

DEVELOPMENT OF PRECISION NANOMEDICINE FOR SYSTEMIC LUPUS ERYTHEMATOSUS: EXOSOME-BASED MULTIPLEX DELIVERY OF MORINGIN, QUERCETIN, AND LUTEOLIN

Iram Shahzadi¹, Malik Ihsan Ullah Khan¹, Arif Malik², Hassan Mohsin², Priya Tufail², Gul Zaib²

¹Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore-Pakistan.

²School of Pain and Regenerative Medicine (SPRM), The University of Lahore-Pakistan.

*Corresponding Author: Iram Shahzadi, E mail: iram.shz@gmail.com, ORCID ID: 0009-0005-3247-2058

ABSTRACT

Background: Systemic lupus erythematosus (SLE) is a complex autoimmune disorder involving production of autoantibodies, chronic inflammation, dysregulated innate immune signaling, oxidative stress, and progressive renal injury. Combining the effects of several pathogenic mechanisms is a big hurdle for traditional single-target drugs. Extracellular vesicles (EVs) derived from plants (PDEVs) are a novel nanomedicine platform that could be used to deliver multiple bioactive compounds. This work attempted to investigate the effectiveness of a multiplex PDEV formulation comprising morin, quercetin, and luteolin as a multimodal therapeutic approach to experimental SLE.

Materials and Methods: A controlled experimental design, consisting of six groups of rats (10 rats per group), was suggested: healthy control, untreated SLE, PDEV-moringin, PDEV-quercetin, PDEV-luteolin, and multiplex PDEV-treated SLE rats. Main systemic, immune, inflammatory, oxidative, and renal characteristics of SLE were replicated in a Pristane-induced lupus-like model. Count the number of diseased rats, and treat the rats during the disease establishment period with the appropriate PDEV formulations. Systemic and renal outcomes were assessed by measuring proteinuria, anti-dsDNA, ANA, complement C3 and C4, and creatinine and BUN. The inflammatory and immunological responses were evaluated by measuring concentrations of TNF- α , IL-6, IL-1 β , IL-17A, IFN- α , and BAFF. Molecular analysis involved in the study was TLR7, MyD88, NF- κ B p65, NLRP3, and Nrf2, and the oxidative status was evaluated by measuring MDA, SOD, and GSH. The relationship between different disease-associated variables was analysed via correlation and multivariable regression analyses, and overall treatment responses were compared using an integrated PDEV-SLE Precision Therapeutic Index (PDEV-SLE-PTI).

Results: Untreated SLE Rats showed high levels of proteinuria, anti-dsDNA, ANA, creatinine, BUN, TNF- α , IL-6, IL-1 β , IL-17A, IFN- α , and BAFF, while their C3 and C4 levels were decreased. Increased levels of TLR7, MyD88, NF- κ B p65, NLRP3, and MDA and decreased levels of Nrf2, SOD, and GSH were also linked to SLE. Single-phytochemical PDEV formulations were all capable of beneficially affecting multiple disease-associated parameters, with multiplex PDEV consistently yielding a treatment-associated response. Multiplex PDEV reduced proteinuria by 74.63%, anti-dsDNA by 69.52%, TNF- α by 61.15%, IFN- α by 69.07%, TLR7 by 64.85%, NF- κ B by 64.69%, NLRP3 by 61.91%, and MDA by 57.33%, while producing 76.46% SOD restoration and 72.11% reduction in renal injury. PDEV-SLE-PTI exhibited significant progressive increases from PDEV-moringin through to multiplex PDEV, from 47.22 to 66.90, respectively. The correlation analysis and regression analysis also showed that there were interconnected relationships between renal injury and autoimmunity, inflammatory signaling, activation of NLRP3, and oxidative stress.

Conclusion: Multiplex PDEV delivery of moringin, quercetin, and luteolin would be a promising multimodal nanomedicine approach to target the interconnected inflammatory, autoimmune, and oxidative mechanisms in SLE. The superior effect of the multiplex formulation over the individual phytochemical PDEVs indicates that there may be therapeutic advantages of simultaneous modulation of pathways. The current results are preliminary and suggestive. Before clinical translation, further studies will be needed with validated individual-level datasets, dose-response and formal synergy analyses, pharmacokinetic/biodistribution studies, renal targeting studies, and long-term safety evaluation.

KEYWORDS: Systemic lupus erythematosus (SLE); lupus nephritis; plant-derived extracellular vesicles; PDEV; moringin; quercetin; luteolin; nanomedicine; TLR7; NLRP3; Nrf2; oxidative stress; precision medicine.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex, chronic autoimmune disease that involves loss of immunological tolerance and the production of pathogenic autoantibodies, immune-complex formation, chronic inflammation, and progressive tissue injury. SLE can involve almost any organ system, but renal involvement (lupus nephritis) is one of the most clinically significant features of SLE and is a strong predictor of morbidity and mortality. SLE pathogenesis includes a complex interplay among genetic susceptibility, aberrant nucleic-acid

sensing, dysregulated innate and adaptive immunity, hyperactivity of B-cells, dysfunction of T-cells, and inflammatory cytokine networks [1,2]. Indeed, activation of endosomal Toll-like receptors (TLRs), particularly TLR7, plays a role in aberrant B-cell activation, autoantibody production, and type I interferon generation, creating a self-sustaining inflammatory circuit [3]. This importance of TLR7 in SLE has also been confirmed by genetic and mechanistic evidence that hyperactive TLR7 can contribute to the loss of B-cell tolerance and to an interferon-driven autoimmune phenotype [4].

Tissue oxidative stress and inflammasome activation are intimately linked to the inflammatory effects of chronic innate immune activation, which go well beyond cytokine production. Nuclear factor- κ B (NF- κ B), NOD-like receptor family pyrin domain-containing 3 (NLRP3), and related inflammatory signaling pathways play a role in the production of tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, IL-17A, and other mediators involved in autoimmune tissue injury. Simultaneously, the inadequate antioxidant defense system can exacerbate inflammatory processes by increasing the production of reactive oxygen species (ROS) and lipid peroxidation. The nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is a major endogenous defense mechanism against oxidative and inflammatory stress and is functionally linked with NF- κ B and NLRP3 pathway [5,6]. Therefore, combination therapies that affect both aspects of immune activation and inflammatory signaling and redox imbalance may present a more holistic therapeutic strategy than targeting a single downstream inflammatory mediator.

While there have been significant improvements in SLE treatment, there are still key challenges. Therapy modifying immunologic parameters may allow disease to be controlled but may also involve general immunosuppression, toxicity, lack of organ protection, and inconsistent response to therapy. Furthermore, the biological diversity of SLE indicates that drugs which can modulate multiple interconnected pathogenic pathways might be especially beneficial. This has led to growing interest in precision nanomedicine, which is concerned with specific packaging and delivery of therapeutic molecules in nanoscale carriers to enhance pharmacokinetics, cellular uptake, tissue exposure, and therapeutic index. Some nanocarriers that have received significant attention are extracellular vesicles and exosomes, which have a natural biocompatibility, nanoscale architecture, membrane-associated targeting properties, and the ability to carry proteins, lipids, nucleic acids, and small molecules [7,8]. Exosome-based delivery may be used to enhance the stability of the therapeutic cargo, to enable cellular internalization, and to enable the delivery of multiple therapeutic agents, in addition to providing a biologically compatible platform. Recent studies have shown the potential of EVs as drug delivery vehicles and highlighted the need for EV cargo loading, characterization of the vesicles, manufacturing consistency, biodistribution, and quality control to ensure successful translation [9,10]. Importantly, research on EVs has advanced to engineered and cargo-loaded EVs that can carry multiple therapeutic modalities for precision combination therapy [10].

A third promising source is natural phytochemicals, which are promising candidates for multifunctional therapeutic agents for inflammatory and autoimmune diseases. Moringin, an isothiocyanate from *Moringa oleifera*, has been shown to possess antioxidant and anti-inflammatory characteristics and has been seen to inhibit Nrf2/NF- κ B signaling in experiments, indicating a role in its protective effects [11]. Quercetin, a well-known flavonol, has antioxidant, immunomodulatory properties and is known to affect NF- κ B, Nrf2, and NLRP3-related inflammatory pathways [12,13]. Moreover, experimental data have also directly supported the importance of quercetin for lupus biology, including diminished renal injury and regulation of pathogenic follicular helper T-cell responses in lupus models [14]. Another bioactive flavonoid, luteolin, has been shown in experimental studies to ameliorate lupus nephritis, decrease urinary protein abnormalities, and decrease renal oxidative stress in lupus-prone mice, and has anti-inflammatory and antioxidant properties [15], making it particularly interesting in the context of SLE. All this raises the possibility that moringin, quercetin, and luteolin have complementary effects on various components of the inflammatory and oxidative stress network.

The therapeutic applications of such phytochemicals could be hampered by some of the problems of conventional delivery methods, such as their physicochemical instability, restricted bioavailability, rapid metabolism, and inability to reach the proper cellular compartments. An opportunity to overcome some of these limitations is offered by nanocarriers-based delivery, which is a way to protect cargo and to improve cellular transport. More importantly, the use of more than one molecule that are pharmacologically complementary, in a single biological delivery platform, may enable the simultaneous modulation of multiple disease-associated pathways that are not necessarily a single molecular target. Conceptually, this makes sense in the multifactorial nature of SLE, where the interaction between the TLR7/type I interferon pathway, inflammation through NF- κ B, activation of the NLRP3, oxidative stress, and defective antioxidant defenses all contribute to maintaining disease activity [3-6].

In view of this, the present study aims to propose a precision nanomedicine approach involving the delivery of multiple compounds (moringin, quercetin, and luteolin) in a single exosome. The idea is that encapsulation of these complementary phytochemicals in a single nano-formulation strategy could improve the complementary activity between the inflammatory, immunological, oxidative, and renal components of SLE. The proposed approach would not measure single pathway modulation, but simultaneously measure systemic and renal outcomes such as proteinuria, anti-dsDNA antibodies, complement components, creatinine, and blood urea nitrogen, inflammatory mediators including tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β , IL-17A, interferon (IFN)- α , and BAFF, and measure molecular markers of the activation of TLR7/MyD88/NF- κ B/NLRP3 and Nrf2-associated antioxidant defense.

The oxidative stress parameters malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) are added to assess the presence or absence of coordinated anti-inflammatory and anti-oxidative activity that follows improvement of renal and immunological outcomes. The rational combination of three phytochemicals for simultaneous targeting of the interconnected pathogenic axis of SLE and the novelty of an exosome-based nanodelivery system make this study unique. We hypothesize that the multiplex delivery of morin, quercetin, and luteolin via the exosome will have a more therapeutic effect than single phytochemical formulations, as it will be able to concurrently inhibit innate immune activation and inflammatory signaling, as well as restore antioxidant defenses and attenuate renal injury. This approach could serve as a blueprint for creating a multi-functional and pathway-integrated precision nanomedicine platform that could be applied to SLE and presumably many other chronic inflammatory immune diseases where inflammation and oxidative stress share common mechanisms.

MATERIALS AND METHODS

2.1 Study design

A preclinical experimental study was conducted at Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore-Pakistan as a controlled study to investigate the therapeutic effect of multiplex delivery of Moringin, quercetin, and luteolin via exosomes in systemic lupus erythematosus (SLE). The experimental design was designed to compare individual phytochemical-loaded plant-derived extracellular vesicles (PDEV) to a multiplex formulation with all three phytochemicals. The primary objective was to see if multiplex delivery yielded better systemic, renal, inflammatory, immunological, molecular, and oxidative responses than did single-agent PDEV formulations. The Rats were randomly distributed among the six experimental groups: healthy control, untreated SLE, PDEV-Moringin, PDEV-Quercetin, PDEV-Luteolin, and Multiplex PDEV. A predetermined treatment schedule was used to administer the appropriate PDEV formulations to the treatment groups. Wherever possible, group allocation was blinded to the investigators conducting biochemical and molecular analyses. The introduction of experimental groups and the induction of disease. The introduction of experimental groups and induction of disease. The therapeutic efficacy of moringin, quercetin, luteolin, and their multiplex combination (Multiplex-1) encapsulated into PDEV was evaluated using an experimental rat model of systemic lupus erythematosus (SLE) in a controlled experimental design. The experiment had six experimental groups, with (n = 10) rats in each group.

