents. The immunosuppressive agents are well-received due to the low cost, simple operation and reasonable repeatability. Cyclosporin A, hydrocortisone, dexamethasone, azathioprine and cyclophosphamide (CY) are common immunity inhibitor but CY is the most widely used among them. CY is an inactive prodrug, mainly metabolized in the liver after oral or intravenous administration and produces less toxic 4-hydroxycyclophosphamide and aldophosphamide. Furthermore, it is degraded to acrolein, phosphamidemustard and normustard. It causes DNA cross-linking that produces cytotoxic lesions. It is a useful drug in chemotherapy for immunosuppressive properties and it is reported that CY could diminish body weight, spleen and thymus index, white blood cell, lymphocyte and platelet counts, the proliferation of peripheral T lymphocytes, the expression of IL-2, IL-6, IL-10, IFN-γ and TNF-α, and the contents of serum TP, ALB, IgG, IgM and IgA (Qi *et al.*, 2019; Shu *et al.*, 2021).

Studies on different metabolites are crucial and to detect the endogenous metabolic changes in biological systems stimulated by endogenous and xenobiotic factors. Through the pattern recognition and metabolic responses are associated with biological systems which locate the target organ, site and identify the biomarkers. As an important biomarker of physiologic or pathologic conditions and a remarkable tool in distinguishing disease phenotypes, the metabolites are more sensitive than conventional biomarkers like complete blood count and serum biochemical tests. The immune system plays a key role in many diseases and defenses against pathogenic microorganisms. Intestinal flora is considered the most significant immune organ. It is a necessary part of human health, affording essential nutrients, synthesizing vitamin K, assisting the digestion of fiber, and improving angiogenesis and enteric nerve functions (Zhang *et al.*, 2015). The disorders in intestinal flora influence normal physiology and susceptibility to disease through collective metabolic activities and host interactions (Lozupone *et al.*, 2012). Metabolomics, a comprehensive study of using analytical techniques to understand how the gut microbiota works through its metabolism and host-microbiota co-metabolisms (Yan *et al.*, 2016). It is extensively used for clinical diagnosis of different diseases such as Alzheimer's disease, cancers, inflammatory bowel disease, and type 2 diabetes mellitus, which show alternations in intestinal flora and metabolites (Zhang *et al.*, 2013). Therefore, metabolomics is a crucial and helpful approach to determine the immune mechanisms of *E. purpurea*.

Although the studies of *E. purpurea* on pharmacology, chemical composition and metabolomics have been conducted, however, the investigation of underlying immunomodulation mechanisms is still unclear. There are many studies on CY-induced immunosuppression in rats and mice, but scanty information is available on the influence of metabolomics. In this study, the GC-MS non-target metabolomics method is used to reveal the abnormal metabolic pathways in rats induced by CY and the pathways that *Echinacea* extract (EE) involves. Therefore, this study provides a new perspective of understanding the immunosuppression of CY and immunoregulation mechanisms of EE and opportunities to explore the potential effects of *E. purpurea* in immunity reinforcement.

MATERIALS AND METHODS

Animal and treatments: A total of 36 active and pathogen-free (SPF) Sprague-Dawley rats having body mass (110 ± 20 g) were purchased from the Center of Experimental Animals of Southern Medical University with approval number (SCXK 2016-0041).

After few days of adaptation to laboratory conditions, the rats were randomly divided into 3 groups, including the normal control group (NC group), immunosuppression group (IS group) and *Echinacea* extract group (EE-IS group). Rats in IS and EE-IS group were given 40mg/kg CY (purchased from Shanghai Ruiyong Technology Co., Ltd) by gavage method after the one-day interval three times. The rats in EE-IS group were gavaged 1g/kg EE (provided by Guangzhou South China Agricultural University Experimental Veterinary Drug Co. Ltd) once a day for 14 days, while the rats in the NC group were given the same dose of normal saline. Six rats were randomly selected from each group on day 14th after treatment. Bodyweight, immune organs indices, serum biochemistry, cytokine levels, and intestinal metabolites were measured.

