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ORIGINAL ARTICLE

Effects of Total Flavonoids Extracted from *Chromolaena odorata* Linn. on Immunosuppression: A Network Pharmacology-Based and Experimental Study

Yao Hu, Fan Yang, Zhen Lin[1], Aftab Qamar, Di-Hua Zhu, Meng-Meng Chen, Min Zhang, Qing-Lang Song, Dan-Ju Kang, Hong-Ying Lin, Zhi-Bao Chen, Jin-Jun Chen

Department of Veterinary Medicine, College of Coastal Agricultural Sciences, [1]Department of Applied Chemistry, College of Chemistry and Environmental Science, Guangdong Ocean University, Zhanjiang, Guangdong, China

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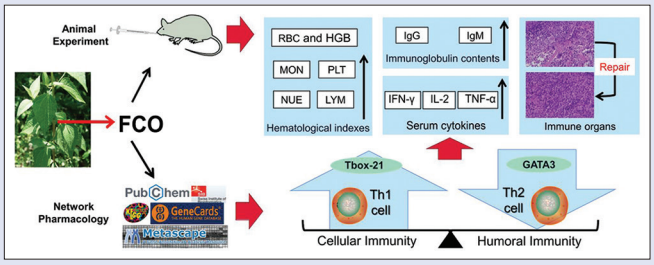
ABSTRACT

Background: *Chromolaena odorata* Linn. (CO) is a perennial herb that is enriched with flavonoids and exhibits immune regulatory activities. For these characteristics, CO can be used as a potential immunoregulator based on the immunosuppression state. **Objectives:** The aim of this study was to assess the effects of flavonoids extracted from CO (FCO) on immune regulation and evaluate their mechanism of action by network pharmacology (NP), followed by *in vivo* confirmation. **Materials and Methods:** FCO were ultrasonically extracted through immersion in alcohol. The potential targets were predicted using a "FCO–immunosuppression–target" network. When the functional enrichment analyses were conducted, a mice model was employed to demonstrate the effects. The hematological indexes and serum levels of immunoglobulin G (IgG), immunoglobulin M (IgM), interferon-γ (INF-γ), interleukin 2 (IL-2), and tumor necrosis factor-α (TNF-α) were measured. The variations observed in immune organs and the changes in expressions of INF-γ, T-box transcription factor 21 (Tbx-21), and GATA Binding Protein 3 (GATA3) were reported. **Results:** NP results showed that a total of 198 targets of FCO were involved in immunosuppression. As indicated by the functional analysis results, FCO impacted T helper (Th) cell differentiation, which might be a vital functional pathway underlying its immune regulatory effects. During animal experiments, the values of hematological indexes, serum levels of IgG, IgM, and TNF-α, and the immune organ indexes increased in FCO groups, and the relative mRNA expressions of INF-γ and Tbx-21 and less damage of the spleen and thymus were reported. **Conclusion:** FCO impacts Th1 and Th2 differentiation pathways and assists in immunosuppression by regulating the secretion of various cytokines and the expression of associated genes, which demonstrates FCO as a promising natural immunoregulator.

Key words: *Chromolaena odorata* Linn., extract, flavonoids, immune regulation, network pharmacology

SUMMARY

- CO is a kind of weed with widespread distribution. CO has been regarded as one of the TCM for containing considerable activities, including immune regulation. The study aims to investigate the effect of FCO on immune regulation and the underlying mechanism. After extraction, we found 38 flavonoids in the leaves of CO by UHPLC-QTOF-MS. NP was employed and 198 targets were found between FCO and immunosuppression. By enrichment and metabolic pathway analysis, we found that FCO affected some factors related to Th1 and Th2 cell differentiation and MAPK pathway. The altered gene levels of relevant factors and recovered organs in the mice model confirmed the ability of its immunoregulation. In detail, the results showed that FCO could increase IgG, IgM, TNF-α, and IFN-γ levels, as well as the expression of Tbox-21. Moreover, the damage of spleen and thymus was partly repaired and the abnormal hematological indexes were recovered. The results of virtual and animal experiments showed that FCO has immune regulation function. This study can deliver a reference for further study on the role of CO as an immunoregulator.



Abbreviations used: CCAS: College of Coastal Agricultural Sciences; CK: Cytokines; *CO: Chromolaena odorata* Linn.; CTX: Cyclophosphamide; FCO: Flavonoids from *Chromolaena odorata* Linn.; GATA3: GATA Binding Protein 3; GDOU: Guangdong Ocean University; H and E: Hematoxylin and eosin; HGB: Hemoglobin; IFN-γ**:** Interferon-γ; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IL: Interleukin; LMS: Levamisole; LYM: Lymphocyte; MAPK: Mitogen-activated protein kinase; MON: Monocyte; NEU: Neutrophil; NP: Network pharmacology; PLT: Platelet; qPCR: Quantitative Real-time Polymerase Chain Reaction; RBC: Red blood cell; Tbx-21: T-box transcription factor 21; TCM: Traditional Chinese medicines; Th: T helper; TNF-α**:** Tumor necrosis factor-α; UHPLC-QTOF-MS: Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry; WBC: White blood cells.