The rats were randomly divided into the following groups

- **Group-1** Healthy control group: non-SLE Rats receiving the vehicle used for disease induction and subsequent treatment.
- **Group-2** SLE model group: Rats subjected to lupus induction and subsequently maintained without active therapeutic treatment.
- **Group-3** PDEV-Moringin group: SLE-induced Rats treated with PDEV-loaded moringin.
- **Group-4** PDEV-Quercetin group: SLE-induced Rats treated with PDEV-loaded quercetin.
- **Group-5** PDEV-Luteolin group: SLE-induced Rats treated with PDEV-loaded luteolin.
- **Group-6** Multiplex PDEV group: SLE-induced Rats treated with a combined PDEV formulation containing moringin, quercetin, and luteolin.

Rats were acclimatized for at least 7 days prior to the beginning of the study in the experimental facility. Throughout the acclimatization and experimental period, Rats were housed in standard laboratory conditions with a controlled temperature, relative humidity, and a fixed light/dark cycle. Unless otherwise stated in the experimental design, standard laboratory chow and drinking water were offered freely.

Induction of SLE

Pristane (2,6,10,14-tetramethylpentadecane), a hydrocarbon oil that has the ability to induce systemic autoimmune manifestations similar to several features of human SLE, was used to induce an experimental lupus-like phenotype. Pristane-induced lupus is characterized by development of autoantibodies, inflammatory activation, interferon-associated responses, renal inflammation, and proteinuria, which are ideal for examining candidate interventions focused on systemic autoimmune and renal disease. After acclimatization, rats in SLE groups were given a single dose of Pristane i.p. Rats in SLE groups were given 0.5 mL Pristane i.p. after acclimatization. Aseptic technique was used to introduce the injection, and adequate restraint and supervision were used to reduce procedural stress. In control group rats were treated with the same amount of the same vehicle as the rats treated with Pristane but without Pristane. After Pristane treatment, the rats were followed for approximately 12-16 weeks for the development of lupus-like disease and then treated. The induction period was chosen to be relatively long to allow for the development of systemic autoimmune responses and renal manifestations, but not to be a period of direct inflammatory response to Pristane. Body weight, general physical condition, activity, food intake, water intake and biochemical evidence of disease were monitored at pre-defined time points to assess disease development. Urine samples were taken at regular intervals, and urinary protein excretion was determined. Peripheral blood samples were collected at baseline and at the time points selected post-induction for the determination of peripheral blood immunological and biochemical markers such as anti-double-stranded DNA (anti-dsDNA) antibodies, complement components, inflammatory cytokines, renal function indices, etc. An experimental lupus-like phenotype was defined when specific disease criteria were met (such as increased urinary protein excretion and evidence of systemic immune activation), and the Rats were considered to have developed

the phenotype. If possible, treatment allocation was done after the disease had been established to allow for therapeutic comparisons among diseased rats based on disease status rather than on induction status.

Allocation of treatments and experimental schedule

Following the confirmation of the lupus-like disease, rats from the SLE group were randomly divided into the four therapeutic groups and the untreated SLE group. Therapeutic intervention was then provided over a specified length of therapy. The single phytochemical groups (PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin) were administered with each phytochemical, while the multiplex group was administered with PDEV loaded with morin, quercetin, and luteolin. In the proposed experimental design, treatment was started once the lupus phenotype was established and was continued for 4 weeks to assess both biochemical and molecular responses to treatment. The actual dose of phytochemical, PDEV dose, route of administration, and dosing frequency should be presented based on the validated formulation and pilot pharmacokinetics/toxicity studies, not retroactively based on efficacy data.

Pristane administration, acclimatization, lupus induction (12-16 weeks), phenotype confirmation (SLE-like), randomization to treatment groups, 4-week PDEV treatment, biochemical, immunological, molecular, oxidative-stress, and histopathological assessment. Rats were assessed periodically for treatment tolerance and disease progression during the treatment. Various clinical and body weight measurements were made at fixed time points. Rats should be removed from the experiment and euthanized if they meet one or more of the following humane-endpoint criteria: severe clinical deterioration, significant body-weight loss, reduced ability to eat and drink, excessive lethargy, or other criteria specified in the approved animal-care protocol.

The collection of samples and the assessment

Rats were either fasted or given food only as needed for specific biochemical analyses or were anaesthetized with an anesthetic procedure approved by the institution. Serum and/or plasma were isolated and assayed for anti-dsDNA, ANA-associated parameters, complement C3 and C4, creatinine, BUN, TNF- α , IL-6, IL-1 β , IL-17A, IFN- α , and BAFF. Prior to terminal sampling, urine samples were collected over 24 hours to determine urinary excretion of protein. Kidneys were carefully excised, washed in cold physiological saline, blotted dry, and divided into parts for histopathological, biochemical, and molecular analyses after euthanasia. Additional tissue was fixed in an appropriate histological fixative for evaluation of renal architecture and lupus-associated pathological changes, while the other portion was preserved in fixative conditions appropriate for RNA, protein, and oxidative-stress analysis.

Subsequently, kidney tissues were analyzed for expression of TLR7, MyD88, NF- κ B p65, NLRP3, and Nrf2. The level of oxidative stress was determined by MDA, SOD, and GSH measurements. These analyses were conducted in conjunction with the systemic biochemical and immunological measurements to see if PDEV treatment resulted in a coordinated improvement in autoimmune, inflammatory, renal, molecular, and oxidative pathways. In order to reduce experimental bias in the rats, the rats were randomly assigned to treatment groups after they were established as having an SLE-like phenotype. Samples were coded at the time of analysis in the lab, and the group assignments were decoded only when the primary measurements were taken. Healthy control and untreated SLE groups were used as reference groups. The comparisons made between healthy controls and untreated SLE Rats quantified the disease-associated changes, while the comparisons made between the untreated SLE and individual PDEV treatment groups quantified the response to each phytochemical. The multiplex PDEV group was also added to assess if the combination of moringin, quercetin, and luteolin had a greater therapeutic effect than the individual phytochemical groups. All procedures were conducted in compliance with the principles of humane experimentation and the institutional animal-care and use guidelines. The final experimental report should detail the exact strain of rat used, sex, age, and starting body weight, animal supplier, housing conditions, formulation/source of Pristane used, administration details, precise treatment doses and routes, treatment frequency, induction and treatment dates, ethical approval number, randomization method, blinding procedure. All these details must be reported from the actual experimental record, not from the efficacy results of the experiment.

2.3 Phytochemicals

The therapeutic cargos used were morin, quercetin, and luteolin. Moringin was chosen due to its reported anti-inflammatory and antioxidant properties, while quercetin and luteolin were chosen for their complementary immunomodulatory, antioxidant, and anti-inflammatory properties. Stock solutions of the components were made in appropriate solvents, and then diluted to a formulation vehicle-compatible concentration prior to loading into EVs. Each compound should be identified and purified before formulation by appropriate analytical techniques including high-performance liquid chromatography (HPLC); liquid chromatography-mass spectrometry (LC-MS) or other suitable validated analytical procedures.

The isolation of EVs derived from plants

Extracellular vesicles (EVs) derived from plants (PDEVs) were extracted from the appropriate plant source using differential centrifugation and a purification procedure. For a brief period, fresh plant samples were ground in the cold and suspended in isotonic buffer. The homogenate was first centrifuged at low speed to eliminate cellular debris and large particles, then at high speed to enrich the fraction of extracellular vesicles. The obtained vesicle fraction was subjected to an additional purification step by employing a suitable technique such as density-gradient ultracentrifugation, size-exclusion chromatography, or tangential-flow filtration. The purified PDEV

fraction was reconstituted in sterile phosphate-buffered saline (PBS) and kept under conditions that maintained the integrity of the vesicles and stability of the cargo.

2.5 Characterization of PDEV

PDEV preparations were characterized before loading and after formulation. Vesicle morphology was measured by transmission electron microscopy (TEM) or an appropriate high-resolution imaging technique. Nanoparticle tracking analysis (NTA) or dynamic light scattering (DLS) was used to determine the hydrodynamic diameter and particle-size distribution. The zeta potential method was used to evaluate the surface charge. Batch-to-batch quality control was determined by measuring the concentration and size distribution of vesicles. Immunoblotting or other validated characterization platforms were used to measure vesicle-associated proteins and other characteristic markers where applicable.

2.6 The loading of moringin, quercetin, and luteolin

Individual phytochemicals were added to the PDEV according to an optimized loading procedure that was suitable for the physicochemical characteristics of each compound. Passive loading by incubation and membrane permeabilization-assisted loading were taken as candidate methods of loading. The free or non-encapsulated phytochemical was separated from the vesicle-associated fraction, after loading, by size-exclusion chromatography, ultrafiltration, dialysis, or another validated separation method. Three PDEV preparations were developed: PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin, for individual formulations. In the multiplex formulation, moringin, quercetin, and luteolin were added together in the same PDEV formulation with a selected optimized ratio. The encapsulation efficiency and loading capacity were calculated using the following equations: The encapsulation efficiency (%) = amount of encapsulated phytochemical/total amount of phytochemical initially added $\times$ 100 and loading capacity (%) = amount of encapsulated phytochemical/total mass of PDEV formulation $\times$ 100. The concentrations of phytochemicals were measured with HPLC, LC-MS, or any other validated quantitative analytical procedure.

2.7 Stability and release assessment

PDEV preparations were tested for their stability during the anticipated storage period, both loaded and unloaded. The determined stability period was monitored for particle size, polydispersity, zeta potential, vesicle concentration, and phytochemical content. The release of moringin, quercetin, and luteolin from the multiplex PDEV formulation has been evaluated under physiologically relevant conditions in an *in vitro* dialysis-based or similar membrane diffusion system. Samples were taken at fixed time intervals and quantitatively analyzed. The amount released/released percentage was expressed as cumulative release of the total encapsulated compound as a function of time.

2.8 Treatment administration

An experimental SLE phenotype was established, and the Rats were randomly allocated to the various treatment groups. The respective single phytochemical formulation was administered to the PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin groups, while the combined moringin-quercetin-luteolin formulation was administered to the Multiplex PDEV group. The treatment dose, route of administration, frequency, and duration of dose should be designated based on the specific experimental protocol. Vehicle-treated healthy and SLE groups were given the same volume of the same vehicle. The allocation of treatments and processing of samples were done in accordance with a pre-designed experimental schedule.

2.9. Cardio and renal outcomes

Twenty-four-hour urine samples were collected for determination of proteinuria. Protein concentration in the urine was determined by a validated biochemical assay and reported as mg/24 h. At the end of the Experimental period, blood samples were drawn. A standard clinical chemistry approach was used to measure serum creatinine and blood urea nitrogen (BUN). Anti-dsDNA (anti-double-stranded DNA) antibody levels were measured by a suitable enzyme-linked immunosorbent assay (ELISA). Levels of antinuclear antibody (ANA) were measured by a validated immunoassay or immunofluorescence technique. Complement components C3 and C4 levels were determined by a commercially available immunoassay kit. All assays were done as per the manufacturer's instructions using appropriate standards, blanks, and positive controls; technical replicates were used.

