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Glabridin Plays Dual Action to Augment the Efficacy and Attenuate the Hepatotoxicity of Methotrexate in Arthritic Rats

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Cite This: *ACS Omega* 2022, 7, 34341−34351

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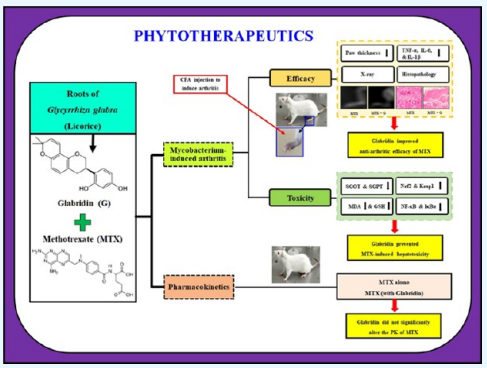
ABSTRACT: Glabridin is chemically an isoflavane class of natural phenols and is found mainly in the roots of *Glycyrrhiza glabra*. It has several beneficial pharmacological actions for the management of inflammatory disorders as well as can counteract drug-induced toxic effects. On the other hand, methotrexate (MTX) is the first-line disease-modifying antirheumatic drug for the treatment of rheumatoid arthritis. However, its treatment is associated with major side effects like hepatotoxicity. In the quest to explore a suitable combination therapy that can improve the efficacy and reduce the hepatotoxicity of MTX, we hypothesized that glabridin might serve the purpose for which there is no literature precedent to date. We explored the antiarthritic efficacy of MTX in the presence or the absence of glabridin using Mycobacterium-induced arthritic model in rats. The results of reduction in paw swelling, inhibition of serum cytokines (TNF-α, IL-6, and IL-1β), and improvement in the bone joints from radiological and histopathological findings suggest that glabridin can substantially augment the antiarthritic efficacy of MTX. Further, results of concomitant glabridin treatment with MTX in the experimental time frame demonstrate that glabridin could considerably prevent the MTX-induced hepatic alteration in serum biochemical markers (SGPT and SGOT) and oxidative stress markers (malondialdehyde (MDA) and glutathione reduced (GSH)). Moreover, glabridin showed a marked effect in impeding the regulation of NF-κB/IκBα and Nrf2/Keap1 pathways in the hepatic tissues. The results of simultaneous administration of glabridin with MTX in the rat model indicate that glabridin had no pronounced effect of causing severe alteration in the pharmacokinetic behavior of MTX. In summary, glabridin can significantly potentiate the antiarthritic efficacy of MTX and can also minimize its hepatotoxicity *via* the inhibition of inflammation and oxidative stress. Further research should be performed to develop glabridin as a phytotherapeutics for the improved efficacy and better tolerability of MTX at the reduced dose level of MTX.



1. INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic joint inflammation.[1] Disease-modifying antirheumatic drugs (DMARDs) are most effective in managing the severity of this disease.[2] Methotrexate (MTX), a conventional synthetic DMARD, is the first-line therapy for RA as per the European Alliance of Associations for Rheumatology (EULAR) recommendations.[3] The possible mechanism for the beneficial action of MTX treatment involves inhibiting cytokine production[4] *via* modulation of signaling pathways such as NF-κB, MMPs, JAK-STAT, and ADORA2A/ADORA3.[5−7] MTX is generally prescribed at a lower dose level for RA treatment than its dose in anticancer therapy.[3] Despite the proven effectiveness of MTX in treating RA, it tends to precipitate significant side effects such as hematological toxicity, liver toxicity, renal toxicity, etc.[8−10] Hepatotoxicity is one of the common adverse effects of MTX, where MTX and its metabolites contribute to the generation of oxidative stress followed by apoptosis that leads to liver damage.[11]

In this context, combination therapy is an attractive approach to improve efficacy and lower the toxic effects of drugs.[12,13] To achieve this goal, researchers throughout the worldwide have focused on the use of plant-based natural products.[14−16] The exploration of plant-based medication may be beneficial in treating chronic diseases because these seem to be safe, and many marketed drugs are directly or indirectly obtained from plant sources.[17−19] Further, it would be beneficial if phytochemicals have their own ability to complement the pharmacological activity of crucial drugs like MTX as well as can counteract the dose-related adverse effects like hepatotoxicity associated with its therapy.[20−23] In this pursuit, we hypothesized that glabridin might serve this purpose.

Received: June 24, 2022
Accepted: September 2, 2022
Published: September 14, 2022

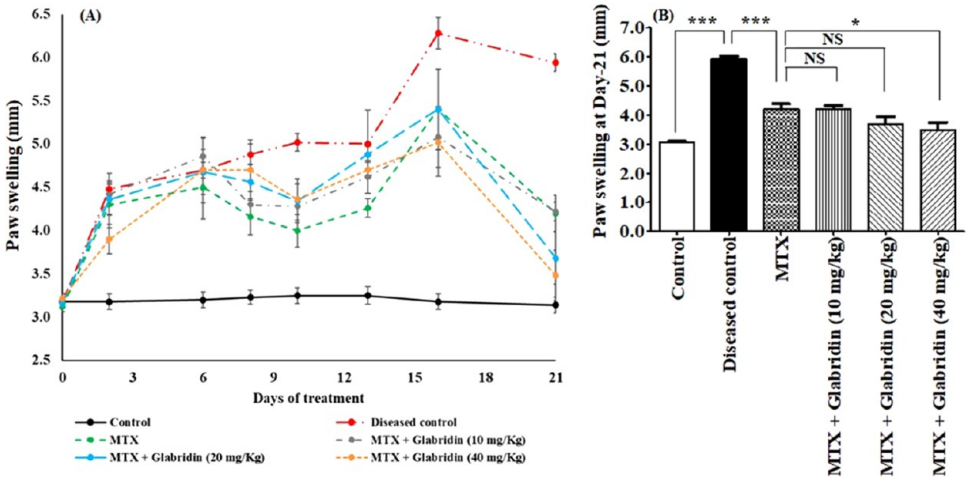


Figure 1. Effect of MTX in the presence of glabridin on the paw swelling changes (A) during the experimental time frame and (B) at the end of the experiment. Each value is expressed as mean ± standard error mean (SEM) (n = 5). Significance level: *p < 0.05 and ***p < 0.001. NS represents statistically insignificant.