Correspondence:

Prof. Jin-Jun Chen,
Department of Veterinary Medicine, College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang 524088 Guangdong, China.
E-mail: chenjj@gdou.edu.cn.
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INTRODUCTION

Immunosuppression refers to inhibition of the immune response. It is one of the side effects associated with many chemotherapeutic agents[1] and is often adopted following organ transplantation (e.g., the high recurrence of hepatitis C after liver transplantation).[2] In this state, drugs or natural substances capable of regulating the immune system

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hold high significance. Having fewer side effects and a wide spectrum of pharmacological activities, some traditional Chinese medicines (TCM), including *Orostachys japonicus* A. Berger,[3] Astragali Radius,[4] and *Chromolaena odorata* Linn. (CO),[5] turn out to be the ideal choices.

CO is an invasive plant with a wide distribution and utilization all around the globe. Previous studies have shown that the extracts of CO exert antibacterial,[6] antioxidative,[7] antidiabetes,[8] anti-inflammatory, and anticataract activities.[9] These effects may be correlated with its high levels of flavonoids, which themselves are known to exhibit anti-inflammatory,[10] antioxidant,[11] immune regulatory activities,[12] and so on. For instance, apigenin may serve as a potential agent to reduce doxorubicin-induced renal injury[13] and the function of quercetin in the respiratory system has become a research hotspot over the past few years as well,[14] whereas the immunomodulatory activity of flavonoids extracted from CO (FCO) has been rarely studied. The subtle correlation between FCO and the immunomodulatory effect of CO in mice is still unclear.[15]

To gain more insights into the effects of FCO on immune regulation and their underlying mechanism of action, FCO targets associated with immunosuppression and their underlying biological processes were screened through network pharmacology (NP), that is, a promising solution following disease–target–drug interaction networks.[16] Subsequently, their efficacies were confirmed in mice in comparison with levamisole (LMS) as a positive drug and by using cyclophosphamide (CTX) as an immunosuppressant.[17] Our study lays a theoretical and experimental basis for FCO to serve as an immunoregulator.

MATERIALS AND METHODS

Chemicals and reagents

Rutin standard substance (R189033, 95%), LMS (L118865, 99%), and CTX (C126004, 98%) were provided by Shanghai Alading Biochemical Technology Co., Ltd (Shanghai, China). Other reagents and chemicals, obtained from Beijing Baoriyi Biotechnology Co., Ltd. and Shanghai Alading Biochemical Technology Co., Ltd (Shanghai, China), were reagents of analytical pure grade.

FCO extraction

The aerial part of CO was collected from Huguangyan, Guangdong Province, China, in summer 2019 and identified by Professor SuQing Liu from College of Coastal Agricultural Sciences (CCAS) of Guangdong Ocean University (GDOU). AAfter the aerial part was dried and ground, 10 g sample was soaked in 70% ethanol (solid:liquid ratio = 1:500) for 24 h and then treated with an ultrasonic cleaner at 30°C, 300 W, 20 kHz for 30 min (KQ 500DE; Kunshan Supersonic Equipment Company, Kunshan, China). After the 6-h treatment using the rotational evaporating device (RE-52AA, Shanghai Yarong Biochemical Equipment Company), the sample was extracted with one-third volume of petroleum ether and then purified with silica gel column chromatography. Once ethanol was removed and the extract was dried, the FCO powder was produced and stored at GDOU (specimen number: CCAS2112004024).

Determination of FCO extraction rate

The FCO extraction rate was determined by adopting the aluminum nitrate colorimetry methods described previously.[18] In brief, FCO solution (1 ml, concentration: 8 g/l) and rutin standard (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 ml; concentration: 0.08 mg/ml) were treated with $NaNO_2$, Al $(NO_3)_3$, and NaOH. The absorbances were measured at 510 nm with blank reagent as the reference.

Determination of FCO composition

The FCO samples were pretreated as per the method described previously by Abdallah *et al.*[19] and then used for ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS; Nexera UHPLC-30A, from Shimadzu Enterprise Management Co., Ltd, Guangzhou, China; Triple TOF 5600, from AB SCIEX Company, State of California, USA). . Briefly, 100 mg of the dried FCO was extracted with 500 µl of 80% solution containing internal standard (concentration: 5 µg/ml), ultrasonically treated after homogenization, and then left to stand for 1 h. Subsequently, the samples were centrifuged and 2 ml supernatant was extracted to perform UHPLC-QTOF-MS. The substance exhibiting a strong intensity (>100) was selected using Analyst TF 1.7.