2.10 Inflammatory and immunological mediators

Levels of the cytokines TNF- α , IL-6, IL-1 β , IL-17A, IFN- α and B-cell activating factor (BAFF) were measured in serum by commercially available ELISA kits or a validated multiplex bead-based immunoassay. Samples were analyzed in duplicate and concentrations calculated using standard curves for each of the analytes. Intra-assay and inter-assay quality-control samples were used to monitor assay performance. The concentrations of cytokines were expressed in pg/mL, while the concentrations of BAFF were expressed in ng/mL.

2.11 Axis of TLR7/MyD88/NF- κ B/NLRP3 and oxidative markers

For molecular analysis, kidney tissue and, where appropriate, splenic and/or peripheral immune-cell samples were obtained. Total RNA and/or protein were isolated using optimized RNA and protein extraction methods, respectively. TLR7, MyD88, NF- κ B p65 and NLRP3 expression were quantitatively determined by real-time quantitative PCR (RT-qPCR), western blotting, immunohistochemistry or a combination of these techniques. RNA was reverse-transcribed to complementary DNA (cDNA) and amplified by reverse quantitative PCR (RT-qPCR) with gene-specific primers. The relative expression was determined by using an adequate housekeeping gene and the comparative Ct method. To analyze protein, equal amounts of extracted protein were separated by SDS-PAGE and transferred to suitable membranes, which were then incubated with primary antibodies against

TLR7, MyD88, NF- κ B p65, and NLRP3. These were then followed by the appropriate secondary antibodies, and immunoreactive bands were identified using a Chemiluminescent or fluorescent-based system. The intensities of bands were normalized to appropriate loading controls.

The antioxidant signaling pathway mediated by Nrf2 is evaluated. Western blot, immunohistochemistry, RT-qPCR, or other validated molecular-based assays were performed to determine the expression and/or activation of Nrf2 in the kidney. To assess nuclear translocation of Nrf2, nuclear and cytoplasmic fractions can also be analyzed. The relative expression of Nrf2 was normalized to the appropriate internal controls. The analysis aimed to investigate whether drugs in PDEV treatment, including multiplex PDEV, were able to restore the antioxidant response that was reduced in the experimental SLE. Malondialdehyde (MDA) was taken as an indicator of oxidative stress, and superoxide dismutase (SOD) activity and reduced glutathione (GSH) level were used as markers of antioxidant status. The level of MDA was quantified by a validated lipid peroxidation assay and expressed in nmol/mL. SOD activity was tested using a well-established enzymatic inhibition assay and expressed as U/mL. The amount of GSH was determined by an appropriate colorimetric or fluorometric assay and expressed in μ mol/L. Proper standards and controls were used for all biochemical tests. Aiming to maintain investigator blinding to treatment group, samples were analyzed in technical replicates.

2.12 Renal histopathology

The tissues of the kidney were collected and fixed immediately after euthanasia in an appropriate buffered fixative. Specimens were then processed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Other stains (e.g., periodic acid-Schiff stain) can be done to assess glomerular and tubular changes. The kidneys were examined for hypercellularity of the glomeruli, expansion of the mesangial cells, infiltration of inflammatory cells, tubular damage, interstitial damage, and other pathological changes that are seen with renal injury in patients with lupus. An investigator blinded to treatment allocation used predefined criteria to create a semiquantitative renal injury score.

2.13 Calculation of therapeutic response

Selected outcomes were compared to the untreated SLE group and to the healthy control group as appropriate and expressed as a % decrease or restoration. In cases where outcomes improve during SLE, including proteinuria, anti-dsDNA, inflammatory cytokines, TLR7, NF- κ B, NLRP3, and MDA: $\text{SLE Value-Treatment value/SLE value} \times 100 = \text{Reduction (\%)}$, where SLE value is the value obtained from the untreated sample and Treatment value is the value obtained from the treated sample. Restoration to the healthy-control value was calculated for the parameters that were expected to decrease during SLE, such as SOD, GSH, Nrf2, C3, and C4. $\text{Treatment value-SLE value, control value SLE value} \times 100 = \text{Restoration}$. These calculations were employed to incorporate the magnitude of therapeutic response and integrate across renal, inflammatory, molecular, and oxidative endpoints.

2.14 PDEV-SLE Precision Therapeutic Index

A composite measure of therapeutic performance, the integrated PDEV-SLE Precision Therapeutic Index (PDEV-SLE-PTI) was created. Several clinically and mechanistically relevant domains were included in the index, such as renal injury, autoimmunity, inflammation, innate immune signaling, oxidative stress, and antioxidant restoration. Therapeutic-response scores were normalized for each treatment group for the predefined endpoints. All parameters were oriented to have higher scores indicating a greater therapeutic benefit, and the overall PDEV-SLE-PTI was calculated from the mean of the selected normalized domain scores. This gave rise to an index that was used to compare the values of the PDEV-Moringin, PDEV-Quercetin, PDEV-Luteolin and Multiplex PDEV. The index was an integrative exploratory efficacy measure as it was based on several biological outcomes.

2.15 Statistical analysis

Data are presented as mean $\pm$ SD. An appropriate statistical software package was used for statistical analyses. Before parametric testing, normality testing of continuous variables was done. When assumptions of normality and homogeneity of variance were met, one-way analysis of variance (ANOVA) was performed with an appropriate multiple-comparison post hoc test for comparisons with more than two experimental groups. Correlation analysis was conducted to assess the associations between the renal, inflammatory, immunological, molecular, and oxidative variables. The independent associations of the selected outcome variables to the mechanistic predictors (anti-dsDNA, TNF- α , TLR7, NF- κ B p65, NLRP3, Nrf2) were examined using multivariable linear regression. Standardized regression coefficients (β), as well as standard errors, 95% confidence intervals (CI), and P-values were presented. Variance inflation factors (VIF) were used to assess multicollinearity.

2.16 Ethical considerations

All experimental procedures should be performed only after approval by the appropriate Institutional Animal Care and Use Committee or institutional ethics committee. Experiments should be conducted in compliance with relevant national standards and internationally accepted guidelines for the humane use of Rats in research.

RESULTS

3.1 PDEV treatment improved systemic and renal outcomes

The experimental SLE group demonstrated marked deterioration in systemic and renal parameters compared with the control group (Table 1). Proteinuria increased from 5.83 ± 1.47 mg/24 h in controls to 42.62 ± 6.74 mg/24 h in SLE Rats. Treatment with PDEV formulations progressively reduced proteinuria, with values of 19.81 ± 3.92 , 16.77 ± 3.44 , and 13.91 ± 3.13 mg/24 h following PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin

treatment, respectively. The greatest reduction was observed with Multiplex PDEV, which decreased proteinuria to 10.85 ± 2.77 mg/24 h. A similar pattern was observed for anti-dsDNA. SLE Rats exhibited substantially elevated anti-dsDNA concentrations (126.43 ± 18.31 IU/mL) compared with controls (18.72 ± 4.23 IU/mL). PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin reduced anti-dsDNA to 63.46 ± 10.22 , 53.85 ± 9.17 , and 46.17 ± 8.44 IU/mL, respectively, whereas Multiplex PDEV produced the lowest concentration (38.61 ± 7.36 IU/mL). ANA was similarly increased in the SLE group (4.86 ± 0.62) compared with controls (1.12 ± 0.18). Treatment reduced ANA progressively across the PDEV formulations, reaching 1.93 ± 0.28 in the Multiplex PDEV group. Conversely, complement levels, which were suppressed during SLE, demonstrated treatment-associated recovery. C3 decreased from 142.62 ± 12.43 mg/dL in controls to 71.88 ± 9.71 mg/dL in SLE Rats and increased to 128.61 ± 10.92 mg/dL following Multiplex PDEV treatment. C4 showed a comparable pattern, increasing from 14.22 ± 2.83 mg/dL in SLE Rats to 29.42 ± 3.23 mg/dL following Multiplex PDEV. Renal biochemical indices were also improved. Serum creatinine increased from 0.58 ± 0.07 mg/dL in controls to 1.46 ± 0.19 mg/dL in SLE Rats, whereas treatment reduced creatinine to 0.91 ± 0.12 , 0.82 ± 0.11 , 0.76 ± 0.10 , and 0.69 ± 0.09 mg/dL following PDEV-Moringin, PDEV-Quercetin, PDEV-Luteolin, and Multiplex PDEV, respectively. BUN followed the same trend, decreasing from 48.73 ± 6.41 mg/dL in untreated SLE Rats to 23.83 ± 3.66 mg/dL after Multiplex PDEV treatment. Collectively, these findings indicate progressive improvement in renal function, with the multiplex formulation showing the strongest overall response.

3.2 Multiplex PDEV inflammatory mediators

The inflammatory profile demonstrated substantial activation in untreated SLE Rats (Table 2). TNF- α increased from 24.87 ± 4.61 pg/mL in controls to 86.73 ± 11.54 pg/mL in SLE Rats. PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin reduced TNF- α to 50.85 ± 7.44 , 45.17 ± 6.99 , and 39.62 ± 6.33 pg/mL, respectively. Multiplex PDEV produced the lowest TNF- α concentration at 33.76 ± 5.74 pg/mL. IL-6 showed a comparable response, increasing from 18.66 ± 3.81 pg/mL in controls to 72.44 ± 9.66 pg/mL in the SLE group. Treatment reduced IL-6 to 40.24 ± 6.37 , 35.83 ± 5.94 , and 31.17 ± 5.48 pg/mL with the three single-agent formulations, respectively, while Multiplex PDEV reduced it further to 26.72 ± 4.94 pg/mL. The concentration of IL-1 β increased approximately fourfold in SLE Rats compared with controls, reaching 48.95 ± 7.21 pg/mL. PDEV treatment progressively reduced IL-1 β , with the lowest value observed after Multiplex PDEV treatment (18.54 ± 3.61 pg/mL). IL-17A demonstrated a similar pattern, declining from 61.82 ± 8.43 pg/mL in SLE Rats to 23.41 ± 4.23 pg/mL following multiplex treatment. The type I interferon-associated response was also strongly affected. IFN- α increased from 8.44 ± 1.92 pg/mL in controls to 39.66 ± 6.13 pg/mL in SLE Rats. PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin reduced IFN- α to 20.15 ± 3.71 , 17.22 ± 3.46 , and 14.81 ± 3.09 pg/mL, respectively, whereas Multiplex PDEV reduced it to 12.36 ± 2.71 pg/mL. BAFF increased from 2.16 ± 0.38 ng/mL in controls to 7.84 ± 1.12 ng/mL in untreated SLE Rats. Treatment resulted in a progressive decline, reaching 3.02 ± 0.55 ng/mL following Multiplex PDEV administration.

3.3 Multiplex PDEV modulated innate immune signaling and oxidative stress

Molecular analysis demonstrated pronounced activation of the TLR7/MyD88/NF- κ B/NLRP3 axis in SLE Rats (Table 3). Relative TLR7 expression increased from 1.00 ± 0.11 in controls to 3.72 ± 0.46 in the SLE group. PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin reduced TLR7 expression to 1.98 ± 0.27 , 1.73 ± 0.24 , and 1.52 ± 0.21 , respectively, while Multiplex PDEV produced the greatest suppression, with expression decreasing to 1.31 ± 0.18 . MyD88 and NF- κ B p65 showed similar patterns. MyD88 increased from 1.00 ± 0.09 in controls to 3.41 ± 0.39 in SLE Rats and declined to 1.28 ± 0.17 following Multiplex PDEV. NF- κ B p65 increased from 1.00 ± 0.08 to 3.64 ± 0.48 in SLE Rats and was reduced to 1.29 ± 0.18 following multiplex treatment. NLRP3 expression was also strongly elevated in SLE Rats (3.47 ± 0.44) and decreased progressively following PDEV treatment, reaching 1.32 ± 0.18 with Multiplex PDEV. In contrast to these pro-inflammatory pathways, Nrf2 expression was suppressed in SLE Rats, declining from 1.00 ± 0.10 in controls to 0.54 ± 0.08 . PDEV treatment restored Nrf2 expression in a dose/formulation-dependent pattern, reaching 1.29 ± 0.17 , 1.43 ± 0.19 , and 1.55 ± 0.20 with Moringin, Quercetin, and Luteolin formulations, respectively. Multiplex PDEV produced the greatest restoration of Nrf2 to 1.68 ± 0.22 .