Bodyweight and immune organ indices: Rats were euthanized and total body weight, spleen, and thymus weight were measured. The spleen and thymus indices were calculated as below:

Spleen index = spleen weight (mg)/body weight (g); Thymus index = thymus gland weight (mg)/body weight (g).

Serum biochemical analysis: Blood was taken from the aorta and centrifuged at 3000 rpm/min for 10 min to collect serum. Alkaline phosphatase (ALP), total protein (TP), albumin (ALB), total bilirubin (T-Bil), creatinine (CREA) and urea (UREA) levels in serum were assessed by an automatic biochemical analyzer (BS-380 Automatic Biochemical Analyzer, Shenzhen Mindray Bio-Medical Electronics Co., Ltd.).

Serum cytokine analysis: IL-6, IFN-γ and TNF-α contents were assessed by using commercial ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd) according to the manufacturer's instructions.

GC-MS Metabolomics Analysis

Sample preparation: Different samples (intestinal contents) from the cecum of experimental rats were collected and prepared according to a previous study (Liu *et al.*, 2021).

Metabolomics analysis: Metabolomics analysis was performed using an Agilent 7890A/5975C GC-MS system (Agilent Technologies Inc., CA, USA). The peak picking, alignment, deconvolution and further processing of raw GC-MS data were referred to the previously published protocols (Gao *et al.*, 2010).

Statistical analysis and identification of differential metabolites: SIMCA software (version 14.1, AB Umetrics, Umeå, Sweden) was chosen for multivariate statistical analysis. The data were preprocessed by UV scaling and mean centering before performing PLS-DA, and OPLS-DA. The variables with VIP values of OPLS-DA model larger than 1 and p values of univariate statistical analysis lower than 0.05 were identified as potential differential metabolites. Fold change (Log2FC) was calculated as binary logarithm of average normalized peak intensity ratio between Group 1 and Group 2. The positive value means that the average mass response of Group 1 is higher than Group 2.

Statistical analysis: For statistical analysis, SPSS 22.0 statistical program was used. Data are presented as Mean ± SD of triplicate measurements. Comparison of two groups of data was analyzed by independent sample T test, multiple groups of data was analyzed by univariate ANOVA, and multiple ratio between each group was analyzed by the least significant difference method (LSD) and Duncan's new complex range test.

RESULTS

Immunosuppressive effects in rats: Results obtained in this study exhibited that the body weight significantly decreased ($P<0.05$) and spleen index increased in IS group ($P<0.05$) compared with NC group. The thymus index had a declining trend. The results showed no significant differences between IS group and EE-IS group among these parameters ($P>0.05$). It was recorded that the spleen index was highest in the EE-IS group (Fig. 1A). The results on serum biochemical parameters showed that the concentrations of T-Bil were increased significantly ($P<0.05$) while TP, ALB and ALP were decreased noticeably ($P<0.05$) in IS group compared to NC group. The values of TP and ALB concentrations in EE-IS group were notably increased ($P<0.05$) while the levels of ALP and T-Bil did not show significant ($P>0.05$) difference compared with IS group. Results showed that the levels of creatinine and urea had no significant difference ($P>0.05$) among the three groups (Fig. 1B). The results on cytokines parameters indicated that the contents of serum IL-6 in IS group were similar to NC group while the serum IFN-γ level showed a decreasing tendency. All these parameters (serum cytokine contents) between IS group and EE-IS group were not significantly different ($P>0.05$), but the EE-IS group showed an increasing tendency (Fig. 1C).

Metabolite partial least squares discriminant analysis (PLS-DA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA): Partial least squares discriminant analysis (PLS-DA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) for metabolite composition indicated a clear separation of metabolic profiling between NC group and IS group, IS group and EE-IS group (Fig. 2). The NC and IS groups model parameters of R^2Y and Q^2 values are 0.986 and 0.805 (Fig. 2A, left), 1 and 0.774 (Fig. 2B, left). The values of IS group and EE-IS group model parameters of R^2Y and Q^2 are 0.924 and 0.726 (Fig. 2A, right), 1 and 0.844 (Fig. 2B, right). PLS-DA permutation test indicated