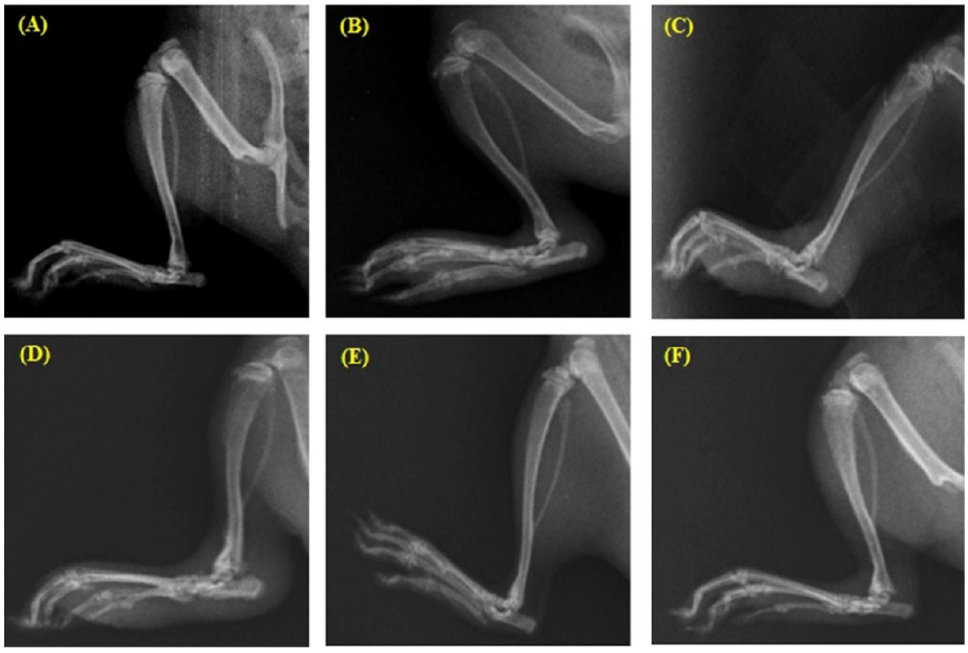


Figure 2. Effect of MTX in the presence of glabridin on the bone joints of the rat's hind limb using radiological examination: (A) control group, (B) diseased control group, (C) MTX alone group, (D) MTX in combination with glabridin (10 mg/kg) group, (E) MTX in combination with glabridin (20 mg/kg) group, and (F) MTX in combination with glabridin (40 mg/kg) group.

We selected glabridin as an experimental candidate in the present study for the following reasons: (a) It is one of the phytoconstituent from traditionally and widely used medicinal plants, namely, *Glycyrrhiza glabra*;[24] (b) the consumption of *G. glabra* in human seems to be safe as a food substance/nutraceutical based on the recommendation by the United States Food and Drug Administration (USFDA) as Generally Recognized as Safe (GRAS)[25] and by Food Safety and Standards Authority of India (FSSAI);[26] and (c) extracts/herbal products of *G. glabra* containing glycyrrhizin have reported for antiarthritic activity. Glabridin is reported to protect human chondrocytes (*in vitro*) from inhibiting osteoarthritis[27] and is also reported to prevent cartilage damage in monosodium iodoacetate-induced arthritis in rats.[28] However, there is hardly any report available to date on the efficacy of MTX with glabridin. (d) Glabridin is reported to have anti-inflammatory activity using the *in vitro*/*in vivo* model[29−31] that may be useful to aid MTX action and (e) several research works are ongoing to use phytochemicals like crocin, resveratrol, chrysin, ellagic acid, rutin, glycyrrhetinic acid, etc. for minimizing the toxic effects of MTX, especially hepatotoxicity.[32−36] We also reported earlier that glabridin could combat the MTX-induced hepatotoxicity via inhibiting oxidative stress, inflammation, and apoptosis using a mice model.[37]

Under these circumstances, it is imperative to investigate the effect of glabridin on the improved efficacy and the lowered hepatotoxicity of MTX. Therefore, we evaluated the same using the Mycobacterium-induced arthritis model in rats. As a part of combination therapy, we also explored further the effect of glabridin on the pharmacokinetics of MTX utilizing a rat model.

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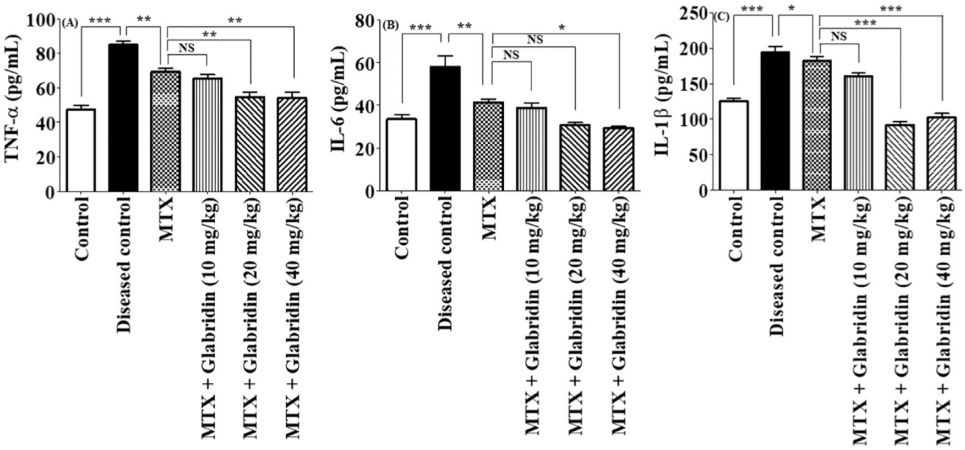

Figure 3. Effect of MTX in the presence of glabridin on serum cytokine levels: (A) TNF-α, (B) IL-6, and (C) IL-1β. Each value is expressed as mean ± SEM (n = 5). Significance level: *p < 0.05, **p < 0.01, and ***p < 0.001. NS represents statistically insignificant.

2. RESULTS

2.1. Glabridin Declined the Paw Swelling. Paw swelling was determined based on paw thickness measurement. The results of change in the paw swelling for the entire experimental time frame are presented in Figure 1A. Comparative paw swelling data on day 21 are shown in Figure 1B. Complete Freund's adjuvant (CFA) treatment resulted in a significant increase in paw swelling compared to the control (p < 0.001). In comparison to the disease control, increased paw swelling was substantially restricted by MTX treatment (p < 0.001). Among all of the experimental dose levels of glabridin, treatment of glabridin at 40 mg/kg in combination with MTX decreased the paw swelling considerably compared to the MTX alone (p < 0.05).