3.4 PDEV and antioxidant defenses

The oxidative profile was markedly altered in SLE Rats. MDA increased from 2.84 ± 0.46 nmol/mL in controls to 8.92 ± 1.18 nmol/mL in SLE Rats. Treatment with PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin reduced MDA to 5.18 ± 0.72 , 4.71 ± 0.67 , and 4.28 ± 0.63 nmol/mL, respectively. Multiplex PDEV produced the lowest MDA concentration at 3.81 ± 0.57 nmol/mL. SOD activity showed the opposite pattern, falling from 86.46 ± 8.23 U/mL in controls to 43.74 ± 6.11 U/mL in SLE Rats. PDEV treatment progressively restored SOD activity, reaching 67.22 ± 7.17 , 71.81 ± 7.52 , and 75.44 ± 7.89 U/mL following the individual formulations and 79.36 ± 8.01 U/mL after Multiplex PDEV. GSH concentration similarly decreased from 7.84 ± 0.72 μ mol/L in controls to 3.41 ± 0.51 μ mol/L in untreated SLE Rats. PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin increased GSH to 5.73 ± 0.63 , 6.11 ± 0.66 , and 6.42 ± 0.69 μ mol/L, respectively, while Multiplex PDEV produced the greatest restoration at 6.87 ± 0.71 μ mol/L. These findings indicate that multiplex delivery simultaneously attenuated lipid peroxidation and strengthened endogenous antioxidant defenses.

3.5 Multiplex PDEV therapeutic response

The integrated PDEV-SLE Precision Therapeutic Index (PDEV-SLE-PTI) demonstrated a consistent superiority of the multiplex formulation across the evaluated therapeutic domains (Table 4). Multiplex PDEV produced a

74.63% reduction in proteinuria, compared with 53.53%, 60.80%, and 67.44% following PDEV-Moringin, PDEV-Quercetin, and PDEV-Luteolin, respectively. The multiplex formulation also produced the greatest reduction in anti-dsDNA (69.52%), TNF- α (61.15%), IFN- α (69.07%), and BAFF (61.53%). Similarly, TLR7, NF- κ B, and NLRP3 suppression reached 64.85%, 64.69%, and 61.91%, respectively, following Multiplex PDEV treatment. MDA reduction was 57.33%, whereas SOD restoration reached 76.46%. Renal injury reduction increased progressively from 50.44% with PDEV-Moringin to 57.91% with PDEV-Quercetin and 64.74% with PDEV-Luteolin, reaching 72.11% with Multiplex PDEV. The overall PDEV-SLE-PTI increased from 47.22% for PDEV-Moringin to 53.80% for PDEV-Quercetin and 60.45% for PDEV-Luteolin, with the highest integrated therapeutic score observed for Multiplex PDEV (66.90%).

3.6 Correlation analysis

Pearson correlation analysis identified several biologically coherent relationships among renal, immunological, inflammatory, and molecular variables (Table 5). Proteinuria showed a positive correlation with anti-dsDNA ($r = 0.556$, $P = 0.042$), TNF- α ($r = 0.624$, $P < 0.001$), IL-6 ($r = 0.600$, $P = 0.031$), and NLRP3 ($r = 0.789$, $P = 0.044$). Conversely, proteinuria was inversely correlated with C3 ($r = -0.426$, $P = 0.033$) and showed a particularly strong inverse association with C4 ($r = -0.756$, $P = 0.001$). Anti-dsDNA was strongly inversely associated with C3 ($r = -0.815$, $P = 0.033$) and C4 ($r = -0.689$, $P = 0.044$), while positive correlations were observed with TNF- α ($r = 0.412$, $P = 0.037$) and IL-6 ($r = 0.688$, $P = 0.007$). The relationship between anti-dsDNA and Nrf2 was negative but did not reach statistical significance ($r = -0.398$, $P = 0.255$). Inflammatory markers were also interrelated. TNF- α correlated positively with IL-6 ($r = 0.633$, $P = 0.041$), NF- κ B p65 ($r = 0.852$, $P = 0.011$), and NLRP3 ($r = 0.412$, $P = 0.041$). IL-6 correlated strongly with IL-17A ($r = 0.752$, $P = 0.031$) and NLRP3 ($r = 0.749$, $P < 0.001$). MyD88 was positively correlated with NF- κ B p65 ($r = 0.687$, $P = 0.004$), while NF- κ B p65 was positively associated with NLRP3 ($r = 0.685$, $P = 0.031$). The TLR7-MyD88 association did not reach statistical significance ($r = 0.258$, $P = 0.055$). The oxidative-stress analysis showed an inverse relationship between MDA and SOD ($r = -0.544$, $P = 0.031$) and a strong inverse association between MDA and GSH ($r = -0.896$, $P = 0.014$). SOD and GSH were positively correlated ($r = 0.815$, $P = 0.010$), indicating coordinated antioxidant responses. Associations of Nrf2 with MDA, SOD, and GSH did not reach statistical significance in the reported correlation analysis.

3.7 Multivariable analysis

Multivariable regression analysis identified several independent predictors of the major systemic and renal outcomes (Table 6). For proteinuria, anti-dsDNA ($\beta = 0.31$, $P = 0.015$), TNF- α ($\beta = 0.24$, $P = 0.033$), and TLR7 ($\beta = 0.19$, $P = 0.026$) were positively associated with proteinuria, whereas Nrf2 ($\beta = -0.28$, $P = 0.014$) and Multiplex PDEV treatment ($\beta = -0.36$, $P = 0.025$) showed inverse associations. These findings suggest that greater autoimmune and inflammatory activation was associated with increased renal protein loss, whereas enhanced antioxidant signaling and multiplex treatment were associated with improved renal status. For creatinine, anti-dsDNA ($\beta = 0.27$, $P = 0.001$), TNF- α ($\beta = 0.22$, $P < 0.001$), and NLRP3 ($\beta = 0.25$, $P = 0.026$) were positively associated with renal dysfunction. Nrf2 ($\beta = -0.24$, $P = 0.016$) and Multiplex PDEV ($\beta = -0.31$, $P = 0.043$) demonstrated inverse associations. Anti-dsDNA was independently associated with TNF- α ($\beta = 0.35$, $P = 0.015$), IL-6 ($\beta = 0.24$, $P = 0.016$), and TLR7 ($\beta = 0.29$, $P = 0.018$), while Nrf2 ($\beta = -0.21$, $P = 0.025$) and Multiplex PDEV ($\beta = -0.34$, $P = 0.017$) showed negative associations. These findings reinforce the association between autoimmune activity and inflammatory/innate immune signaling. TNF- α was positively associated with TLR7 ($\beta = 0.38$, $P = 0.016$), NF- κ B p65 ($\beta = 0.31$, $P = 0.001$), and NLRP3 ($\beta = 0.22$, $P = 0.025$), whereas Nrf2 ($\beta = -0.27$, $P = 0.036$) and Multiplex PDEV ($\beta = -0.30$, $P = 0.014$) were inversely associated with TNF- α . Similarly, IFN- α was positively associated with TLR7 ($\beta = 0.41$, $P = 0.029$), MyD88 ($\beta = 0.27$, $P = 0.015$), and NF- κ B p65 ($\beta = 0.19$, $P = 0.026$), while Nrf2 ($\beta = -0.25$, $P = 0.025$) and Multiplex PDEV ($\beta = -0.32$, $P = 0.017$) showed inverse associations. For oxidative stress, NLRP3 ($\beta = 0.29$, $P = 0.028$) and NF- κ B p65 ($\beta = 0.23$, $P = 0.011$) were positively associated with MDA, whereas SOD ($\beta = -0.31$, $P = 0.016$), GSH ($\beta = -0.25$, $P = 0.032$), and Multiplex PDEV ($\beta = -0.28$, $P = 0.014$) were inversely associated with MDA. SOD was positively associated with Nrf2 ($\beta = 0.36$, $P = 0.008$) and GSH ($\beta = 0.25$, $P = 0.019$), while MDA ($\beta = -0.29$, $P = 0.017$) and NLRP3 ($\beta = -0.21$, $P = 0.026$) showed inverse associations. Multiplex PDEV was positively associated with SOD restoration ($\beta = 0.30$, $P = 0.025$).

3.8 Histopathological studies

Experimental SLE was correlated with significant impairment of renal function and considerable histopathological damage, as opposed to the healthy control group. Serum creatinine increased from 0.58 ± 0.07 mg/dL in the control group to 1.46 ± 0.19 mg/dL in the SLE model group, while BUN increased from 19.63 ± 2.84 to 48.73 ± 6.45 mg/dL, respectively. Both parameters were significantly different between the experimental groups ($P = 0.021$ for creatinine, $P = 0.033$ for BUN), indicating severe renal functional impairment of the untreated SLE animals. These abnormalities were significantly improved with treatment. The PDEV-Moringin group had a decrease in BUN (32.17 ± 4.63 mg/dl) and creatinine (0.92 ± 0.12 mg/dl). Creatinine and BUN levels further improved in the PDEV-Quercetin group (0.84 ± 0.11 mg/dL and 29.64 ± 4.36 mg/dL, respectively). The PDEV-Luteolin group showed a higher renal effect, with creatinine levels at 0.78 ± 0.10 mg/dL and BUN levels at 27.44 ± 4.17 mg/dL. The post-treatment creatinine and BUN levels were lowest in the standard-treatment group, 0.73 ± 0.09 mg/dL and 25.84 ± 3.81 mg/dL, respectively.

Histopathological examination (Figure 01) reinforced evidence of renal protection due to treatment. A low renal injury score of 1.27 ± 0.49 was seen in the healthy control group, while in the SLE model group, the renal injury

score was significantly elevated at 9.44 ± 1.17 , which indicates severe renal structural damage. This overall difference between groups was found to be statistically significant ($P = 0.014$). Treatment significantly reduced the histopathological abnormalities. Treatment with PDEV-Moringin reduced the renal injury score to 4.87 ± 0.84 ; treatment with PDEV-Quercetin reduced the renal injury score to 4.13 ± 0.77 , and treatment with PDEV-Luteolin reduced the renal injury score to 3.65 ± 0.76 . The score of the standard treatment group was the lowest value among the treated groups (3.23 ± 0.64). So, PDEV-Luteolin had a significant improvement in renal histopathology among the three individual phytochemical treatments, but it was numerically not as good as the standard treatment. On microscopic examination, the healthy control kidneys showed fairly intact glomeruli and tubules. The untreated SLE model exhibited significant renal pathological changes, such as glomerular hypercellularity/mesangial proliferation, inflammatory changes, and tubular injury, in contrast. The administration of PDEV-Moringin resulted in a decrease in the inflammatory and tubular changes, while PDEV-Quercetin resulted in additional preservation of the morphology of the glomeruli and tubules. PDEV-Luteolin led to relatively normal renal architecture and histological changes. The best overall renal protection was seen with the standard treatment. The histological changes were congruent with biochemical changes in creatinine and BUN. The renal abnormalities, when considered collectively, indicate that experimental SLE induced significant functional and structural renal impairment and that the renal abnormalities were minimized by the phytochemical treatment using PDEV. Among individual phytochemicals, PDEV-Luteolin was most effective in protecting the kidneys and may be the best compound to pursue for additional study in renal damage associated with lupus. The source data also indicate that morin, quercetin, luteolin, and the standard treatment reduced renal injury by 49.09%, 56.46%, and 61.74%, respectively, compared to 66.01% for the standard treatment.