The chromatographic column UPLC BEH C18 (1.7 µm × 2.1 × 100 mm) was used with an injection volume of 5 µl, 15 secondary spectra every 50 ms, bombardment energy of 40 eV, as well as a collision energy difference of 20 V. By referencing an existing paper on the parameter setting,[19] the mobile phase and electrospray ion source (ESI) ion source parameters are shown in Tables s1 and s2. The original mass spectrometry

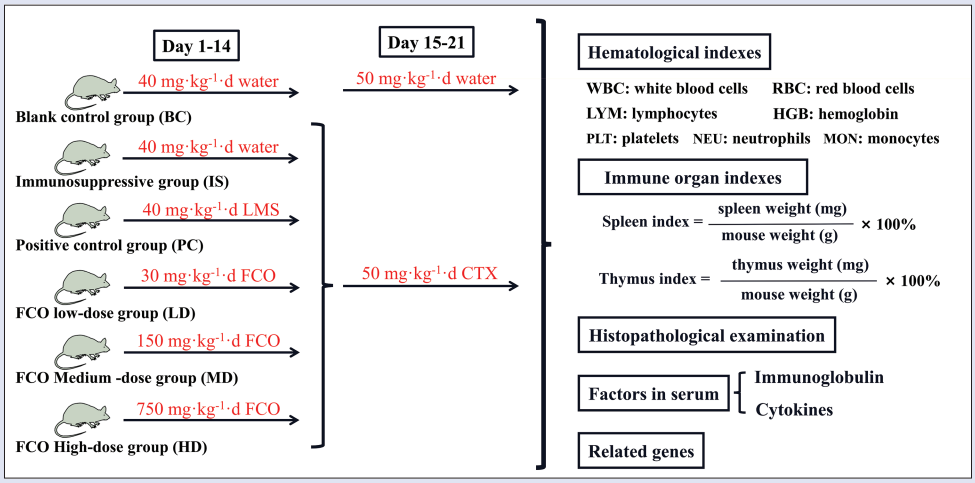

Figure 1: The reagents and operation of animal experiment. Note All the mice received the reagents by oral administration. CTX = cyclophosphamide, FCO = flavonoids extracted from *Chromolaena odorata* Linn., LMS = levamisole

was imported by using Progenesis QI 3.0 software. The peaks were found based on the self-built secondary mass spectrometry database by using the corresponding pyrolysis law matching method.

Identification of repeat proteins between FCO and immunosuppression

The flavonoids obtained were searched online. The 2D frameworks of the flavonoids were obtained using PubChemRDF 1.7 β (https://pubchem.ncbi.nlm.nih.gov/).[20] The flavonoids were selected with Swiss ADME 2021 (http://www.swissadme.ch/index.php) based on a bioavailability score threshold ≥30%,[21,22] and Swiss Target Prediction 2021 (http://www.swisstargetprediction.ch/index.php) was used to predict the FCO targets.[23] All the proteins associated with immunosuppression were searched with the use of GeneCards 5.2 (https://www.genecards.org/),[24] and Wayne map (Venny 2.1.0 (csic.es)) was used to screen immunosuppression and FCO targets. The "FCO–targets–immunosuppression" net was built using Cytoscape 3.7.0 software.

Bioinformatic annotation

By referencing the operation of an existing study,[25] Metascape 3.5 (https://metascape.org/gp/index.html#/main/step1)[26] and KEGG Mapper tools 98.0 (http://www.genome.jp/kegg/)[27] were employed to conduct enrichment and metabolic pathway analysis of the screened targets above. Terms with a *P* value <0.01, a minimum count of 3, and an enrichment factor >1.5 were captured and then integrated into clusters in accordance with their membership similarities.[25]

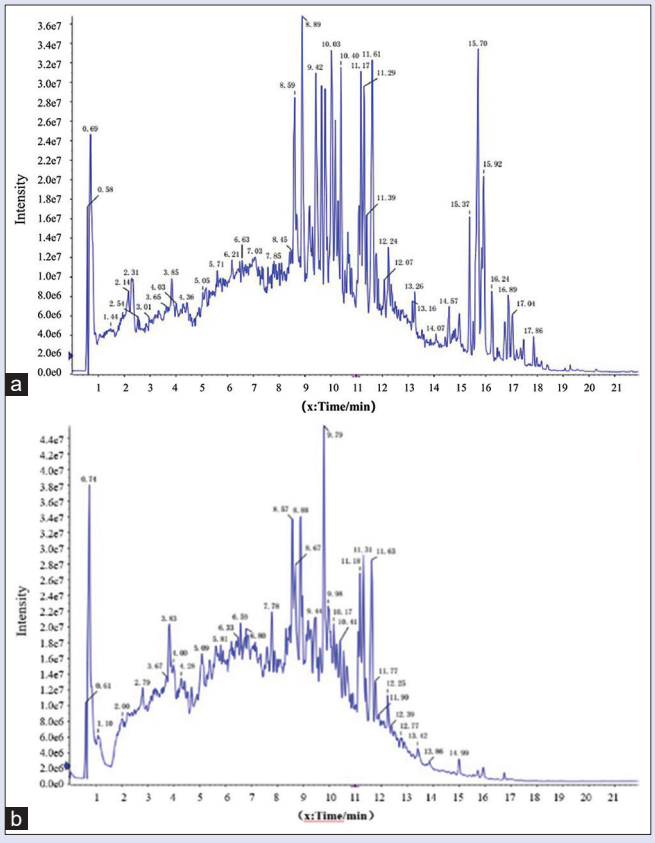

Figure 2: Results of FCO composition after UHPLC-QTOF-MS. Note: a: positive ion mode, b: negative ion mode; FCO = flavonoids extracted from *Chromolaena odorata* Linn., UHPLC-QTOF-MS = ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

Animals and treatment

A total of 72 Kunming mice (average weight: 20 ± 2 g) were obtained from Tianqin Biotechnology Co., Ltd (Hunan province, China) (certificate number: 43006700018844; license number: SCXK, Xiang) and were fed adaptively for 1 week based on a 12 L: 12 D light cycle. After undergoing the 1-week acclimatization period, the mice stochastically fell into six groups [Figure 1], with 12 mice in each group (male:female = 1:1; average age: 6 weeks). All the reagents were endotoxin free. The experimental protocols involving animals were approved by the Institutional Committee for Animal use and Ethics of the CCAS, GDOU (protocol approval number: GDOU2019052A).