2.2. Glabridin Restricted Radiological Changes in the Joints. X-ray imaging of the right hind paws was performed to observe any changes related to severe soft tissue swelling, bone proliferation, and reduction in the joint space of metatarsals as well as phalanges, which occurred due to CFA treatment. The above-mentioned remarkable changes were observed in the diseased control group compared to the control (Figure 2). MTX treatment considerably reduced the alterations mentioned above compared to disease control. Concomitant glabridin therapy with MTX elicited a marked improvement based on the changes in the paw architecture, especially bone proliferation/erosion.

2.3. Glabridin Inhibited the Inflammatory Cytokines in the Serum. The level of TNF-α, IL-6, and IL-1β was significantly elevated in the diseased control group compared to the control (p < 0.001) (Figure 3). Treatment of MTX reduced the levels of TNF-α (p < 0.01), IL-6 (p < 0.01), and IL-1β (p < 0.05) markedly compared to the diseased control. The decline in all of the cytokine levels by glabridin at 10 mg/kg compared to only MTX treatment lacks statistical significance. However, glabridin at 20 mg/kg with MTX notably dropped the levels of TNF-α (p < 0.01) and IL-1β (p < 0.001) compared to MTX alone. Concomitant treatment of glabridin at 40 mg/kg with MTX reduced the levels of TNF-α (p < 0.01), IL-6 (p < 0.05), and IL-1β (p < 0.001) substantially compared to MTX alone.

2.4. Glabridin Prevented the Histopathological Alterations in the Bone Joints. Histopathological examination of the bone joints was performed through the hematoxylin and eosin (H&E) staining method (Figure 4). The histological scoring was also performed using the important histopathological features of bone joints, such as inflammatory cell infiltration, bone erosion, synovial proliferation, and pannus formation (Figure 5). All of the parameters mentioned above, were notably elevated in the diseased control compared to the control (p < 0.001). MTX treatment exhibited a marked lessening of all the parameters such as inflammatory cell infiltration (p < 0.05), synovial proliferation (p < 0.05), and pannus formation (p< 0.001), except bone erosion compared to the disease control. Glabridin treatment at 10 mg/kg with MTX did not substantially affect all of the parameters compared to MTX alone. Upon concomitant treatment of glabridin with MTX at 20 mg/kg considerably checked the inflammatory cell infiltration and synovial proliferation (p < 0.05) as well as bone erosion and pannus formation (p < 0.01). Glabridin (40 mg/kg) with MTX noticeably lowered all of the parameters like inflammatory cell infiltration (p < 0.01), bone erosion (p < 0.001), synovial proliferation (p < 0.001), and pannus formation (p < 0.001).

2.5. Glabridin Declined the Hepatic Marker Enzyme Levels in the Serum. The hepatic marker enzymes such as SGOT and SGPT were considerably elevated (p < 0.001) by MTX treatment compared to the diseased control. Concomitant treatment of glabridin at all of the dose levels with MTX significantly dropped both the SGOT (Figure 6A) and SGPT (Figure 6B) levels compared to the MTX alone (p < 0.05).

2.6. Glabridin Lowered the Oxidative Stress Marker Levels in the Hepatic Tissues. The content of malondialdehyde (MDA) and glutathione reduced (GSH) was significantly augmented and declined, respectively, upon MTX treatment compared to the diseased control (p < 0.05). Simultaneous treatment of MTX with glabridin at all of the dose levels exerted a substantial drop in MDA content compared to only MTX treatment (p < 0.001) (Figure 6C). Among all of the dose levels of glabridin, concurrent administration of MTX with glabridin at only 40 mg/kg showed a marked effect in preventing the MTX-induced depletion in the GSH level (p < 0.05) (Figure 6D).

2.7. Glabridin Downregulated the NF-κB/ IκBα and Nrf2/Keap1 Levels in the Hepatic Tissues. Western blotting (WB) was performed to assess the alteration in the protein expression of NF-κB, IκBα, Nrf2, and Keap1 in the hepatic tissues (Figure 7). Results displayed the significant upregulation of NF-κB expression in the diseased control compared to the control (p < 0.01). Only MTX treatment considerably

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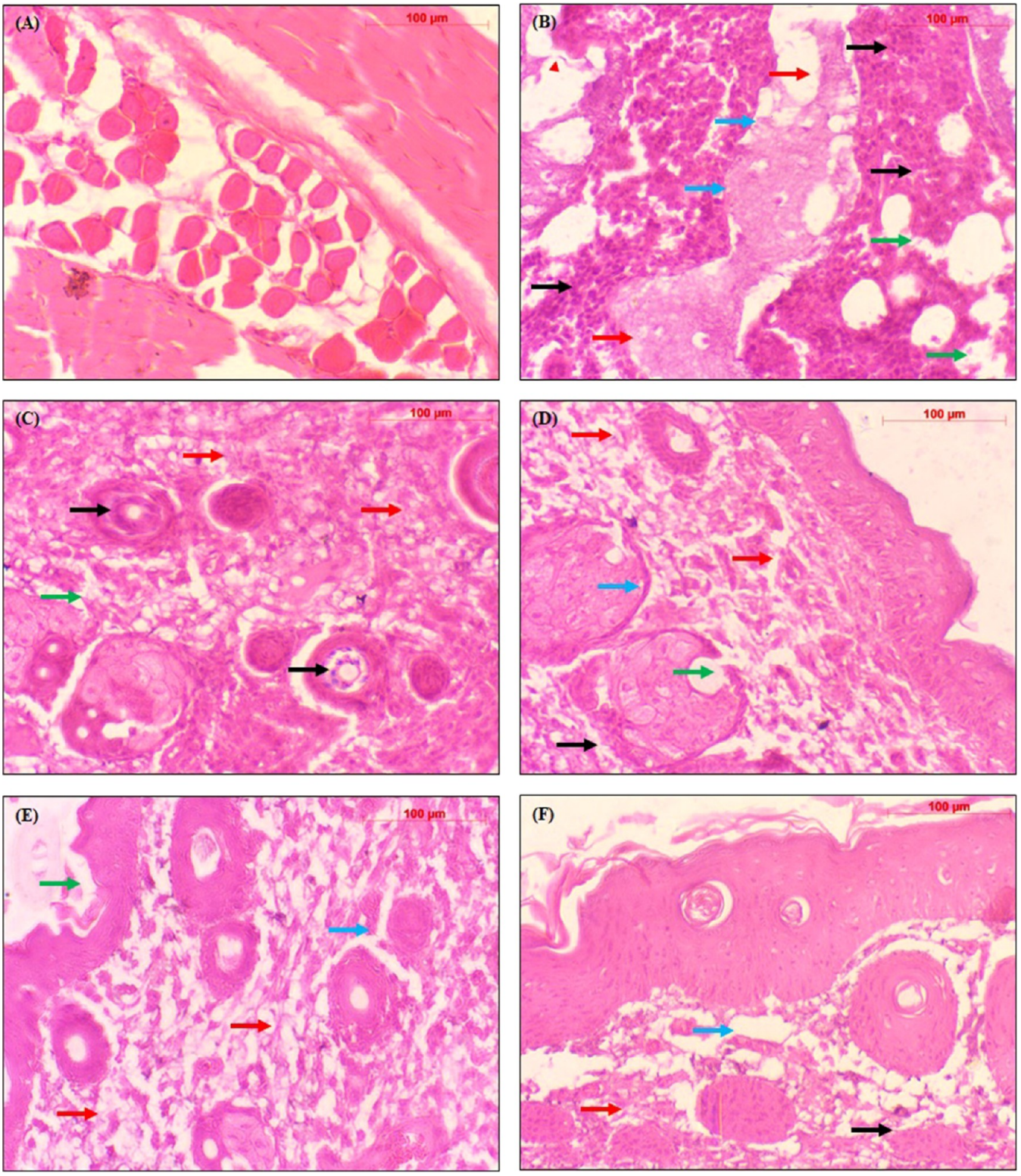