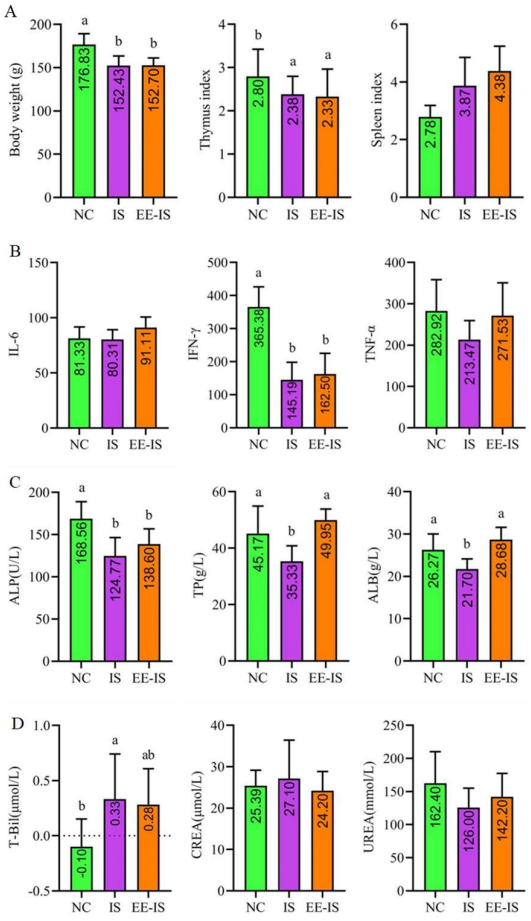

Fig. 1: The effects of EE on body weight and immune organ indices (A), serum biochemical profile (B), and cytokines (C) parameters in immunosuppressive rats induced by CY.

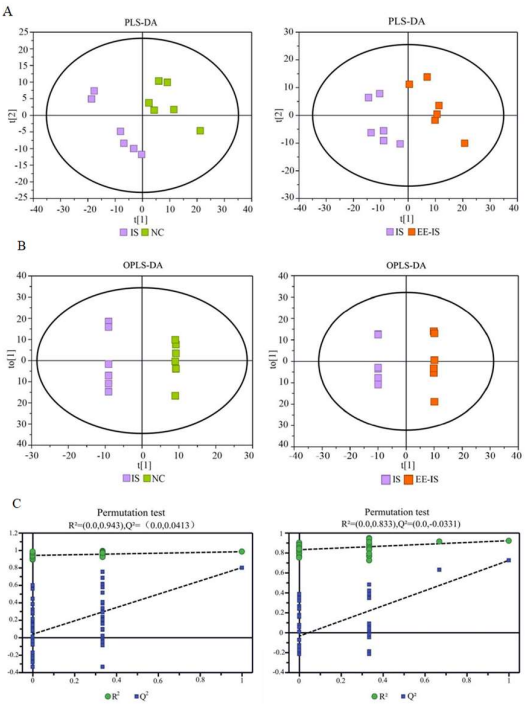

Fig. 2: PLS-DA, PLS-DA permutation test, and OPLS-DA of metabolites (n = 6). (A) PLS-DA score plot. (B) PLS-DA permutation test based on PLS-DA score plot. (C) OPLS-DA score plot.

the current model is valid (Fig. 2C). Combined with these values, it showed that the quality of the model is good and can explain the difference between the two groups.