Hematological indexes

All mice were euthanized on day 22 with CO_2 inhalation. Hematological indexes and immune organ index have been found as the routine targets to measure the immunity level of mice.[17,28] One hundred microliters of blood sample was obtained from the tail veins of the respective group and placed into a centrifuge tube containing the anticoagulant Ethylenediaminetetraacetic acid (EDTA)-Na_2. The numbers of white blood cells (WBC), red blood cells (RBC), lymphocytes (LYM), hemoglobin (HGB), platelets (PLT), neutrophils (NEU), and monocytes (MON) were analyzed with the use of the automated blood cell analyzing device (URIT 5180; Youlite Electronic Group Company, Guilin, China).

Immune organ indexes

The spleen and thymus were obtained from each member in the respective group. After the surface liquid on organs was dried with

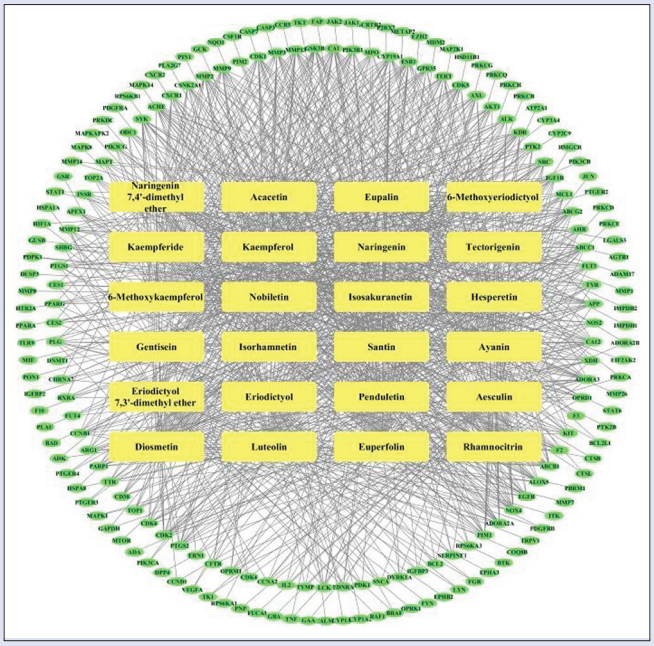

Figure 3: Network analysis of FCO–immunosuppression targets. Note: The inner ring: degree >3. FCO = flavonoids extracted from *Chromolaena odorata* Linn.

a filter paper, an analytical balance (Ar3130, Ohouse International Trading Company) was used to record the weights of spleen, thymus, and body.

Pathological examination of immune tissues

The effects of FCO at the histological level were assessed.[1] The thymus and spleens of all the mice were extracted. Next, 10% formaldehyde solution was used to fix the extracted thymus and spleen for over 24 h and was washed using running water. After being treated with ethanol and xylene, the samples were subjected to paraffin embedment and then sectioned. The tissue samples were stained with hematoxylin and eosin (H and E) and examined under a microscope (Eclipse Ci-E, Nikon Instruments Shanghai Co., Ltd).

Immunoglobulin and cytokines

Five hundred microliters of blood sample was collected and centrifuged for 10 min at 4°C at 3000 rpm. The supernatant was used to assess the levels of immunoglobulin G (IgG), immunoglobulin M (IgM), interleukin 2 (IL-2), tumor necrosis factor-α (TNF-α), and immune interferon-γ (INF-γ) in the serum using the enzyme-linked immunosorbent assay (ELISA) kit (Enzyme linked Biotechnology Co., Ltd., Shanghai, China).[1]

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

The RNA abstraction kit (Nanjing Novozan Biotechnology Co., Ltd, Nanjing, China) was leveraged to abstract the total RNA from the

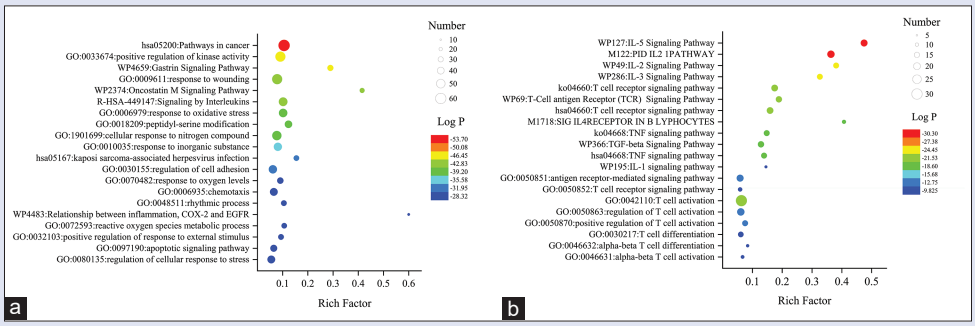

Figure 4: Bioinformatic analyses of the intersection proteins. (a) Top 20 enriched terms in each term cluster; (b) Top 20 terms related to immunosuppression.