Figure 4. Representative H&E-stained images of bone joints at 40× magnification with a scale bar (100 μm) to evaluate the effect of MTX in the presence of glabridin: (A) control group, (B) diseased control group, (C) MTX alone group, (D) MTX in combination with glabridin (10 mg/kg) group, (E) MTX in combination with glabridin (20 mg/kg) group, and (F) MTX in combination with glabridin (40 mg/kg) group. Arrow indicators: black—inflammatory cell infiltration, green—bone erosion, red—synovial proliferation, and blue—pannus formation.

downregulated the NF-κB expression compared to the diseased control ($p < 0.01$). Simultaneous treatment of MTX with glabridin at 40 mg/kg significantly downregulated the protein expression of NF-κB ($p < 0.05$) (Figure 7A). It is pertinent to mention that we measured the phosphorylated form of NF-κB instead of NF-κB. Based on the literature reports, the present study plan is mainly because of these two reasons: (a) the phosphorylated form is the active form for subsequent action on inflammation and (b) the total NF-κB is unaltered.[38,39] We also

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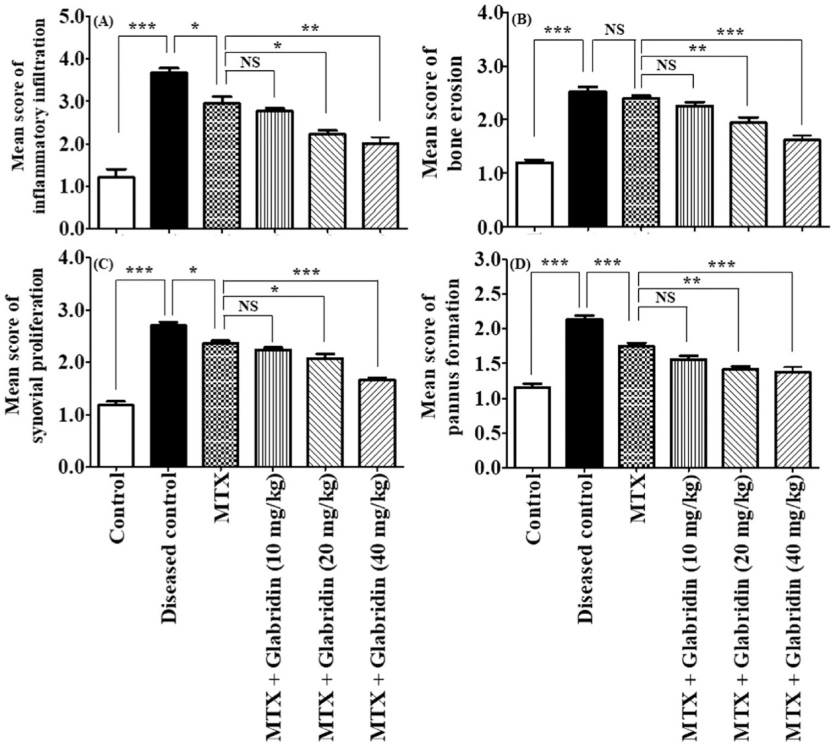

Figure 5. Bar graph for scoring histopathological parameters to evaluate the effect of MTX in the presence of glabridin: (A) inflammatory cell infiltration, (B) bone erosion, (C) synovial proliferation, and (D) pannus formation. Each value is expressed as mean ± SEM ($n = 5$). Significance level: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. NS represents statistically insignificant.