Differential metabolite analysis: Combined with the first principal component VIP value (threshold >1) of the OPLS-DA model and the P value (threshold <0.05) of univariate test, the differential metabolites were identified by searching the reference material database including chromatographic retention time and mass spectrometry. There are 18 differential metabolites between IS group and NC group showed by histogram and heatmap (Fig. 4A and Fig. 5A). Compared with NC group, results showed 15 down-regulated and 3 up-regulated while the content of gluconic acid, 3,4-dihydroxyphenylacetic acid and gamma-aminobutyric acid increased in IS group. The level of N-acetylaspartic acid, rhamnose, ribose, xylulose, (3,4-dihydroxyphenyl) butyric acid, azelaic acid, pyruvic acid, 5-hydroxyindole, 2-ketoglutaric acid, 2-ketoisocaproic acid, 3-methyl-2-oxovaleric acid, 4-pyridoxic acid, 3-methyl-2-ketobutyric acid, 3-hydroxybenzoic acid and hexanoic acid decreased. The results showed the presence of 38 differential metabolites between IS group and EE-IS group. Compared with IS group, 28 down-regulation and 10 up-regulation, the content of glucose, glycolic acid, phenylacetic acid, p-Cresol, (3,4-Dihydroxyphenyl) butyric acid, 4-hydroxyphenylacetic acid, 2-hydroxyisobutyric acid, 4-hydroxypyridine, palmitic acid, stearic acid increased in EE-IS group. The level of orotic acid, myo-inositol, histidine, creatinine, uric acid, ethanolamine, 3-hydroxybutyric acid, citric acid, oleic acid, 4-hydroxyproline, lactic acid, aminomalonic acid, xanthine, linoleic acid, 4-hydroxyphenylethanol, 3-hydroxypropionic acid, cysteine, serine, pseudouridine, proline, hypoxanthine, gamma-aminobutyric acid, threonine, glycine, uracil, pyroglutamic acid, alanine, glutamic acid decreased (Fig. 4B and Fig. 5B).

Analysis of metabolic pathways: The results on different metabolites determined by MetaboAnalyst 4.0 (Table 1 and 2) showed that 18 metabolic pathways were involved in IS group and NC group while 41 metabolic pathways were involved in IS group and EE-IS group. The metabolites analysis demonstrated the differences of metabolic pathways between IS group and NC group, mainly including vitamin B6 metabolism, pantothenate and coenzyme A(CoA) biosynthesis, valine, leucine and isoleucine degradation, pentose phosphate pathway, arginine and proline metabolism, and alanine, aspartate, and glutamate metabolism (Fig. 5A). The results on the differences of metabolic pathways between IS group and EE-IS group mainly included inositol phosphate metabolism, phosphatidylinositol signaling system, ascorbate and aldarate metabolism, pyruvate metabolism and valine, leucine and isoleucine biosynthesis (Fig. 5B).

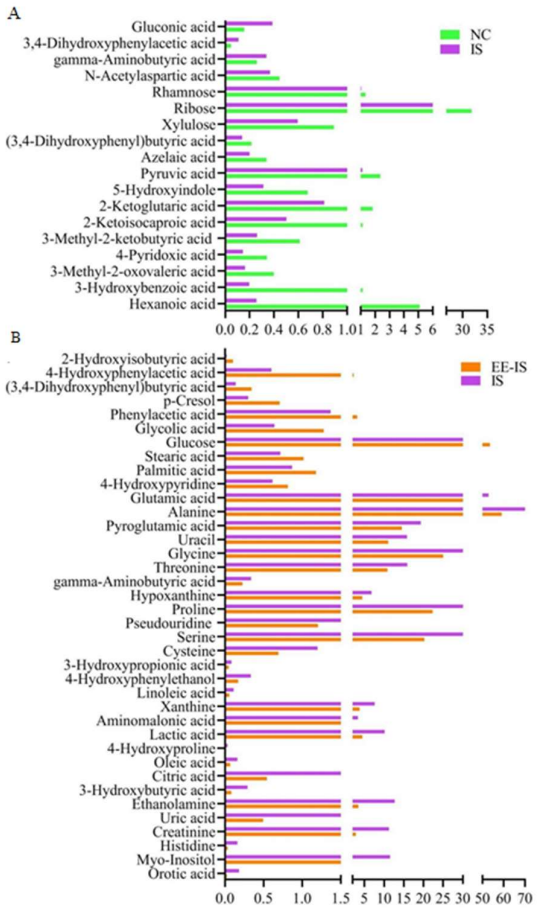

Fig. 3 Histogram of differentially expressed metabolites. (A) IS group and NC group. (B) EE-IS group and IS group.