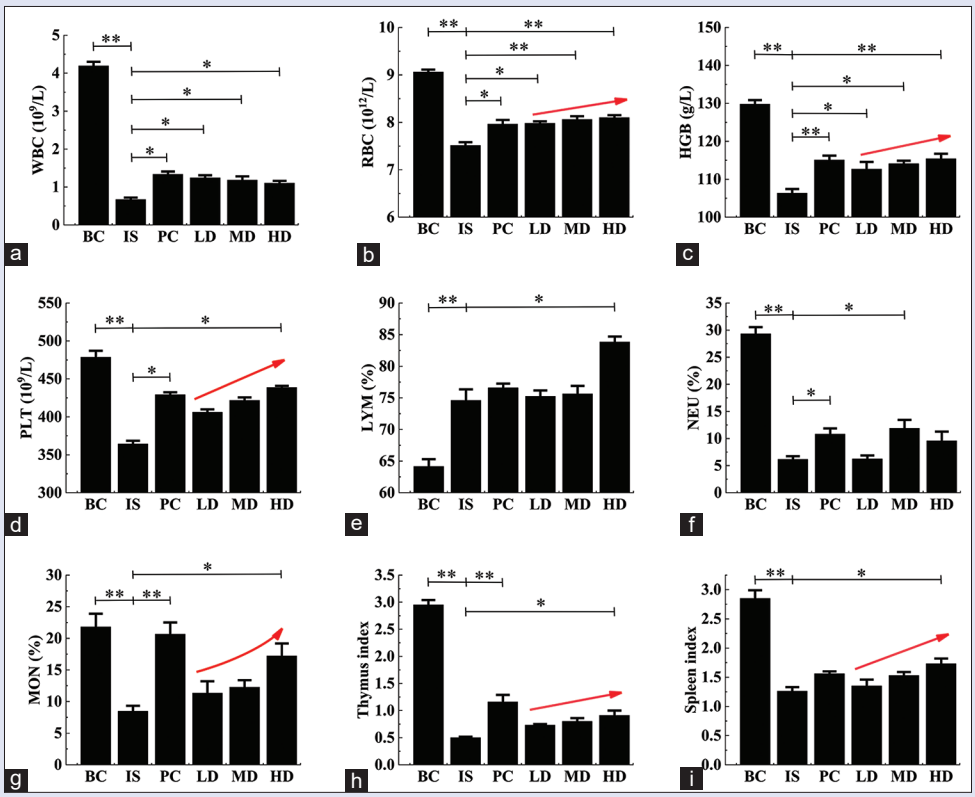

Figure 5: (a) white blood cells (WBC); (b) red blood cells (RBC); (c) hemoglobin (HGB); (d) platelets (PLT); (e) lymphocytes (LYM); (f) neutrophils (NEU); (g) monocytes (MON); (h) Thymus index; (i) Spleen index. FCO = flavonoids extracted from *Chromolaena odorata* Linn.; BC: blank control group; IS: immunosuppressive group; PC: positive control group; LD: FCO low-dose group (30 mg•kg-1•d); MD: FCO medium-dose group (150 mg•kg-1•d); HD: FCO high-dose group (750 mg kg^{-1} d). *: $P<0.05$, **: $P<0.01$, in contrast to IS

extracted spleens in accordance with the manufacturer's instructions. To analyze the gene expression, the extracted RNA was transcribed into cDNA for Quantitative Real-time Polymerase Chain Reaction (qRT-PCR / qPCR). The primer sequences (Shanghai Biotechnology Co., Ltd) used for qPCR are presented in Table s3. The relative expressions of the target genes were determined using the $2^{-\Delta\Delta Ct}$ method.

Statistical methods

All data were processed using Statistical Package for the Social Sciences (SPSS) 22.0 software (SPSS Inc., Chicago, IL, USA) for statistical analyses. One-way analysis of variance (ANOVA) test and Least—Significant Difference approach (LSD) approach were employed for comparison of significance and the results were displayed as average ± standard deviation (SD). $P < 0.05$ indicated statistical significance. $P < 0.01$ indicated remarkable differences. The outcomes were presented with the use of Origin 2018 software (OriginLab, Northampton, MA, USA).

RESULTS

Extraction rate and components of FCO

The equation for the standard curve of rutin by linear regression analysis was as follows: y = 8.1362x − 0.0159 (R^2 = 0.988), indicating a linear relationship between absorbance and rutin level [Figure s1].

FCO extraction rate (%) = (A + b)× 25 × 250 × 100/(k × 1000 × 2)

where A is the extinction of FCO and the measured extinction of FCO was 0.20, B is the intercept of the equation, and k is the coefficient of the equation. The extraction rate of FCO was obtained as 8.29% after calculation.

As indicated by Figure 2a and b, UHPLC-QTOF-MS found 22 flavonoids in a positive ion mode and 16 flavonoids in the other mode (total: 38 flavonoids).