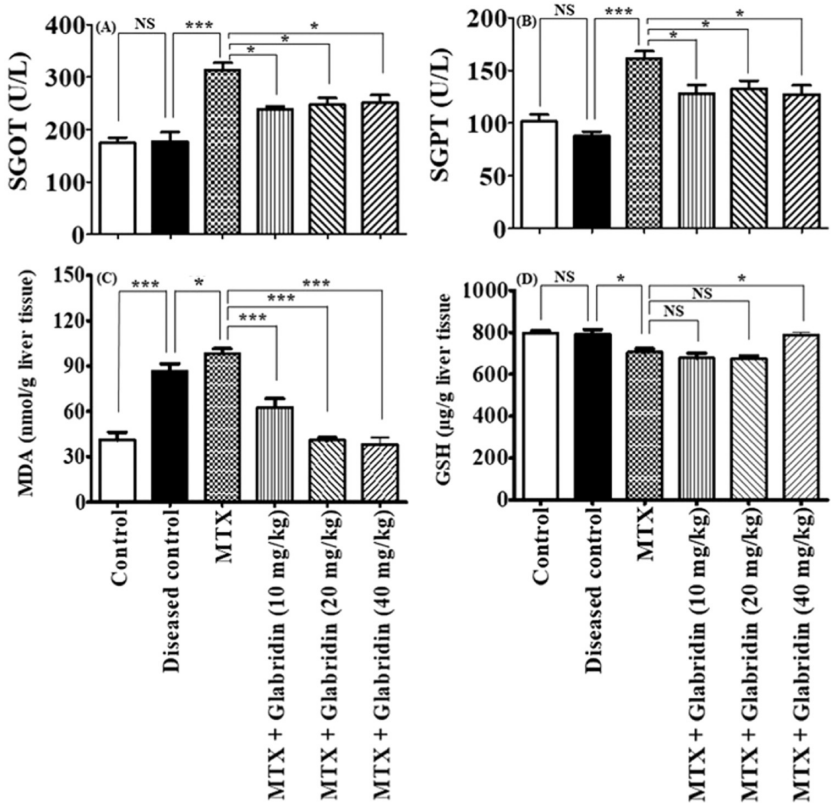

Figure 6. Effect of MTX in the presence of glabridin on hepatic marker enzymes in the serum and oxidative stress markers levels in the hepatic tissues: (A) SGOT, (B) SGPT, (C) malondialdehyde (MDA) content, and (D) glutathione reduced (GSH). Each value is expressed as mean ± SEM ($n = 5$). Significance level: *$p < 0.05$ and ***$p < 0.001$. NS represents statistically insignificant.

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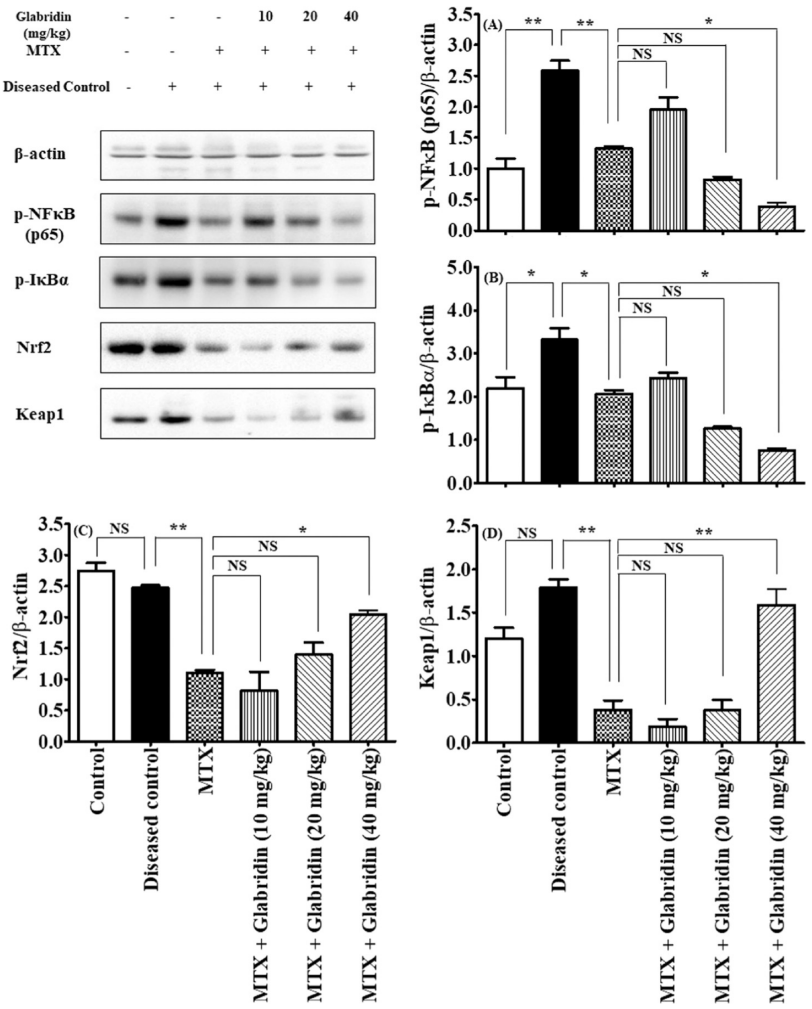

Figure 7. Effect of MTX in the presence of glabridin on protein expressions in the hepatic tissues using WB analysis: (A) *p*-NF-κB (p65), (B) *p*-IκBα, (C) Nrf2, and (D) Keap1. Each value is expressed as mean ± SEM ($n = 5$). Significance level: $^{*}p < 0.05$ and $^{**}p < 0.01$. NS represents statistically insignificant.

estimated the protein expression of IκBα, which elicited similar behavior to NF-κB (Figure 7B).

The Nrf2 expression did not alter to a significant extent in the diseased control compared to the control. MTX treatment considerably downregulated the Nrf2 protein expression compared to the diseased control ($p < 0.01$) (Figure 7C). Concomitant treatment of MTX with glabridin at 40 mg/kg significantly upregulated the Nrf2 expression ($p < 0.05$). We also determined the protein expression of Keap1, where the observed effects are similar to the alterations of the Nrf2 protein expression (Figure 7D).

2.8. Glabridin did not Alter the MTX Level in the Plasma. We investigated the impact of concurrent glabridin treatment at 40 mg/kg on the oral pharmacokinetics of MTX using a normal rat model. The mean plasma concentrations versus time profiles of MTX are shown in Figure 8, whereas the pharmacokinetic parameters of MTX are summarized in Table 1. No statistically significant difference in C_{max} of MTX was observed due to treatment of glabridin compared to MTX alone. However, the concomitant administration of glabridin with MTX substantially delayed the T_{max} of MTX compared to only MTX administration. Nevertheless, the coadministration of glabridin with MTX did not cause any considerable changes in the oral exposure of MTX compared to MTX alone. Moreover,

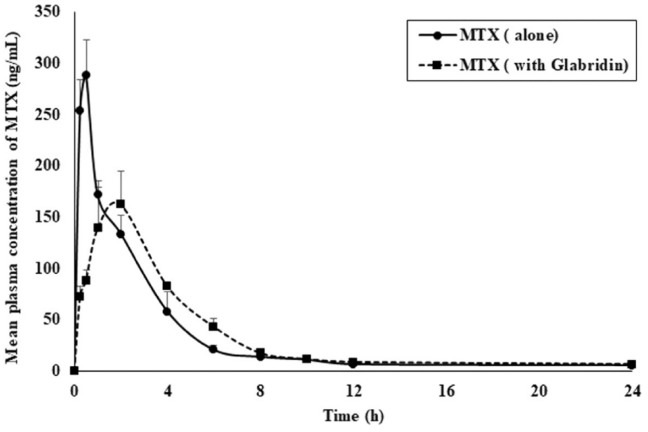

Figure 8. Mean plasma concentration versus time profile of MTX after oral administration in the rats for the following study groups: (A) MTX (3 mg/kg) as alone and (B) MTX (3 mg/kg) in the presence of glabridin (40 mg/kg). Each value is expressed as mean ± SEM ($n = 6$).