Table 1: Differential metabolite pathway between IS group and NC group

Pathway	Raw *p*	-LOG(*p*)	Impact
Vitamin B6 metabolism	0.000531	7.5399	0
Pantothenate and CoA biosynthesis	0.007377	4.9094	0
Valine, leucine and isoleucine degradation	0.013896	4.2762	0.02168
Valine, leucine and isoleucine biosynthesis	0.013896	4.2762	0
Pentose phosphate pathway	0.0146	4.2267	0.05183
Arginine and proline metabolism	0.017793	4.029	0.02385
Tyrosine metabolism	0.017908	4.0225	0.00635
Pyruvate metabolism	0.018033	4.0155	0.20684
Glycolysis / Gluconeogenesis	0.018033	4.0155	0.10044
Glycine, serine and threonine metabolism	0.018033	4.0155	0
Cysteine and methionine metabolism	0.018033	4.0155	0
Glyoxylate and dicarboxylate metabolism	0.018033	4.0155	0
Alanine, aspartate and glutamate metabolism	0.019662	3.9291	0.22116
Citrate cycle (TCA cycle)	0.019936	3.9152	0.1049
Butanoate metabolism	0.030236	3.4987	0.03175
Arginine biosynthesis	0.030746	3.482	0
D-Glutamine and D-glutamate metabolism	0.030746	3.482	0
Pentose and glucuronate interconversions	0.032074	3.4397	0.125

DISCUSSION

The body weight of rats decreased due to exposure to CY. The lower body in our experimental study has also been recorded in previous research. The reduced body mass of rats of EE-IS group might be because by taste aversion and appetite via EE metabolites such as alkamides and caffeine (Khattab *et al.*, 2019) . The thymus and spleen are the important and vital immune organs responsible for the proliferation of immunological cells that directly influence the immune system. The indices of the spleen and thymus reflect the morphogenesis and disease resistance ability of

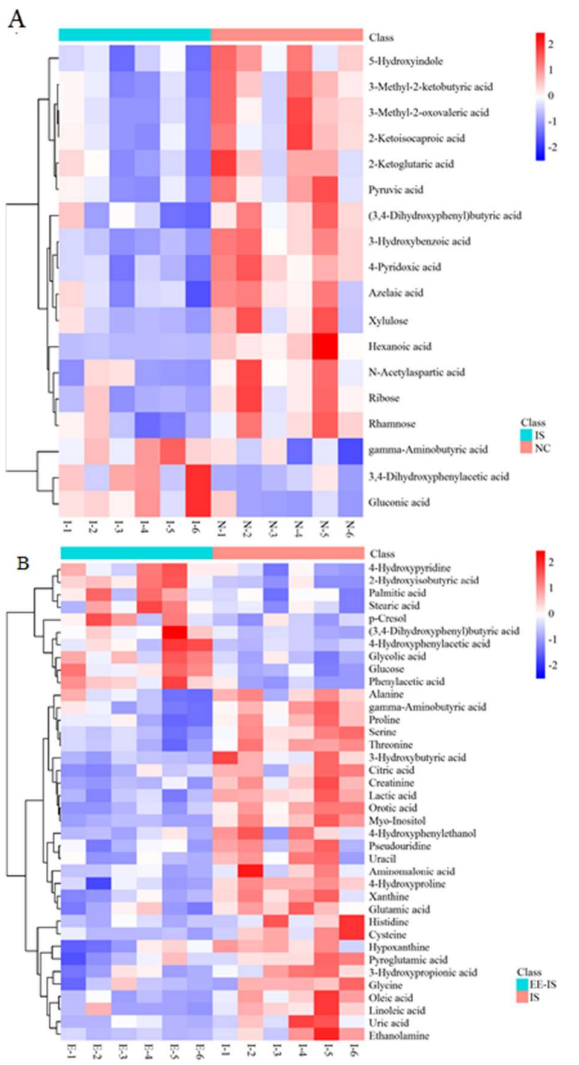

Fig. 4: Heatmap of differentially expressed metabolites. (A) IS group and NC group. (B) EE-IS group and IS group.