Identification of repeat proteins between FCO and immunosuppression

In this study, only 24 flavonoids exhibited satisfactory bioavailability and predictability after NP [Table s4]. Thus, 413 targets in FCO were collected [Table s5], of which 198 key targets were associated with immunosuppression [Figure s2, Figure 3].

Bioinformatic analyses of FCO–immunosuppression intersection proteins

As shown in Figure 4a, the 198 key targets associated with immunosuppression were primarily correlated with cancer, kinase activity, wound healing, oxidation and toxic substance, signaling by interleukins, inflammation, and others [Figure 4a]. Moreover, enrichment analysis revealed that numerous pathways of interleukins, T-cell receptors, and T helper (Th) cell differentiation were influenced by those targets [Figure 4b].

Figure s3 shows that FCO can change the expression of some factors involved in the Th1 and Th2 cell differentiation pathway, which certainly affects the balance of Th cell subsets to a certain extent under immunosuppression.

According to Figure 5, WBC (Figure 5a), RBC (Figure 5b), and HGB (Figure 5c) in all FCO test groups increased significantly ($P < 0.05$), while LYM (Figure 5e), MON (Figure 5g), and PLT (Figure 5d) increased significantly in FCO high dose group (HD; $P < 0.05$). In conclusion, all the hematological indexes, expect WBC (Figure 5a) and NEU (Figure 5f), increased in proportion with the FCO dose.

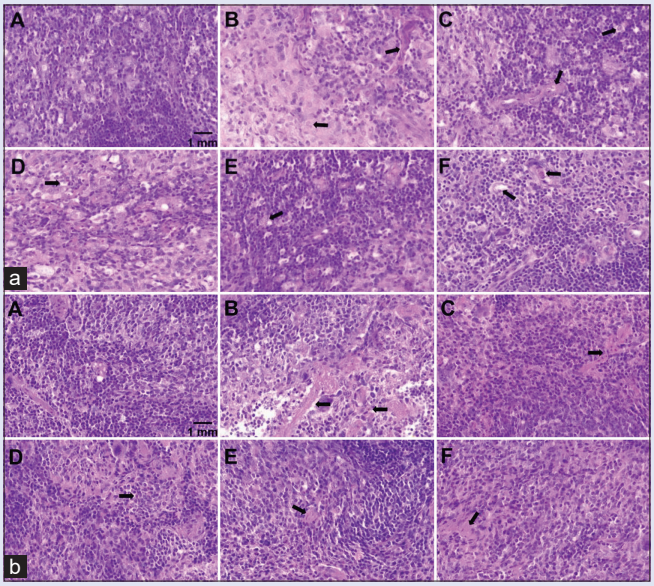

Figure 6: (a) Effect of FCO on thymus histopathology in mice (400×). A: blank control group; B: immunosuppressed group, the thymocytes of cortex decreased, in which the thymocytes were broken and the interlobular septum was widened (arrow); C: positive control group, the cortical thymocytes were dense and the thymic corpuscle was intact, whereas the interlobular septum was widened (arrow); D: FCO low-dose group, fewer thymocytes in cortex and thymocyte fragments (arrow); E: FCO medium-dose group, the thymocytes of cortex and the thymic corpuscle were normal (arrow); F: FCO high-dose group, the boundary between cortex and medulla was significant with intact thymic corpuscle structure and clear vein structure (arrow). All the pictures are ×400 magnification. (b) Effect of FCO on spleen histopathology in mice (400×). A: blank control group; B: immunosuppressed group, LYM decreased with disordered trabecular structure and hemorrhagic spots were found (arrow); C: positive control group, the trabecula were normal (arrow); D: FCO low-dose group, the LYM in white pulp had a decreased number (arrow); E: FCO medium-dose group, the ellipsoid structure was intact (arrow) and the boundary between the white and red pulp was clear; F: FCO high-dose group, the ellipsoid structure was intact (arrow) with no other lesions. All the pictures are ×400 magnification. FCO = flavonoids extracted from *Chromolaena odorata* Linn., LYM = lymphocytes

Repair of the thymus and spleen in mice

As shown in Figure 5h and i, compared with the immunosuppressive group (IS), the thymus index of positive control group (PC) increased 1.82 times ($P < 0.01$) and the thymus and spleen indexes of the HD increased as well ($P < 0.05$). Moreover, the immune organ indexes of mice in FCO groups increased in proportion with increase in the dose.

According to Figure 6, compared with IS, the thymocytes and LYM significantly increased in the FCO groups with improved histological structures. The pathological sections above and the increased immune organ indexes indicated the resistance of FCO to immunosuppression.

Level of serum factors and expression of transcription factors

According to Figure 7a and b, the concentration of serum immunoglobulins increased as well ($P < 0.05$ in HD). In addition, the IgM content of HD was 24.57% higher than that in PC.