$T_{1/2}$ and clearance of MTX were also insignificantly affected in the presence of glabridin.

https://doi.org/10.1021/acsomega.2c03948
ACS Omega 2022, 7, 34341−34351

Table 1. Pharmacokinetic Parameters of MTX as Alone and in Combination with Glabridin after Oral Administration in the Rats[a]

pharmacokinetic parameters	MTX (alone)	MTX (with Glabridin)
C_{max} (ng/mL)	268.9 ± 24.8	202.8 ± 38.6
T_{max} (h)	0.4 ± 0.1	1.4 ± 0.3[b]
$T_{1/2}$ (h)	2.7 ± 0.4	2.9 ± 0.4
AUC_{0-t} (ng h/mL)	699.1 ± 68.9	755.1 ± 65.8
$AUC_{0-\infty}$ (ng h/mL)	722.6 ± 68.8	805.0 ± 78.3
V_d/F (L/kg)	18.7 ± 4.1	15.4 ± 1.3
Cl/F (L/h/kg)	4.5 ± 0.5	3.9 ± 0.4

[a]Footnote: C_{max}, maximum plasma concentration; T_{max}, time to reach C_{max}; AUC_{0-t}, area under the curve for plasma concentration from zero to last measurable sample time; $AUC_{0-\infty}$, area under the curve for plasma concentration from zero to infinity; V_d/F, volume of distribution after oral administration; and Cl/F, clearance after oral administration. Each value is expressed as mean ± SEM (n = 6). [b]Statistical significance at $p < 0.05$.

3. DISCUSSION

MTX is the key DMARD for the effective treatment of RA, which is a chronic autoimmune disorder. Unlike all the DMARDs, having concern of severe adverse effects, MTX treatment is also associated with several toxicities. Regular use of MTX is associated with hepatotoxicity, leading to liver damage.[11,32,35] In this context, MTX is one of these crucial life-saving drugs available in the market despite drug-induced liver injury (DILI) concerns. Therefore, it is labeled as a "Box warning" under the category of "most DILI concern".[26] In modern treatment strategies, combination therapy is an attractive approach to dealing with chronic inflammatory disorders.[40,41] To identify a phytochemical having an inherent ability to complement the drug action as well as can combat the drug-induced hepatotoxicity in parallel, we investigated the antiarthritic efficacy of MTX in the presence of glabridin using the Mycobacterium-induced arthritis model. This rat model is an extensively used model for inducing arthritis as it mimics RA's clinical and histological features. It is reported that CFA injection causes severe swelling and hyperalgesia, followed by the loss of bone joint integrity.[42] Bone erosion, synovial proliferation, inflammatory cell infiltration, and pannus formation are the important pathological hallmarks of RA.[43] Chronic inflammation is associated with RA, where elevations of several cytokine levels occur.[38] Selective TNF-α inhibitors are plunged into the market, but their use is limiting day by day due to severe side effects.[3] Current research is ongoing on selective TNF-α and/or IL-6 inhibitors with lesser side effects. MTX mainly acts on inhibiting cytokines through its anti-inflammatory and immunosuppressive actions. We found in the present study that glabridin, especially at 40 mg/kg, potentiates the antiarthritic efficacy of MTX based on the observations on paw edema formation and cytokine inhibition (TNF-α and IL-6). We also investigated the serum level of IL-1β, which is mainly associated with chronic inflammation. Although MTX has a degree of action against IL-1β, we observed that concomitant treatment of glabridin markedly augments the inhibitory potential of MTX. The radiological data and histopathological findings also suggest the effectiveness of glabridin in improving MTX efficacy. Glabridin's effect on potentiating the MTX action is possibly linked to the cytokine-mediated anti-inflammatory pathway of MTX. Inhibitory activity of glabridin on various cytokines using diverse *in vitro*/*in vivo* models is also evident in the literature.[44−48] The tentative pathway for the anti-inflammatory activity of glabridin must be elucidated further.

If any candidate can counteract drug-induced toxicity in addition to the improved efficacy of the same drug, it would be an added advantage. MTX is reported to cause hepatotoxicity, leading to liver damage. Leakage of hepatic biochemical enzyme markers occurs due to MTX treatment-associated alteration in the hepatic transport and membrane permeability during liver injury.[32,35] Further, MTX treatment elevates oxidative stress involving the Nrf2 pathway, leading to apoptosis and organ damage.[39,49] In the present study, we also found similar observations upon MTX treatment. It is reported that glabridin did not cause cytotoxicity using various cell lines (*in vitro*) even at high concentrations up to 100 μM.[28,50,51] Yuan et al. described that glabridin has the ability to restrict toxicological alterations in the liver using monosodium iodoacetate-induced osteoarthritic rats (*in vivo*).[28] We reported earlier that pretreatment of glabridin can prevent the MTX-induced hepatotoxicity upon a single high-dose administration of MTX using a mice model (*in vivo*).[37] In the current investigation, glabridin and MTX were treated simultaneously in the arthritic rats for a longer time period. Results suggest that glabridin can restrict the enhancement in hepatic marker enzyme levels (SGOT and SGPT) and minimize oxidative stress (MDA and GSH). Further, glabridin treatment is found to be beneficial in lessening the oxidative stress conditions via restricting inflammatory pathway genes (NF-κB/IκBα) and restoring the antioxidant defense phenomenon (Nrf2/Keap1). Similar effects of glabridin treatment to downregulate the NF-κB pathway using the *in vitro* model are reported in the literature.[39,49] Ma et al. reported that glabridin could protect acetaminophen-induced liver injury by targeting the PI3K/Akt/Nrf2 pathway.[52] The possible mechanistic pathway of action should be explored further.