Table 2: Differential metabolite pathway between EE-IS group and IS group

Pathway	Raw *p*	-LOG(*p*)	Impact
Inositol phosphate metabolism	0.000064	9.655	0.12939
Phosphatidylinositol signaling system	0.000064	9.655	0.03736
Ascorbate and aldarate metabolism	0.000064	9.655	0
Pyruvate metabolism	0.000068	9.5964	0.08398
Valine, leucine and isoleucine biosynthesis	0.000325	8.0316	0
Sphingolipid metabolism	0.000817	7.1103	0
Cysteine and methionine metabolism	0.000817	7.1093	0.11776
Glyoxylate and dicarboxylate metabolism	0.001093	6.8187	0.1799
Glycine, serine and threonine metabolism	0.001504	6.4998	0.50186
Aminoacyl-tRNA biosynthesis	0.001581	6.4497	0.16667
Fatty acid degradation	0.002073	6.1786	0
Galactose metabolism	0.002328	6.0627	0.03499
Arginine and proline metabolism	0.002442	6.0149	0.24864
Purine metabolism	0.002524	5.982	0.07078
Phenylalanine metabolism	0.002571	5.9636	0
Synthesis and degradation of ketone bodies	0.002904	5.8418	0
Starch and sucrose metabolism	0.00424	5.4633	0.4207
Neomycin, kanamycin and gentamicin biosynthesis	0.00424	5.4633	0
Glutathione metabolism	0.006329	5.0626	0.11891
Porphyrin and chlorophyll metabolism	0.007612	4.878	0
Butanoate metabolism	0.008103	4.8155	0.03175
Histidine metabolism	0.008125	4.8129	0.22131
D-Glutamine and D-glutamate metabolism	0.008133	4.8118	0.5
Arginine biosynthesis	0.008133	4.8118	0.11675
Nitrogen metabolism	0.008133	4.8118	0
Citrate cycle (TCA cycle)	0.009202	4.6884	0.09038
Taurine and hypotaurine metabolism	0.010935	4.5158	0
Thiamine metabolism	0.010935	4.5158	0
Linoleic acid metabolism	0.013636	4.295	1
Propanoate metabolism	0.013744	4.2872	0
Glycerophospholipid metabolism	0.01514	4.1904	0.01324
Alanine, aspartate and glutamate metabolism	0.016616	4.0974	0.28366
Primary bile acid biosynthesis	0.01862	3.9835	0.02239
Pantothenate and CoA biosynthesis	0.021833	3.8243	0
Pyrimidine metabolism	0.022609	3.7894	0.12249
beta-Alanine metabolism	0.022627	3.7886	0
Biosynthesis of unsaturated fatty acids	0.029608	3.5197	0
Tyrosine metabolism	0.032509	3.4262	0
Selenocompound metabolism	0.046639	3.0653	0
Fatty acid biosynthesis	0.047442	3.0482	0.01472
Fatty acid elongation	0.047442	3.0482	0

the organisms. In this study, CY increased the spleen index, which is in contrast to some previous studies and may be due to the stimulation of extramedullary hematopoiesis as a compensatory reaction (Zhu *et al.*, 2019; Chen *et al.*, 2021). In our study, splenomegaly occurred in rats of EE-IS group. Previous research has indicated that *E. purpurea* roots elevate the number of activated lymphocytes in spleen. Thymic atrophy in our study correspond with past finding and improvement was not showed after EE administration. Results showed that there is lack of evidence showing the effects of *E. purpurea* on the thymus. It has been reported that Cichoric acid, one of the EE had been found to reduce the thymus index in the collagen-induced arthritis model (Jiang *et al.*, 2014). Moreover, CY can cause liver damage indicated by a significant difference of ALP, TP, ALB and T-Bil indices of liver function. TP and ALB are important proteins derived from liver and have been identified by substantial studies that *E. purpurea* improves these parameters which is in line with our results (Sadigh-Eteghad *et al.*, 2011; Oskoii *et al.*, 2012; Rezaie *et al.*, 2013). Biochemistry indexes of kidneys, including urea and creatinine, are within a normal reference range. The contents of IL-6 were similar in rats among all groups and were increased under EE treatments. There is no consistent conclusion in the study of *E. purpurea* in the level of IL-6 that might be associated with the difference of the main components of *E. purpurea* and animal model (Park *et al.*, 2018). TNF-α is the valuable and useful element in the modulation of the immune system and can be unregulated through important bioactive components in *E. purpurea* and alkylamides that blinds CB2 receptors resists the expression of TNF-α mRNA. This study investigated that CY inhibited the level of TNF-α and EE had the tendency for increase.