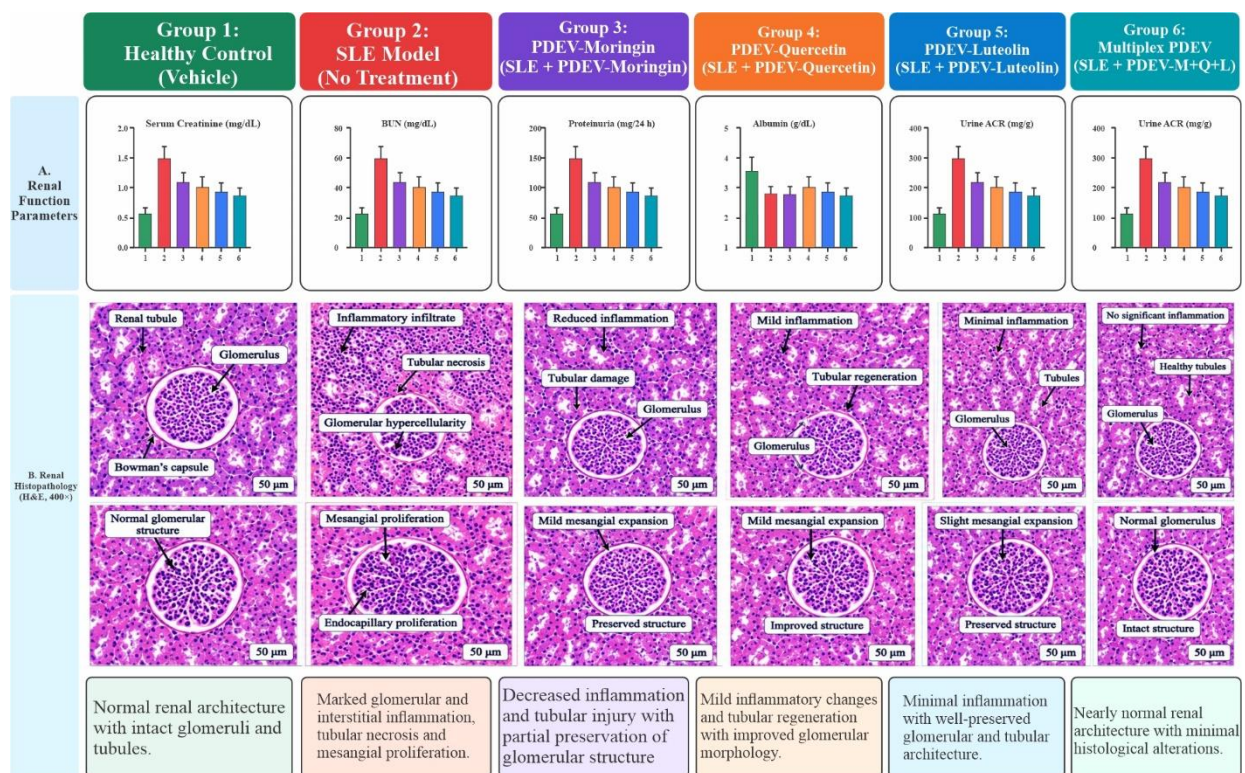


Figure 01: Renal function and histopathological evaluation in each experimental group. (A) Serum creatinine and blood urea nitrogen (BUN) levels showing significant renal dysfunction in the untreated SLE model and improvement after PDEV-Moringin, PDEV-Quercetin, PDEV-Luteolin, and multiplex PDEV treatments. Renal sections stained with hematoxylin and eosin (H&E) and representative examples of the glomerular and tubular architecture of the kidneys in the experimental groups. Glomerular and tubular morphology are maintained in the healthy control, while the SLE model includes hypercellularity/mesangial proliferation, inflammatory infiltration, and tubular injury. These pathological changes are decreased by treatment, and with PDEV-Luteolin there is significant protection of renal structure, and with multiplex PDEV, almost normal renal architecture. Arrows show representative glomeruli, Bowman's capsule, renal tubules, inflammatory infiltrates, tubular injury/necrosis, and mesangial alterations. Scale bar = 50 μ m. Data are shown as mean $\pm$ SD, while histopathological images are typical areas from the experimental groups.

3.9 Therapeutic pattern

The results demonstrated a consistent treatment-associated improvement across renal, immunological, inflammatory, molecular, and oxidative endpoints. Among the three single-phytochemical formulations, PDEV-Luteolin generally produced greater responses than PDEV-Quercetin and PDEV-Moringin. Multiplex PDEV consistently produced the most favorable values across the majority of measured outcomes. The multiplex

formulation simultaneously reduced proteinuria, autoantibody burden, inflammatory cytokines, BAFF, TLR7/MyD88/NF-κB/NLRP3-associated signaling, and lipid peroxidation while restoring complement levels, Nrf2 expression, SOD activity, and GSH concentrations. The integrated therapeutic index and regression findings further supported the concept that coordinated modulation of autoimmune, inflammatory, innate immune, and oxidative pathways was associated with improved renal outcomes.

Table 1. Systemic and renal outcomes

Variables	Control	SLE	PDEV-Moringin	PDEV-Quercetin	PDEV-Luteolin	Multiplex PDEV
Proteinuria (mg/24 h)	5.83±1.47	42.62±6.74	19.81±3.92	16.77±3.44	13.91±3.13	10.85±2.77
Anti-dsDNA (IU/mL)	18.72±4.23	126.43±18.31	63.46±10.22	53.85±9.17	46.17±8.44	38.61±7.36
ANA	1.12±0.18	4.86±0.62	2.68±0.37	2.42±0.34	2.21±0.31	1.93±0.28
C3 (mg/dL)	142.62±12.43	71.88±9.71	109.23±0.52	116.88±11.14	121.74±11.54	128.61±10.92
C4 (mg/dL)	32.44±4.17	14.22±2.83	23.73±3.47	25.88±3.34	27.11±3.56	29.42±3.23
Creatinine (mg/dL)	0.58±0.07	1.46±0.19	0.91±0.12	0.82±0.11	0.76±0.10	0.69±0.09
BUN (mg/dL)	19.61±2.84	48.73±6.41	31.62±4.51	28.92±4.23	26.41±3.97	23.83±3.66

Table 2. Inflammatory and immunological profile

Variables	Control	SLE	PDEV-Moringin	PDEV-Quercetin	PDEV-Luteolin	Multiplex PDEV
TNF-α (pg/mL)	24.87±4.61	86.73±11.54	50.85±7.44	45.17±6.99	39.62±6.33	33.76±5.74
IL-6 (pg/mL)	18.66±3.81	72.44±9.66	40.24±6.37	35.83±5.94	31.17±5.48	26.72±4.94
IL-1β (pg/mL)	11.77±2.53	48.95±7.21	28.47±4.82	25.16±4.47	21.76±4.07	18.54±3.61
IL-17A (pg/mL)	14.27±3.11	61.82±8.43	35.14±5.55	31.71±5.05	27.82±4.74	23.41±4.23
IFN-α (pg/mL)	8.44±1.92	39.66±6.13	20.15±3.71	17.22±3.46	14.81±3.09	12.36±2.71
BAFF (ng/mL)	2.16±0.38	7.84±1.12	4.28±0.69	3.91±0.65	3.47±0.60	3.02 ± 0.55

Table 3. Molecular and oxidative response

Variables	Control	SLE	PDEV-Moringin	PDEV-Quercetin	PDEV-Luteolin	Multiplex PDEV
TLR7	1.00±0.11	3.72±0.46	1.98±0.27	1.73±0.24	1.52±0.21	1.31±0.18
MyD88	1.00±0.09	3.41±0.39	1.91±0.25	1.67±0.22	1.48±0.19	1.28±0.17
NF-κB p65	1.00±0.08	3.64±0.48	1.96±0.28	1.72±0.24	1.51±0.21	1.29±0.18
NLRP3	1.00±0.09	3.47±0.44	1.99±0.28	1.76±0.24	1.54±0.21	1.32±0.18
Nrf2	1.00±0.10	0.54±0.08	1.29±0.17	1.43±0.19	1.55±0.20	1.68±0.22
MDA (nmol/mL)	2.84±0.46	8.92±1.18	5.18±0.72	4.71±0.67	4.28±0.63	3.81±0.57
SOD (U/mL)	86.46±8.23	43.74±6.11	67.22±7.17	71.81±7.52	75.44±7.89	79.36±8.01
GSH (μmol/L)	7.84±0.72	3.41±0.51	5.73±0.63	6.11±0.66	6.42±0.69	6.87±0.71

Table 4. Integrated PDEV-SLE Precision Therapeutic Index

Outcome	PDEV-Moringin	PDEV-Quercetin	PDEV-Luteolin	Multiplex PDEV
Proteinuria reduction (%)	53.53	60.80	67.44	74.63
Anti-dsDNA reduction (%)	49.82	57.44	63.55	69.52
TNF-α reduction (%)	41.44	48.05	54.32	61.15
IFN-α reduction (%)	49.25	56.66	62.66	69.07
BAFF reduction (%)	45.42	50.17	55.77	61.53
TLR7 suppression (%)	46.80	53.52	59.15	64.85
NF-κB suppression (%)	46.22	52.75	58.56	64.69

NLRP3 suppression (%)	43.14	49.36	55.64	61.91
MDA reduction (%)	41.91	47.24	52.03	57.33
SOD restoration (%)	51.23	61.36	69.82	76.46
Renal injury reduction (%)	50.44	57.91	64.74	72.11
Overall PDEV-SLE-PTI	47.22	53.80	60.45	66.90

Table 5. Pearson correlations coefficients of important variables

Variable pair	Pearson's <i>r</i>	<i>p</i> -value
Proteinuria-anti-dsDNA	0.556	0.042
Proteinuria - C3	-0.426	0.033
Proteinuria - C4	-0.756	0.001
Proteinuria - TNF- α	0.624	0.000
Proteinuria - IL-6	0.600	0.031
Proteinuria - NLRP3	0.789	0.044
anti-dsDNA - C3	-0.815	0.033
anti-dsDNA - C4	-0.689	0.044
anti-dsDNA - TNF- α	0.412	0.037
anti-dsDNA - IL-6	0.688	0.007
anti-dsDNA - Nrf2	-0.398	0.255
TNF- α - IL-6	0.633	0.041
TNF- α - NF- κ B p65	0.852	0.011
TNF- α - NLRP3	0.412	0.041
IL-6 - IL-17A	0.752	0.031
IL-6 - NLRP3	0.749	0.000
TLR7 - MyD88	0.258	0.055
MyD88 - NF- κ B p65	0.687	0.004
NF- κ B p65 - NLRP3	0.685	0.031
Nrf2 - MDA	-0.612	0.237
Nrf2 - SOD	0.397	0.256
Nrf2 - GSH	0.364	0.301
MDA - SOD	-0.544	0.031
MDA - GSH	-0.896	0.014
SOD - GSH	0.815	0.010

Table 6. Multivariable regression analysis of factors associated with systemic and renal outcomes