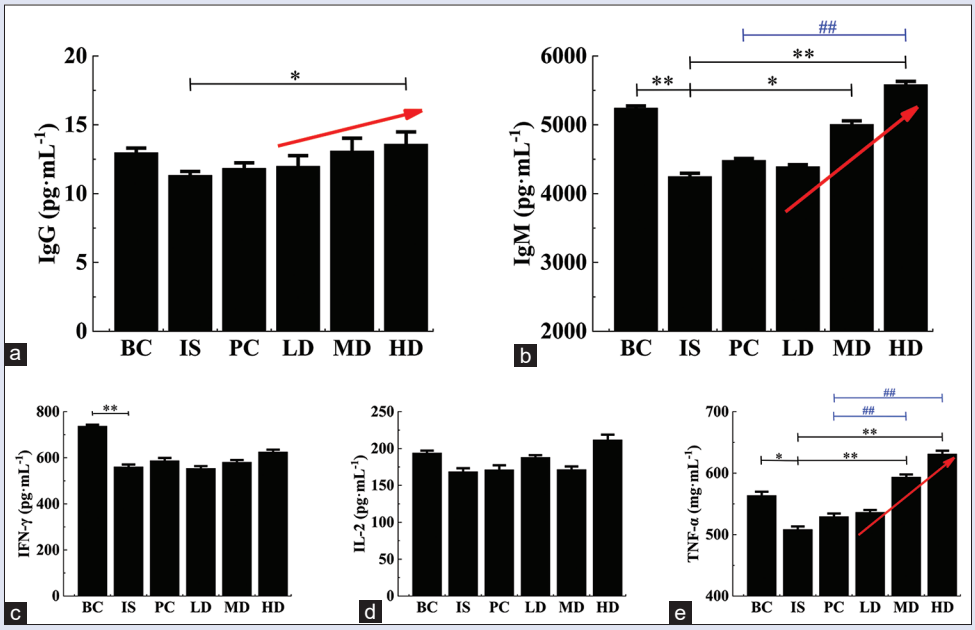

Figure 7: (a) immunoglobin G (IgG); (b) immunoglobin M (IgM); (c) immune interferon interferon-γ (INF-γ); (d) interleukin 2 (IL-2); (e) tumor necrosis factor (TNF-α). FCO = flavonoids extracted from *Chromolaena odorata* Linn.; BC: blank control group; IS: immunosuppressive group; PC: positive control group; LD: FCO low-dose group (30 mg kg^{-1} d); MD: FCO medium-dose group (150 mg kg^{-1} d); HD: FCO high-dose group (750 mg•kg-1•d). **P*<0.05, ***P*<0.01, compared with IS; #*P*<0.05, ##*P*<0.01, compared with PC

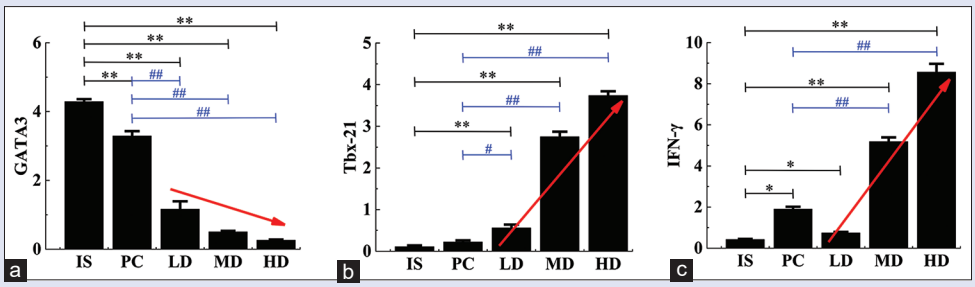

Figure 8: (a) GATA Binding Protein 3 (GATA3); (b) T-box transcription factor 21 (Tbx-21); (c:) immune interferon interferon-γ (INF-γ). FCO = flavonoids extracted from *Chromolaena odorata* Linn.; BC: blank control group; IS: immunosuppressive group; PC: positive control group; LD: FCO low-dose group (30 mg•kg-1•d); MD: FCO medium-dose group (150 mg kg^{-1} d); HD: FCO high-dose group (750 mg kg^{-1} d). **P*<0.05, ***P*<0.01, in contrast to IS; #*P*<0.05, ##*P*<0.01, in contrast to PC

As indicated by Figure 7c–e, no significant changes were reported in IL-2 content among the six groups ($P > 0.05$), whereas IFN-γ and TNF-α increased with increase in the concentration of FCO. Moreover, the TNF-α content in FCO medium-dose group (MD) and HD was significantly higher ($P < 0.01$) compared with IS and even the PC group, indicating that FCO can stimulate some cytokines and change their levels to varying degrees.

The results of qPCR were reliable [Figures s4–s7]. Figure 8 shows that the relative mRNA expressions of T-box transcription factor 21 (Tbx-21) and INF-γ were threefold higher in HD compared with IS ($P < 0.01$), while GATA Binding Protein 3 (GATA3) decreased noticeably in all FCO groups ($P < 0.01$). Compared with PC, FCO upregulated the relative expressions of INF-γ and Tbx-21 ($P < 0.05$), whereas it significantly downregulated the Th2-related transcription factor GATA3 ($P < 0.01$).

DISCUSSION

NP has been extensively employed in numerous fields of TCM due to its ability to screen traditional herbs for natural active substances and discover the underlying mechanism.[29,30] The mechanism of 12 flavonoids from sea buckthorn on hyperlipidemia has been explored through NP analysis and verified at cellular levels.[31] In addition, some of the 32 flavonoids from *Radix scutellariae* exhibited a strong α-glucosidase inhibitory activity and might impact type II diabetes via Peroxisome proliferator-activated receptor (PPAR) signaling pathway only with the assistance of NP.[32] In a previous study, 13 flavonoids were extracted from CO and their ethanol extracts were found to exhibit no cytotoxicity within a concentration.[33] Combined with the previous studies, FCO is considered as a potential natural immunoregulator for its extractability, low toxicity, and the related pharmacological activities revealed by NP.