Moreover, the levels of IFN-γ significantly reduced in IS group but were similar to EE group. Previous studies showed that *E. purpurea* had the potential to improve IFN-γ production (Mishima *et al.*, 2004). However, it was also reported that *E. purpurea* did not significantly increase TNF, IL-2, IL-6 and IFN-γ peak levels compared with placebo (Wenner *et al.*, 2015). The results of our experimental study confirmed that CY can damage immunity and EE can reverse this change. These data support further metabolomics research for the mechanisms for *E. purpurea.*

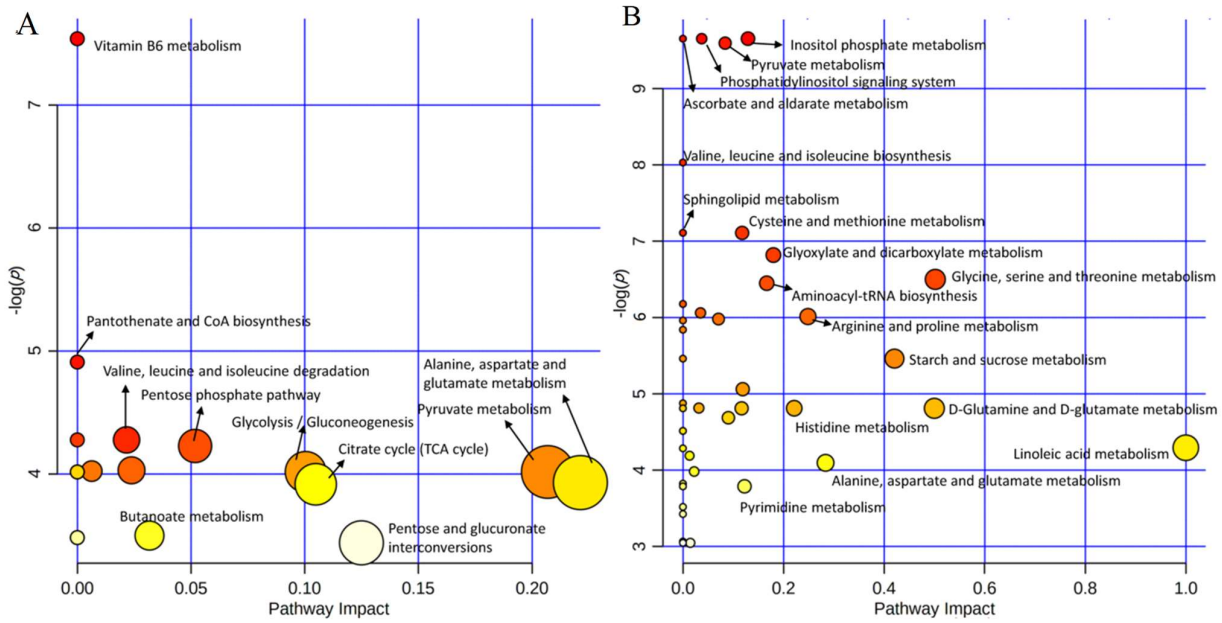

Fig. 5: Pathway analysis of metabolites. (A) IS group and NC group. (B) EE-IS group and IS group.

Metabolomic analysis was used to better understand how EE intervention improves immunosuppression in rats and 18 metabolites were significantly altered in CY-induced rats while EE noticeably changed 38 metabolites. As shown in this study, the differential metabolites in IS group and NC group primarily participate in vitamin B6 metabolism, CoA biosynthesis, valine, leucine and isoleucine degradation, pentose phosphate pathways, arginine and proline metabolism and alanine, aspartate and glutamate metabolism. These metabolic changes were found to be mainly involved in amino acid metabolism and energy metabolism, which are consistent with several studies (Qu *et al.*, 2016; Tian *et al.*, 2019). It was documented that the differential metabolites under CY model, indicated that amino acids, energy metabolism, and organic acids metabolism were the major metabolic pathways. In this study, vitamin B-6 metabolism, pantothenate and CoA biosynthesis are major differential metabolic pathways and the former is most important in metabolome view. The levels of 4-pyridoxic acid and 3-methyl-2-ketobutyric acid were significantly reduced in IS group compared with NC group while the intermediate products of Vitamin B6 metabolisms, pantothenate and CoA biosynthesis, respectively. Vitamin B-6 includes pyridoxal, pyridoxine, and pyridoxamine, which involved reactions like amino acid, carbohydrate, and fat metabolism. Low vitamin B-6 concentrations are associated with inflammation, higher oxidative stress, and metabolic conditions, including cardiovascular disease, inflammatory bowel disease and diabetes (Shen *et al.*, 2010). Pantothenate is an important precursor for the biosynthesis of CoA and acyl carrier proteins (ACP), the necessary materials for the synthesis of steroids by fatty acids and the intermediates for the citric acid cycle, antibody synthesis and choline acetylation. CoA, CoA thioesters, or 49-phosphopantetheine are the cofactors in about 4% of all enzymes, the important material for bacteria to build cell wall. These results suggested that CY could disturb vitamin B6 metabolism and pantothenate and CoA biosynthesis in rats.