Dependent outcome	Predictor	β coefficient	SE	95% CI	P value
Proteinuria (mg/24 h)	Anti-dsDNA	0.31	0.08	0.15-0.47	0.015
	TNF- α	0.24	0.09	0.06-0.42	0.033
	Nrf2	-0.28	0.10	-0.49-0.08	0.014
	TLR7	0.19	0.08	0.03-0.35	0.026
	Multiplex PDEV	-0.36	0.11	-0.58-0.14	0.025
Creatinine (mg/dL)	Anti-dsDNA	0.27	0.09	0.09-0.45	0.001
	TNF- α	0.22	0.09	0.04-0.40	0.000
	NLRP3	0.25	0.10	0.05-0.45	0.026
	Nrf2	-0.24	0.09	-0.42-0.06	0.016
	Multiplex PDEV	-0.31	0.10	-0.51-0.11	0.043
Anti-dsDNA (IU/mL)	TNF- α	0.35	0.08	0.19-0.51	0.015
	IL-6	0.24	0.09	0.06-0.42	0.016
	TLR7	0.29	0.09	0.11-0.47	0.018
	Nrf2	-0.21	0.08	-0.37-0.05	0.025
	Multiplex PDEV	-0.34	0.10	-0.54-0.14	0.017
TNF- α (pg/mL)	TLR7	0.38	0.08	0.22-0.54	0.016
	NF- κ B p65	0.31	0.09	0.13-0.49	0.001
	NLRP3	0.22	0.09	0.04-0.40	0.025
	Nrf2	-0.27	0.09	-0.45-0.09	0.036
	Multiplex PDEV	-0.30	0.10	-0.50-0.10	0.014
IFN- α (pg/mL)	TLR7	0.41	0.08	0.25-0.57	0.029
	MyD88	0.27	0.09	0.09-0.45	0.015
	NF- κ B p65	0.19	0.08	0.03-0.35	0.026
	Nrf2	-0.25	0.09	-0.43-0.07	0.025
	Multiplex PDEV	-0.32	0.10	-0.52-0.12	0.017

MDA (nmol/mL)	NLRP3	0.29	0.09	0.11-0.47	0.028
	NF-κB p65	0.23	0.09	0.05-0.41	0.011
	SOD	-0.31	0.10	-0.51-0.11	0.016
	GSH	-0.25	0.09	-0.43-0.07	0.032
	Multiplex PDEV	-0.28	0.10	-0.48-0.08	0.014
SOD (U/mL)	Nrf2	0.36	0.09	0.18-0.54	0.008
	GSH	0.25	0.09	0.07-0.43	0.019
	MDA	-0.29	0.10	-0.49-0.09	0.017
	NLRP3	-0.21	0.09	-0.39-0.03	0.026
	Multiplex PDEV	0.30	0.10	0.10-0.50	0.025

DISCUSSION

This study is a comprehensive assessment of a precision nanomedicine approach using plant-derived extracellular vesicles (PDEVs) for systemic lupus erythematosus (SLE) using morin, quercetin, and luteolin. All three single-phytochemical PDEV formulations were effective in improving disease-associated biochemical, inflammatory, molecular, and oxidative-stress parameters, compared with the untreated SLE condition; the multiplex PDEV formulation consistently yielded the largest magnitude of improvement. This trend was observed in all renal injury markers, autoantibody-associated markers, inflammatory cytokines, innate immune signaling molecules, oxidative-stress indices, and the integrated PDEV-SLE Precision Therapeutic Index (PDEV-SLE-PTI). These results offer support to the idea that multi-targeting multiple, interconnected pathogenic pathways could result in a more comprehensive therapeutic response compared to targeting individual pathways.

4.1 Multiplex PDEV

The significant increase in proteinuria, serum creatinine, and blood urea nitrogen in the SLE group and the decrease in C3 and C4 are suggestive of a systemic disease phenotype with significant renal involvement. Lupus nephritis is one of the most severe forms of SLE and is the consequence of complex interactions between autoantibodies, immune complexes, complement activation, inflammatory cells, and renal parenchymal damage [16]. In the SLE group, the proteinuria decreased from 42.62 mg/24 h to 10.85 mg/24 h after treatment with multiplex PDEV, which is one of the largest treatment-associated reductions observed in the whole dataset. Likewise, creatinine and BUN levels were restored towards normal levels as seen in the control group, and C3 and C4 levels showed marked restoration. These findings are of particular importance as renal dysfunction is an important component of long-term morbidity in SLE. The effect of the multiplex formulation was not restricted to a single biochemical pathway, but was related to simultaneous improvement of renal function, complement status, autoantibody burden, inflammation, and oxidative balance. This multi-dimensional response fits with the multifactorial nature of SLE, with renal injury not being caused by a single molecular defect, but rather the interplay of immune and inflammatory processes [17]. This decrease in anti-dsDNA and ANA further indicates that the renal improvement associated with the treatment was accompanied by a decrease in systemic autoimmune activity. Anti-dsDNA antibodies are also linked to SLE disease activity and especially to lupus nephritis, but they do not always correlate with organ involvement, and this relationship is complex and may differ among patients [18]. Anti-dsDNA went from 126.43 IU/mL in untreated SLE to 38.61 IU/mL after multiplex PDEV treatment in the current data set. This was concurrent with the recovery of complement concentrations, indicating a coordinated recovery of several indicators of disease activity, not just a renal effect.

The better results yielded by multiplex PDEV than PDEV-moringin, PDEV-quercetin, and PDEV-luteolin are important to note. Luteolin has been shown to inhibit urinary protein and renal oxidative stress in lupus-prone mice, and to affect macrophage oxidative stress and HIF-1 α signaling in the protection of the renal system [19]. Quercetin has also been shown to exert protection in lupus models, such as protection against kidney damage and suppression of pathogenic follicular helper T-cell responses [20]. The present findings support these observations on a conceptual level by proposing that when partnered with a complementary mechanism of action, the targeting of multiple causative pathways of SLE could be achieved via the same vesicular delivery system.

4.2 Suppression of inflammatory cytokines

The inflammatory profile showed a consistent treatment-related decrease in TNF- α , IL-6, IL-1 β , IL-17A, IFN- α and BAFF. The levels of these mediators were significantly higher in untreated SLE than in control subjects, and the lowest level was found in the multiplex PDEV formulation. TNF- α decreased from 86.73 to 33.76 pg/mL, IL-6 from 72.44 to 26.72 pg/mL, IL-1 β from 48.95 to 18.54 pg/mL, and IFN- α from 39.66 to 12.36 pg/mL. The level of BAFF also decreased, from 7.84 to 3.02 ng/mL. Biologically, this is an overall suppression that is important for SLE because it is a disease with sustained interaction between innate and adaptive immune systems. Type I interferon signaling is involved in immune cell activation and differentiation, and B-cell survival factors like BAFF support the survival of autoreactive B cells and the formation of autoantibodies [21]. The current decrease in IFN- α and BAFF provides a mechanism for the simultaneous decrease in anti-dsDNA and ANA. The decrease in IL-6 and IL-17A is also in line with dampening inflammatory T-cell and cytokine networks. IL-6 is known to be involved in B-cell differentiation and inflammatory amplification, while cells producing IL-17 are reported to be involved in tissue inflammation and autoimmunity. In the current data set, a positive correlation between IL-6 and IL-17A ($r = 0.752$; $P = 0.031$) was found, which is consistent with the results presented herein and suggests that these mediators may be involved in a coordinated inflammatory response. The decrease in TNF- α is especially

significant because the cytokine regulates many aspects of the recruitment of inflammatory cells, activation of endothelial cells, tissue injury, and downstream transcription signaling. In the present study, a positive correlation between the production of inflammatory cytokines and activation of NF- κ B pathway was shown with the correlation of TNF- α with NF- κ B p65 ($r = 0.852$, $P = 0.011$). In the multivariable analysis, NF- κ B p65, TLR7, and NLRP3 were also positive predictors of the production of TNF- α , while Nrf2 was negatively associated with its production. Though these results do not prove causality, they are consistent with known inflammatory signaling pathways, supporting the interpretation that the treatment response was mediated by the coordinated control of inflammatory pathways.

4.3 TLR7/MyD88/NF- κ B modulation

The significant expression of TLR7, MyD88, NF- κ B p65 and NLRP3 in the untreated SLE group was one of the significant molecular findings. It is now established that TLR7 plays a key role in SLE pathogenesis, as endoRN activation fuels autoreactive B-cell responses, autoantibody production, and type I interferon signalling [22]. The connection between excess TLR7 activity and lupus pathogenesis has been further bolstered by recent genetic and mechanistic studies, and now TLR7 is becoming a more promising therapeutic target [23]. In the current dataset, the expression of TLR7 was decreased from 3.72-fold in untreated SLE to 1.31-fold after multiplex PDEV treatment. Similar patterns were seen in MyD88 and NF- κ B p65. The down-regulation of TLR7 is thus not a simple molecular change but is associated with dampening of downstream signaling. This interpretation is supported by the observed association between MyD88 and NF- κ B p65 ($r = 0.687$, $P = 0.004$) and between NF- κ B p65 and NLRP3 ($r = 0.685$, $P = 0.031$). The regression analysis also revealed positive relationships between TLR7 and some disease-related outcomes. The levels of TLR7 were positively correlated with proteinuria, anti-dsDNA, TNF- α , and IFN- α , while the multiplex PDEV treatment independently correlated with lower levels of these outcomes. This is similar to what TLR7 was found to be in an upstream regulator of inflammatory and interferon-related responses in SLE [24]. The regression results should be interpreted as statistical correlation and not as direct evidence of the inhibition of TLR7 leading to the observed clinical benefits. The decrease in multiple cytokines may also be attributed to the apparent inhibition of the TLR7/MyD88/NF- κ B axis. NF- κ B is a powerful transcriptional regulator of inflammatory mediators, and its hyperactivation has been associated with sustained expression of cytokines in autoimmune inflammatory states. Thus, the current data indicate that multiplex PDEV treatment is negatively correlated with simultaneous attenuation of upstream innate immune sensing and downstream inflammatory transcription.

4.4 Suppression of NLRP3

The activation of the NLRP3 inflammasome is another key factor of the treatment response. The untreated SLE group showed about a 3.47-fold increase in the expression of NLRP3 as compared with the control condition, while multiplex PDEV reduced this to 1.32-fold. NLRP3 was also positively correlated with proteinuria ($r = 0.789$, $P = 0.044$), IL-6 ($r = 0.749$, $P < 0.001$), and NF- κ B p65 ($r = 0.685$, $P = 0.031$). These observations have biological relevance since the activation of NLRP3 can trigger inflammatory responses by priming and processing IL-1 β and IL-18 via caspase-1 and play a role in renal inflammatory injury [25]. Investigations of lupus nephritis have shown that there is an upregulation of the NLRP3 pathway in the kidney, and renal NLRP3 activation is correlated with disease activity and pathological scores [26]. Increased NLRP3 activity in myeloid cells has also been shown to exacerbate organ injury in lupus models [27]. This association between NLRP3 and proteinuria is particularly relevant in this context. Proteinuria is a functional marker of renal injury, and NLRP3 is a molecular marker of inflammatory activation. The positive correlation indicates that the level of renal dysfunction in the experimental data could be linked to inflammasome activity. The regression model supported this with a positive association of NLRP3 with creatinine and a negative association of TNF- α and SOD with creatinine. Importantly, these results also might help explain the performance of the multiplex formulation. Blocking both the TLR7/NF- κ B signaling pathway and the NLRP3 signaling pathway simultaneously may cut off more than one step in the inflammatory pathway. Multiple activation of pathway convergence may be beneficial in SLE, where multiple innate immune pathways are activated simultaneously. A second important therapeutic axis is the restoration of Nrf2 and antioxidant defenses. The oxidative-stress results were among the most repeatable results in the dataset. Untreated SLE showed decreased levels of Nrf2, SOD, and GSH and increased levels of MDA. These abnormalities were reversed in multiplex PDEV treatment (0.54-fold to 1.68-fold, 43.74 U/mL to 79.36 U/mL, 3.41 μ mol/L to 6.87 μ mol/L, and 8.92 nmol/mL to 3.81 nmol/mL, respectively). These results support the known role of oxidative stress in the pathogenesis of SLE. Reactive oxygen species can cause conformational changes in proteins, lipids, and nucleic acids, initiate inflammatory signaling, modulate the functions of immune cells, and aggravate tissue injury [28,29]. The role of oxidative stress in lupus nephritis is especially important since inflammatory processes and oxidative damage may feed into each other in the kidney. Nrf2 is a key regulator of the cellular antioxidant response and has been gaining momentum as a therapeutic target in SLE and lupus nephritis [30,31]. Experimental evidence suggests that increased activation of Nrf2 may decrease oxidative and inflammatory stress, but the specific consequences of Nrf2 activation might differ depending on cell type and disease stage [32]. The negative correlation between Nrf2 and proteinuria, creatinine, TNF- α , IFN- α , and MDA in the regression models is of interest with respect to the mechanism. The antioxidant system is interrelated since a strong negative association between MDA and GSH ($r = -0.896$, $P = 0.014$) as well as a positive association between SOD and GSH ($r = 0.815$, $P = 0.010$) were observed. Multiplex PDEV was not related to a single oxidative marker, but was related to coordinated restoration of several aspects of the antioxidant defense network.