The evaluation of pharmacokinetic interaction is an integral part of proposed combination therapy. Thus, the proposed candidate should not influence the pharmacokinetics of drugs. However, simultaneous administration can be done for any beneficial aspect through intended pharmacokinetic interaction. In the present study, glabridin exhibited a lack of any significant effect in modifying the pharmacokinetics of MTX. Alteration in the T_{max} may happen during concomitant oral administration, for which several shreds of evidence also exist in the literature.[53,54]

4. CONCLUSION

The potential of glabridin was investigated under the purview of phytotherapy with MTX in RA using a widely used adjuvant-induced arthritic rat model. The present study results suggest that glabridin could potentiate the MTX action by reducing paw swelling, inhibiting cytokines (TNF-α, IL-6, and IL-1β) along with improvement in the bone joints based on radiological and histopathological examination. The current experimental results further indicate that glabridin could lessen the MTX-induced hepatotoxicity through declining hepatic marker enzymes (SGPT and SGOT), attenuating oxidative stress (MDA and GSH), and downregulating the protein expressions for inflammatory genes (NF-κB/IκBα) and normalizing the protein expressions for the antioxidant defense phenomenon (Nrf2/Keap1). Based on the overall outcomes of the present study, glabridin is found to potentiate the antiarthritic efficacy and lower the hepatotoxicity of MTX via inhibition of cytokines and oxidative stress. Additionally, lack of pharmacokinetic inter-

https://doi.org/10.1021/acsomega.2c03948
ACS Omega 2022, 7, 34341−34351

action of MTX with glabridin is also suitable for concomitant therapy. It is the first-time report of glabridin for combination therapy potential with MTX. USFDA and FSSAI recommend *G. glabra* as a safe food additive, and therefore, encouraging results by its one of the phytoconstituents, namely, glabridin, warrants further research toward augmented efficacy and better tolerability of MTX in RA.

5. MATERIALS AND METHODS

5.1. Chemicals and Reagents. MTX (purity >99%), MDA (purity ≥99%), GSH (purity >98%), and the incomplete Freund's adjuvant (Lot no. SLBM9341V) was procured from Sigma-Aldrich. Heat-killed *Mycobacterium tuberculosis* (Lot no. 9044274) was obtained from DIFCO. The anti-p-NF-κB p65 antibody (Lot no. K1219), anti-p-IκBα antibody (Lot no. L2319), and anti-Nrf2 antibody (Lot no. L1614) were obtained from Santa Cruz Biotechnology, whereas the Keap1 antibody (Lot no. WB3190738) was procured from Invitrogen. Poly-(ethylene glycol)-200 (PEG-200) was purchased from HiMedia Laboratories. MS-grade materials were used for bioanalysis, and the remaining chemicals/reagents were of bioreagent grade or above. Ultrapure water from a water purification system (Make: Merck-Millipore; Model: Direct Q3) was used for dose preparation and analysis.

5.2. Animals Maintenance. Adult female Wistar rats (120−180 g of body weight) were used for the present study. Animals were kept in individually ventilated cages (IVC) (Make: Techniplast) under a controlled environment of temperature (25 ± 2 °C), humidity (50 ± 20%), and light (12 h light/12 h dark). Rat pellet diet and water were freely available to animals unless otherwise mentioned.

5.3. Ethical Approval. Animal experiments were accomplished as per the "Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA) guidelines (Government of India, New Delhi) with mandatory approval from the Institutional Animal Ethics Committee (IAEC) of our institute (IAEC approval No: 2074/78/2/2021, and 255/79/8/2021).

5.4. Test Article Dose and Dose Formulation. Glabridin in a pure form (purity >99%) was obtained from the NPMC Division of our institute.[51] The dose of glabridin was selected at three dose levels from 10 to 40 mg/kg body weight daily based on our previous experimentation.[37] MTX dose was selected at 3 mg/kg body weight once a week following the earlier reported literature.[38,55] An individual dose of glabridin or MTX was prepared freshly using 5% DMSO, 45% PEG-200, and q.s. water (v/v) and administered at a 10 mL/kg dose volume.

5.5. Effect of Glabridin on the Antiarthritic Efficacy of MTX. *5.5.1. Induction of Arthritis.* Arthritis was induced in rats by injecting 50 μL of CFA into the subplantar region of the right hind paw on day 1. CFA was prepared as a suspension by triturating 5 mg/mL heat-killed *M. tuberculosis* in the incomplete Freund's adjuvant. It caused the paw edema formation within 24 h of CFA administration with progressive arthritic conditions. The experiment was performed using earlier reported protocols.[38,55]

5.5.2. Study Design. On day 0, all the normal rats were divided randomly into six groups containing five animals each as follows: control, diseased control, MTX alone (3 mg/kg), MTX (3 mg/kg) in combination with glabridin (10 mg/kg), MTX (3 mg/kg) in combination with glabridin (20 mg/kg), and MTX (3 mg/kg) in combination with glabridin (40 mg/kg). On day 1, all of the experimental animals were treated with CFA to induce arthritis, except animals in the control group. The oral treatment of MTX and glabridin was given from day 0 and continued up to day 21. In combination groups, MTX was administered after 30 min of the glabridin treatment.

Paw swelling and animal body weight were checked intermittently from day 1 to day 21. After 2 h of MTX treatment on day 21, the radiological investigation of the paws was performed. After 4 h, blood samples were taken from the retro-orbital plexus into the microcentrifuge tubes. Serum was separated by centrifugation for 10 min at 8000 rpm and was used to estimate biochemical markers and inflammatory cytokines. In the end, animals were sacrificed by carbon dioxide euthanasia, dissected to obtain liver and hind paws, followed by rinsing with ice-cold normal saline and blotted dry. The liver-to-body weight ratio was also estimated (Figure S1). Tissues of bone joints were stored in a neutral buffered formalin solution (10%, v/v) to fix at first for histopathological examination, followed by snap-freezing of liver tissues under liquid nitrogen and further stored at −80 °C for subsequent studies.

5.5.3. Paw Swelling Measurement. Paw swelling was monitored for the progression and severity of arthritis. It was measured based on the alteration in the paw thickness. The swelling of the inflamed and control paw was measured using a digital caliper (Make: Generic; Model: LSHAZI03590).

5.5.4. Radiological Investigation. All of the animals were anesthetized by injecting ketamine hydrochloride (100 mg/kg) through the intraperitoneal route.[56] Further, animals were placed on the radiological plate, and imaging of the hind paws was obtained using an X-ray instrument for veterinary use (Make: Siemens; Model: Heliophos-D) to evaluate the severity of arthritis.

5.5.5. Cytokine Estimation. The serum cytokines levels of TNF-α (Rat TNF-α ELISA Kit, Lot no. 1818268B, Invitrogen), IL-6 (Rat IL-6 ELISA Kit, Lot no. 166226040, Invitrogen), and IL-1β (Rat IL-1β ELISA Kit, Lot no. 162075024, Invitrogen) were quantified as per the manufacturer's protocol.