E. purpurea polysaccharides could strength the immune system, reverse the adverse effect of CCl4 in AML12 cells and the damage of H_2O_2 in mice and remarkably promote the enzyme activities of catalase, glutathione peroxidase, superoxide dismutase while reduce the content of malondialdehyde in serum and liver tissues homogenate (Hou *et al.*, 2020). The studies of *E. purpurea* in metabonomics indicate that triazoles inhibits gibberellic acid (GA3) biosynthesis which enhances the resistance of *E. purpurea* to pathogens due to modification of phenylpropanoid pathways, the biosynthetic pathways of caffeic acid derivatives. The values of GA3, paclobutrazol, and uniconazole can increase the level of caftaric acid in the roots (Jones *et al.*, 2010). Alkylamides are the important element in *Echinacea* that inhibits the cytochrome P450 3A4 which is the main pathway for the metabolism of xenobiotics and endobiotics (Modarai *et al.*, 2009). Previous study has reported that EE can improve amino acids in serum and urine with age like tyrosine, aspartate, ornithine, serine, alanine, beta-alanine, glutamate, threonine and phenylalanine except glycine in urine and can down-regulate most sugar, long-chain fatty acids and their derivatives (Wang *et al.*, 2017).

The previous studies about immunostimulants in metabolomics showed that low relative molecular mass seleno-aminopolysaccharide could improve immune system through amino acid, nucleotide, and nitrogen metabolism, aminoacyl-transfer ribonucleic acid biosynthesis. It was found that the concentrations of histidine, proline, serine, glutamic acid, and creatine increased in treated groups (Zhou *et al.*, 2019). Interestingly, the metabolites above mitigated in EE-IS group in this study, which might be associated with the difference of animal and treatments. However, the results of differential metabolites indicated myo-inositol decreased in the EE-IS group, a key biomarker in inositol

phosphate metabolism, phosphatidylinositol signaling system and ascorbate and aldarate metabolism. Phospoinositide 3 kinase/Protein kinase B (PI3K-AKT) signaling pathway is most wide attention, involved in a variety of important cellular functions on metabolism, inflammation, and tumor (Huang *et al.*, 2011; Yan *et al.*, 2016). Moreover, results showed that lactic acid was the significant metabolite in pyruvate metabolism and was reduced by EE treatment. Most ATP-dependent processes, such as the membrane-bound pumps, are key factors to maintain the homeostasis of impaired metabolites or ions. The contents of pyruvate and lactate are also severely influenced. Pyruvate is regarded as an antioxidant that could inhibit the generation of free radicals and promote elimination from the mitochondrial electron transport chain. These results suggested that EE could affect inositol phosphate metabolism, phosphatidylinositol signaling system, ascorbate and aldarate metabolism, pyruvate metabolism in CY-induced rats and play important role in immune regulation.

Conclusion: In conclusion, this study showed that EE could improve CY-induced immunosuppression indicated by an elevated level of serum IFN-γ, TNF-α, TP and ALB and regulating the expression of inositol phosphate metabolism, phosphatidylinositol signaling system, ascorbate, and aldarate metabolism, pyruvate metabolism and valine, leucine and isoleucine biosynthesis in immunosuppressive rats.

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