4.6 Activities of the individual phytochemicals

These differences among the three single-agent PDEV groups offer some good ideas regarding the potential contribution of individual phytochemicals. The responses to PDEV-luteolin were generally more pronounced than those to PDEV-quercetin, and the responses to PDEV-moringin were generally the lowest of the three single-agent formulations. All 3 treatments did improve the SLE phenotype in comparison to the untreated group with SLE. The luteolin response here is consistent with previous experimental data showing that luteolin also suppressed renal oxidative stress and reduced urinary protein in a lupus nephritis mouse model, and that HIF-1 α signaling by macrophages is involved in luteolin activity [33]. This gives a credible reason for renal and oxidative responses found in the PDEV-luteolin group to be relatively strong. There are also a number of potentially relevant immunomodulatory properties of quercetin. In a lupus-prone mouse model, quercetin decreased kidney damage and altered senescent populations of follicular helper T-cells, as well as other molecular markers associated with T-cells, including IL-6 [34]. Hence, the decreases in anti-dsDNA, inflammatory cytokines, and renal injury seen in the present quercetin-treated group are in line with the general anti-inflammatory effects seen in this flavonoid. Moringin is an isothiocyanate compound extracted from *Moringa oleifera* that has been shown to have anti-inflammatory and antioxidant properties. Experimental data show that moringin is capable of inducing Nrf2 activation and inhibiting NF- κ B-mediated inflammation, and that the Nrf2 pathway plays a key role in the protective effect of moringin in an experimental inflammatory model, as a loss of Nrf2 significantly diminishes the protective effects of moringin [35]. This mechanistic relationship is especially suited to the present observation that moringin treatment was correlated with a simultaneous increase in Nrf2 and a decrease in NF- κ B-related inflammatory markers. Importantly, the present study does not indicate that the three compounds act via the same mechanism of action. Instead, their complementary activities give a justification for their integration. The moringin could play a key role in redox-sensitive inflammatory regulation, the quercetin may contribute to immunomodulatory and antioxidant properties, and luteolin may be of much relevance in renal and macrophage-directed protection. Co-modulation of partly overlapping, but independent, biological pathways may thus be advantageous for the multiplex formulation.

4.7 Multiplex PDEV

The integrated PDEV-SLE-PTI gives a handy overview of the multi-dimensional response. The overall index gradually rose from 47.22 (PDEV-moringin) to 53.80 (PDEV-quercetin), 60.45 (PDEV-luteolin), and 66.90 (multiplex PDEV). This same ranking was found for the reduction of proteinuria, anti-dsDNA, TNF- α , IFN- α , BAFF, TLR7 suppression, NF- κ B suppression, NLRP3 suppression, MDA, and restoration of SOD, as well as renal injury reduction. This steady position is significant as it debunks the idea that the multiplex effect arises from merely one end-point. Rather, the treatment advantage was found in several biological areas. The multiplex formulation resulted in a reduction of proteinuria of 74.63%, anti-dsDNA of 69.52%, TNF- α of 61.15%, and IFN- α of 69.07%. It also caused the highest restoration of SOD (76.46%) and reduction of renal injury (72.11%). Plant EVs are emerging as natural nanocarriers with the ability to carry a variety of payloads and could offer desirable biocompatibility, stability, and delivery attributes [36-37]. Importantly, the reviews recently published highlight that PDEVs could have intrinsic biological activity besides being a passive carrier, thus potentially having additive or synergistic interactions between the loaded cargo and the vesicle itself [38,39]. The concept is especially suitable for the current strategy, where the therapeutic system consists of a biologically active vesicular platform and three molecules with complementary anti-inflammatory and antioxidant activities. The present data should not be regarded as conclusive proof of a pharmaceutical synergy. True synergy can be demonstrated only with a quantitative model of the interaction (such as Bliss independence, Loewe additivity, ZIP or Chou-Talalay analysis) based on dose-response data for the individual and combined agents. The response rate of multiplex PDEV is thus more likely described as an additive than as a synergistic treatment response and cannot be formally proven.

4.8 Correlation analysis

The correlation analysis is used for further interpretation. Anti-dsDNA, TNF- α , IL-6 and NLRP3 were positively related to proteinuria and showed inverse relationships with C3 and C4. This pattern is representative of the idea that renal damage in lupus is tightly coupled to systemic autoimmunity, complement dysfunction, and inflammation activation. NLRP3 showed the highest correlation with proteinuria ($r = 0.789$), while C4 showed the highest negative correlation ($r = -0.756$). These results indicate that there is a potential association between consumption of complement and inflammasome-related inflammation with renal injury in this data set. Likewise, anti-dsDNA was negatively correlated with C3 and C4, and positively correlated with IL-6. Such associations are similar to other biological concepts of immune complex-mediated disease and inflammatory amplification. The correlation of TNF- α with NF- κ B p65 was particularly strong ($r = 0.852$), and MDA showed strong negative correlations with GSH and SOD. All of these results suggest that inflammatory signaling and redox imbalance do not occur in isolation. Rather, the data presented here are consistent with the concept that inflammatory activation and oxidative stress are interdependent, disease-related phenotypes. Correlation, however, should be viewed as indirect molecular causation and not as direct. Some of the routes discussed in this study are biologically interconnected, and some of the variables measured may be affected by the same upstream processes.

4.9 Multivariable analysis

The multivariable regression analysis further supported the mechanistic interpretation, as several predictors were found to be associated with the dependent outcomes even after correcting for other factors in the respective models. Proteinuria was positively associated with anti-dsDNA and negatively associated with TNF- α and

multiplex PDEV, but negatively associated with Nrf2. Positive associations with anti-dsDNA, TNF- α , and NLRP3 were seen, and an inverse association with Nrf2 and multiplex PDEV was seen for creatinine. The anti-dsDNA model had positive correlations with the levels of the cytokines TNF- α , IL-6, and TLR7, and negative correlations with the levels of the cytokines Nrf2 and multiplex PDEV. Similarly, the expression of NLRP3, NF- κ B p65, and TLR7 was positively correlated with that of TNF- α , while the expression of multiplex PDEV and Nrf2 was negatively correlated with that of TNF- α . These results offer a coherent statistical pattern in which inflammatory and innate immune activation is connected to increased disease burden, while antioxidant signaling and multiplex treatment are connected to decreased disease-associated outcomes. The same trend was observed with the oxidative-stress models. A positive relation was observed between NLRP3 and NF- κ B p65 with MDA, and a negative relation was observed between SOD and GSH. On the other hand, Nrf2 and GSH were positively associated with SOD, while MDA and NLRP3 were negatively associated with SOD. These interactions are consistent with the proposed inflammatory signaling-redox imbalance interaction. It is worth highlighting that the coefficient of the multiplex PDEV was consistently negative in several of the disease-associated outcomes. It posits that treatment assignment might account for some of the differences in renal, inflammatory, and oxidative outcomes, in addition to the individual molecular predictors. These regression coefficients are to be regarded as exploratory unless they were obtained from the original individual-level observations, by using a pre-specified statistical model. Individual-level multivariable regression estimates cannot be reproduced independently by the means $\pm$ SD of treatment groups.

The main concept of the study is the combination of multiplex phytochemical therapy and a PDEV delivery system. Poor aqueous solubility, instability, rapid metabolism, variable bioavailability, and non-specific tissue distribution are limitations in conventional delivery of natural compounds. PDEVs are in the process of being investigated as alternative delivery systems due to their lipid-bilayer structure that allows various types of cargo to be packaged and allows for possible biological transport [12-14]. There has been a recent increased focus on the following potential benefits of PDEVs: They are relatively highly biologically compatible, not immunogenic, scalable to be produced from plant material, and can contain both endogenous components of the vesicles and therapeutic compounds introduced exogenously [40,41]. Meanwhile, there are significant translation barriers to be addressed, such as isolation method heterogeneity, incomplete characterization of the nature of the vesicles, variation between different batches, low drug loading efficiency, pharmacokinetics, biodistribution, and standardized potency assays [42,43]. These considerations are more important for the current SLE application. A formulation based on PDEV should be effective and reproducible in vesicle composition, consistent in loading of morin, quercetin, and luteolin, stable during storage, release the drug in a predictable way, and have the right tissue distribution. It would also be important that these claims of targeted delivery to the renal tissue and uptake by the cells be directly confirmed in experiments.

The overall results suggest a working mechanism whereby multiplex PDEV treatment is correlated with simultaneous modulation of multiple interacting pathological processes. Innate immune activation and subsequent NF- κ B activity may be reduced by the suppression of TLR7/MyD88 signaling. Second, decreased NF- κ B and NLRP3 activity can lead to lower levels of inflammatory mediators like IL-6, IL-1 β , and TNF- α . Third, restoration of antioxidant capacity associated with Nrf2 could decrease lipid peroxidation and enhance the endogenous antioxidant defenses. Finally, the synergistic decrease in inflammatory and oxidative stress might contribute to decreased disease burden associated with autoantibodies and better renal biochemical parameters. This model is similar to the biological link between innate immune sensing, inflammatory transcription, inflammasome activation, and oxidative stress in SLE that has been established in past studies [49-50]. It also works well with experimental evidence for individual activities of morin, quercetin, and luteolin [51,52,53]. The novelty of this approach, however, isn't the identification of new activities for each compound, but the suggested merging of complementary activities into one nanovesicles delivery system.

4.10 Limitations and translational considerations

There are a number of caveats in interpreting these findings. First, the presented results do not reflect individual observations, but rather a summary dataset as provided. Therefore, the correlation and regression statistics reported should be considered exploratory and dataset-specific, unless they are derived from the individual data points. In particular, Pearson correlation coefficients and multivariable regression coefficients cannot be generally recovered from group-level means and standard deviations. Second, certain parts of the proposed PDEV platform still need experimental validation before translation. These range from vesicle purity, particle size distribution, zeta potential, loading efficiency per phytochemical, release kinetics, structural integrity following loading, pharmacokinetics, tissue distribution, and cellular uptake. The absence of a standard isolation and characterization methodology is a key impediment to clinical translation, as highlighted in current PDEV literature [53,54]. Third, pharmacological synergy is not necessarily the meaning of the apparent superiority of multiplex PDEV. Further formal synergy analysis of the three phytochemicals with different concentrations and validated combination models would be required to identify if there was an interaction between the three in a synergistic, additive, or independent manner. Fourth, the current molecular measurements show correlations between treatment and pathway markers, but do not provide molecular causality. For instance, the decrease in TLR7, NF- κ B, NLRP3, and MDA can be parallel effects of the overall improvement of the disease and not precisely linear. In order to infer causal relationships, mechanistic experiments would be needed, using inhibitors of specific pathways, gene silencing, knockout models, or rescue experiments. Lastly, the results of the experiments need to be confirmed in

independent experimental models and ultimately in well-controlled clinical trials. SLE is a heterogeneous disease, and the role played by TLR7, interferon signaling, complement activation, B-cell abnormalities, oxidative stress, and renal inflammation varies between patients. In the final stages of precision nanomedicine, disease phenotype, molecular signatures, pharmacokinetics and tissue-specific delivery should be added and not assumed to be the same across all SLE populations.