5.5.6. Histopathological Examination. For histopathological examination, the hind paw was fixed in the neutral buffered formalin solution (10%, v/v). After that, the specimen was dehydrated using a gradually changing composition of ethanol and embedded in paraffin, followed by decalcification, section cutting (5 μm), and staining with H&E dyes. The slides were evaluated for inflammatory changes under a light microscope using 40× magnification (Make: Magnus; Model: INVI). The slides were evaluated using important histopathological parameters (inflammatory cell infiltration, bone erosion, synovial proliferation, and pannus formation) to assess the disease severity. Scoring was done using the parameters mentioned above.[43,57]

5.6. Effect of Glabridin against the MTX-Induced Hepatotoxicity. *5.6.1. Hepatic Marker Enzymes.* The activity of two hepatic enzymes, namely, SGOT and SGPT, in the serum was estimated using an automated biochemical analyzer (Make: Erba Mannheim; Model: EM360).

5.6.2. Oxidative Stress Markers. MDA content was measured in the hepatic tissue. The liver tissue homogenate (100 mg/mL) was prepared using 1.15% KCl in water (w/v). The GSH content was also determined in the hepatic tissue. Liver tissue (50 mg each) in 100 mM sodium phosphate buffer containing 5 mM EDTA (750 μL) and 25% of orthophosphoric acid (200 μL) was homogenized at first for the estimation of GSH. The studies mentioned above were performed using our earlier reported protocol.[37,51]

https://doi.org/10.1021/acsomega.2c03948
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5.6.3. Western Blotting. In the hepatic tissues, the protein expressions of NF-κB, IκBα, Nrf2, and Keap1 were determined using Western Blot analysis. The tissue homogenate was prepared in radioimmunoprecipitation buffer containing a protease inhibitor cocktail, phenylmethylsulfonyl fluoride (2 mM), sodium orthovanadate (0.5 mM), and sodium fluoride (50 mM), followed by protein estimation using Bradford's method. The protein was separated using sodium dodecyl sulfate−polyacrylamide gel electrophoresis, then transferred to the poly(vinylidene difluoride) membrane (100 volts, 120 min, 4 °C), which was blocked with 3% bovine serum albumin and then incubated overnight at 4 °C with the corresponding primary antibodies. Afterward, the membrane was washed thrice with Tris-buffered saline and then reincubated for 2 h at room temperature with a chemiluminescent horseradish peroxidase-conjugated secondary antibody. Finally, the membrane was again washed thrice with Tris-buffered saline, followed by imaging through the Chemidoc imaging system (Make: Syngene; Model: G: BOX, XT-4), and then densitometry analysis was carried out by Image J software.[37,58]

5.7. Effect of Glabridin on the Pharmacokinetics of MTX. *5.7.1. Study Design.* Pharmacokinetic studies were performed to elucidate the effect of glabridin on the oral exposure of MTX in normal rats. Overnight fasted animals were randomly divided into two groups containing four subgroups (n = 6). Each study arm is composed of two subgroups to accomplish a sparse sampling technique. The study arms were: MTX (3 mg/kg) alone and MTX (3 mg/kg) in combination with glabridin (40 mg/kg). MTX was administered after 30 min of glabridin treatment in case of combination group. After MTX administration, the blood samples were collected from the retro-orbital plexus into microcentrifuge tubes with the anticoagulant (aqueous EDTA solution) at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h. Blood samples were centrifuged for 10 min at 8000 rpm to obtain plasma samples (50 μL each) and then stored further at −80 °C for analysis.[59]

5.7.2. Sample Processing. The stock solution of MTX and internal standard (IS) phenacetin were prepared and diluted further with methanol to make individual standard solutions. These solutions were spiked into the blank plasma to prepare matrix-match calibration standards (7.8 to 4000 ng/mL). Experimental plasma samples were processed with methanol (200 μL) containing IS (50 ng/mL) for plasma protein precipitation, followed by centrifugation for 10 min at 14,000 rpm. The sample was decanted into the vial and injected onto the liquid chromatography with tandem mass spectrometry (LC-MS/MS) system.

5.7.3. Bioanalysis. An LC-MS/MS system (Make: Thermo Fisher Scientific; Model: Ultimate 3000 for high-performance liquid chromatography (HPLC) and TSQ Endura for MS) was used to quantitate MTX in the processed plasma samples. The chromatographic separation was accomplished in a Kinetex EVO C_{18} (50 mm × 2.1 mm, 5 μm) column (Phenomenex) using the isocratic mobile phase composition of formic acid in water (0.1%, v/v) and acetonitrile (80:20, v/v) at a 0.3 mL/min of flow rate. The autosampler and column oven temperatures were 15 and 45 °C, respectively. The detection of MTX and IS was performed in a heated-ESI source using a triple quad mass spectrometer. Representative mass spectra, chromatograms, and optimized LC-MS/MS parameters for the quantitation of MTX are given in Figures S2, S3, and Table S1, respectively. The acquisition and processing of data were done using LCQUAN software.

5.7.4. Pharmacokinetic Data Evaluation. Pharmacokinetic parameters were calculated based on plasma concentration versus time data by a noncompartmental method using software (PK solution, Summit Research Services).

5.8. Statistical Evaluation. One-way analysis of variance (ANOVA) with post hoc Tukey's test was used for statistical evaluation of efficacy/toxicity data (GraphPad Prism 5 software). Unpaired Student's t-test using online software was used for pharmacokinetic data analysis (QuickCalcs, GraphPad). Experimental data were presented as mean ± standard error mean (SEM), and data were compared between the following groups: control vs. disease control and disease control vs MTX alone or MTX in combination with glabridin. The data were considered statistically significant if the p-value was less than 0.05, 0.01, and 0.001.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03948.

Liver-to-body weight ratio; selected reaction monitoring (SRM) chromatograms, and LC-MS/MS conditions for analysis of experimental samples (PDF)

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https://doi.org/10.1021/acsomega.2c03948
ACS Omega 2022, 7, 34341−34351

Funding

Financial support was obtained from the Council of Scientific and Industrial Research, New Delhi, India (MLP6006 & HCP38).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

A.D., M.B., and D.K. are thankful to UGC/CSIR (New Delhi, India) for providing necessary research fellowship support. With profound sorrow and grief, we must acknowledge the contribution of Late Dr. Payare Lal Sangwan for providing pure glabridin to accomplish the present research work. IIIM publication number: CSIR-IIIM/IPR/00421.

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