CTX negatively affects the immune function,[34] whereas LMS can significantly regulate the immune system.[35] Ideal results were achieved for CTX and LMS in our study. The effect of flavonoids on the hematological index discovered previously[36] were found to be consistent with the results of the current study. FCO can normalize the hematological index without causing any hemolysis phenomenon. The increase in LYM and MON was in a direct correlation with the

immune enhancement; they resisted immunosuppression together with elevated RBC, HGB, and PLT. Thymus and spleen are the main immune organs of mice; their indexes have been found to decrease in the CTX group.[37] Among the flavonoids, apigenin significantly improved the mice's relative spleen weight[38] and genistein exerted an identical effect to apigenin in immunosuppressed broilers.[12] Moreover, it has been demonstrated that the flavonoids of *Astragalus* could possibly increase the thymus and spleen indexes.[39] FCO achieved the same effect in this study. With the increase in tissue cells, the indexes of immune organs in FCO test groups also increased. This phenomenon was hinted by NP – the wound response pathway was affected. On the whole, FCO has a relatively potent ability to repair immune organs in immunosuppressed mice at a dose of 750 mg/kg/day, with the effect almost consistent with that of the LMS group. Similar tendencies were found in the levels of IgG and IgM, which are the major proteins involved in immune response and produced by activated B lymphocytes.[40] It has been demonstrated that quercetin administered with ovalbumin can significantly help increase serum IgG titers in mice, and it serves as an adjuvant.[41] Kaempferol exhibits identical adjuvant property.[14] However, our results showed that flavonoids can also be effective when used alone. The increased levels of IgG and IgM in FCO groups refer to one of the characteristics of immune enhancement and functional recovery of immune organs, especially the thymus capable of regulating B lymphocytes.

When immunosuppression happens, immune regulation is extremely crucial, and the Th cells significantly help regulate and balance the immune system, of which Th1 cells primarily impact cellular immunity and Th2 cells are involved in humoral immunity.[42] As indicated by Metascape's findings, FCO could disrupt certain signaling pathways involved in Th cell differentiation. Among these pathways, the Mitogen-activated protein kinase (MAPK) pathway is the most affected pathway; it is involved in the development of IFN-γ and impacts Th cell differentiation.[43] However, Th1 and Th2 cell differentiation has been found as the most intuitive pathway for immune balance among the affected pathways and it has a clear transcriptional regulation.[44] T-bet and GATA3 are found to be two transcription factors primarily impacting the transcriptional program of differentiation. To be specific, T-bet affects the Th1 cells and GATA3 affects the Th2 cells. Furthermore, some cytokines can affect Th cell differentiation. For instance, IL-2 and INF-γ primarily affect Th1 cells, while IL-4 and IL-5 affect Th2 cells.[40] TNF-α is correlated with Th1 response.[45]

The most crucial compounds in our findings were aesculin and eupalin, which linked with both IL-2 and TNF by network analysis. The studies on eupalin are relatively limited, while aesculin has multiple activities and can affect the MAPK pathway.[46] In addition, some effects of other flavonoids on these factors have also been investigated. Genistein, associated with 20 targets of immunosuppression by NP, is found to downregulate IL-4 and IL-5 levels and upregulate INF-γ level in mice.[47] Genistein inhibited Th2 cells by downregulating GATA-3 level and upregulating T-bet level. This finding was consistent with the findings of current study, with upregulation of IL-2, INF-α, and TNF-γ levels. Cytokines associated with Th2 cells could not be found, but it could be speculated that FCO decreased Th2 cell–associated cytokines via the relevant pathways and by significantly decreasing GATA3 levels. Besides, baicalin, a flavonoid other than FCO, reduced the ratio of T-bet/GATA-3.[48] However, other studies had mixed results with respect to these factors. As indicated by an existing paper, the mice were immunized by kaempferol with or without ovalbumin. Kaempferol, different from other flavonoids, did not affect GATA-3 expression,[41] though it has been linked with 51 targets in this paper. This is because the immunosuppression due to CTX follows a Th2 cell–dominated state[49] and the addition of FCO can upregulate Tbx-21 expression level. Since ovalbumin already stimulates immune enhancement as an antigen, kaempferol is not required to decrease GATA-3 level, but it serves as an adjuvant to increase the levels of both Th1 and Th2 cells to improve the body's overall immune ability, which is a flexible type of immune regulation.

CONCLUSION

In conclusion, this study demonstrated that FCO can suppress immunosuppression in mice through adjustment of the delicate balance between Th1 and Th2 cell subgroups by NP and *in vivo*, and its underlying mechanism of action is significantly dependent on the regulated Tbx-21 expression and some other factors in some signaling pathways, especially the Th1 and Th2 cell differentiation pathway as well as the MAPK pathway.

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Conflicts of interest

There are no conflicts of interest.

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