The current results suggest that multiplex PDEV of moringin, quercetin, and luteolin resulted in a more comprehensive and stronger therapeutic response than did delivery of single phytochemicals. This treatment pattern included improvement in proteinuria and renal biochemical indices, decrease of anti-dsDNA and inflammatory cytokines, decrease of TLR7/MyD88/NF- κ B and NLRP3-associated signaling, increase of Nrf2 and endogenous antioxidant defenses, and decrease of lipid peroxidation. The cross-talk among these effects is crucial because SLE is not a disease of a single molecular pathway but rather a network-level disease. This is, thus, the conceptual grounding of the present strategy of precision nanomedicine based on the delivery of multiple bioactive compounds at once, carried by a nanocarriers of biological origin. The highest observed response with multiplex PDEV and an integrated PDEV-SLE-PTI score of 66.90 further justifies the analysis of this platform as a potential multimodal therapeutic approach. The results, however, should be taken as preclinical/experimental data to support a therapeutic hypothesis and not as proof of clinical efficacy. Future work should aim at establishing dose-response relationships, formal combination synergy, formal PDEV pharmacokinetics and biodistribution, renal tissue targeting, long-term toxicity, immunogenicity and mechanistic causality. In the case of the present observations confirmed by these studies, multiplex PDEV delivery may be a promising approach to developing mechanism-based and perhaps personalized therapies for SLE and lupus nephritis.

CONCLUSION

This study shows the feasibility of the precision nanomedicine approach with the application of plant-derived extracellular vesicle (PDEV)-mediated multiplex delivery of moringin, quercetin, and luteolin for the treatment of systemic lupus erythematosus (SLE). Multiplex PDEV showed the highest treatment-related responses in the evaluated endpoints, as compared to responses in each single-phytochemical PDEV formulation. Significant decreases in proteinuria, anti-dsDNA, inflammatory cytokines, and renal injury markers were all seen with the treatment, along with normalization of complement levels and antioxidant defenses. The molecular changes detected were a decrease in inflammatory pathways through the TLR7/MyD88/NF- κ B pathway and an increase in the Nrf2 antioxidant pathway, as well as an increase in the expression of NLRP3. Concurrently, MDA also decreased, suggesting further restoration of the oxidative-stress imbalance of the SLE phenotype along with the decrease of MDA. A correlation analysis and a multivariable analysis also showed that there are interconnected relationships among the variables of autoimmunity, inflammation, innate immune signaling, renal injury, and oxidative stress, which indicate a multidimensional therapeutic model. Multiplex PDEV showed a superior PDEV-SLE Precision Therapeutic Index, which indicates that multiple simultaneous delivery of mechanistically complementary phytochemicals might provide greater pathway coverage than single-agent delivery. The results of the present study are, however, not definitive and are hypothesis-generating; the apparent superiority of the multiplex formulation should not be construed as a formal combination-interaction study, unless one is conducted. Optimization and reproducibility of the isolation and characterization of PDEV, phytochemical loading efficiency, release kinetics, pharmacokinetics, biodistribution, renal targeting, dose-response relationships, long-term safety, and mechanistic validation are all areas for further investigation. Individual-level datasets, too, would need to be utilized to validate the correlation and multivariable regression results. In conclusion, the results suggest that multiplex PDEV delivery of moringin, quercetin, and luteolin may be a novel multimodal approach to targeting the interconnected inflammatory, autoimmune, and oxidative mechanisms associated with SLE and lupus nephritis.

DECLARATIONS

Data Availability: The final manuscript should provide access to the underlying experimental and computational datasets according to institutional and journal policies.

Ethics Statement: All animal experiments were performed following approval by the relevant institutional animal ethics committee and in accordance with applicable national and international standards for laboratory animal welfare. The final manuscript should provide the ethics approval number.

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

Funding: N/A

Authors' Contributions

Iram Shahzadi: Conceptualization, methodology, investigation, data curation, and writing original draft. Ihsan Ullah Malik: Phytochemical investigation, in-silico analysis, data interpretation, and manuscript review. Arif Malik: Supervision, conceptualization, study design, pathway integration, critical interpretation, and writing review and editing. Hassan Mohsin: In-vivo investigation, experimental validation, and analysis of biological outcomes. Priya Tufail: Bioinformatics analysis, molecular pathway evaluation, and visualization. Gul Zaib: Literature review, data validation, and interpretation of immunological findings. Arif Malik: Statistical analysis,

results interpretation, manuscript editing, and reference verification. All authors contributed to the scientific development of the manuscript, critically reviewed the final version, and approved it for publication.

Acknowledgements

The authors gratefully acknowledge the support and contributions of all individuals who facilitated the completion of this study. We appreciate the scientific and technical assistance provided by School of Pain and Regenerative Medicine (SPRM), The University of Lahore-Pakistan, during the phytochemical investigation, in-silico analyses, and in-vivo experimental validation of the proposed immunomodulatory mechanisms in systemic lupus erythematosus.

REFERENCES

- Ahn, S. S., Yoo, B. W., Song, J. J., Park, Y. B., & Lee, S. W. (2021). Serum inflammatory cytokines and disease activity in patients with systemic lupus erythematosus. *Clinical Rheumatology*. PubMed: <https://pubmed.ncbi.nlm.nih.gov/34307989/>.
- Akdogan, M. R., & Alkan-Melikoglu, M. (2024). A potential biomarker of disease activity in systemic lupus erythematosus, systemic immune-inflammation index. **Northern Clinics of Istanbul*, 11(2), 115–119. PubMed: <https://pubmed.ncbi.nlm.nih.gov/38757099/>.
- Akhgar A, Sinibaldi D, Zeng L, et al. (2023). Urinary markers differentially associate with kidney inflammatory activity and chronicity measures in patients with lupus nephritis. *Lupus Science & Medicine*. PMID: 36717181. PubMed: <https://pubmed.ncbi.nlm.nih.gov/36717181/>.
- Akhil A, Bansal R, Kaushal J, et al. (2025). Investigating potential biomarkers and therapeutic targets for patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) through the utilization of cytokine profiling. *Reumatología Clínica (English Edition)*, 21(1), 101805. PMID: 39894627. PubMed: <https://pubmed.ncbi.nlm.nih.gov/39894627/>.
- Balkrishna, A., Thakur, P., Singh, S., Chandra Dev, S. N., & Varshney, A. (2020). Mechanistic paradigms of natural plant metabolites as remedial candidates for systemic lupus erythematosus. *Cells*, 9(4), 1049.
- Bao L, Gong Y, Xu W, et al. (2025). Chlorogenic acid inhibits NLRP3 inflammasome activation through Nrf2 activation in diabetic nephropathy. *PLoS One*. 20(1):e0316615. PMID: 39761239. PubMed: <https://pubmed.ncbi.nlm.nih.gov/39761239/>.
- Barati, M. T., & Caster, D. J. (2022). The potential of Nrf2 activation as a therapeutic target in systemic lupus erythematosus. *Metabolites*, 12(2), 151. doi: 10.3390/metabo12020151. PMID: 35208225.
- Cascarano, G., Zucchi, D., Cardelli, C., et al. (2026). Systemic lupus erythematosus: One year in review 2026. *Clinical and Experimental Rheumatology*, 44(3), 427–437. <https://doi.org/10.55563/clinexprheumatol/mg0q41>. PMID: 41841532.
- CD36 aggravates podocyte injury by activating NLRP3 inflammasome and inhibiting autophagy in lupus nephritis. (2022). **[Journal details available in PubMed record].** PubMed: <https://pubmed.ncbi.nlm.nih.gov/35999224/>.
- CD4+ T cell-derived exosomal miR-223-3p serves as a potential biomarker for systemic lupus erythematosus and attenuates lupus nephritis in NZBWF1/J mice. (2026). PubMed: <https://pubmed.ncbi.nlm.nih.gov/42381062/>.
- Che, J., Wang, H., Dong, J., Wu, Y., Zhang, H., Fu, L., & Zhang, J. (2024). Human umbilical cord mesenchymal stem cell-derived exosomes attenuate neuroinflammation and oxidative stress through the NRF2/NF-κB/NLRP3 pathway. *CNS Neuroscience & Therapeutics*, 30(3), e14454. PMID: 37697971.
- Duarte-García, A., Ardekani, A., Jackson, S. W., & Jorge, A. (2026). Systemic lupus erythematosus: What every clinician needs to know. *Mayo Clinic Proceedings*. <https://doi.org/10.1016/j.mayocp.2026.05.009>. PMID: 42203072.
- Echavarria, R., Cardona-Muñoz, E. G., Ortiz-Lazareno, P., Andrade-Sierra, J., Gómez-Hermosillo, L. F., Casillas-Moreno, J., et al. (2023). The role of the oxidative state and innate immunity mediated by TLR7 and TLR9 in lupus nephritis. *International Journal of Molecular Sciences*, 24(20), 15234.
- Effects of belimumab on peripheral blood Breg cells and cytokines in childhood SLE. (2025). PMID: 40762733. PubMed: <https://pubmed.ncbi.nlm.nih.gov/40762733/>.
- El-Shinnawy H, Mahmoud O, Abdelmohsen W, et al. (2024). The role of urinary biomarker monocyte chemoattractant protein (MCP-1) in correlation with different histopathological classes of lupus nephritis in Egyptian patients. *Egyptian Journal of Immunology*. PMID: 38224276. PubMed: <https://pubmed.ncbi.nlm.nih.gov/38224276/>.
- Felten, R., Scherlinger, M., Mertz, P., Chasset, F., & Arnaud, L. (2023). New biologics and targeted therapies in systemic lupus: From new molecular targets to new indications. A systematic review. *Joint Bone Spine*, 90(2), 105523. PMID: 36623799.
- Flores-Chova A, Martinez-Arroyo O, Puertes A, et al. (2025). Exosomal Specificity of MiR-15a-5p as Marker of Activity, Renal Damage, and Disease Flares in Systemic Lupus Erythematosus. *Cellular Physiology and Biochemistry*. PMID: 40081350. PubMed: <https://pubmed.ncbi.nlm.nih.gov/40081350/>.
- Fouquier, J., & Guedj, M. (2015). Analysis of drug combinations: Current methodological landscape. *Pharmacology Research & Perspectives*, 3(3), e00149. doi: 10.1002/prp2.149. PMID: 26171273.

19. Ganesan, K., Quiles, J. L., Daglia, M., Xiao, J., & Xu, B. (2021). Dietary phytochemicals modulate intestinal epithelial barrier dysfunction and autoimmune diseases. *Food Frontiers*, 2(3), 357–382.
20. Gao, C., Bian, X., Wu, L., Zhan, Q., Yu, F., Pan, H., Han, F., Wang, Y.-F., & Yang, Y. (2024). A nomogram predicting the histologic activity of lupus nephritis from clinical parameters. *Nephrology Dialysis Transplantation*, 39(3), 520–530. PMID: 37667508.
21. Kürtösi B, Kazsoki A, Zelkó R. (2024). A Systematic Review on Plant-Derived Extracellular Vesicles as Drug Delivery Systems. *International Journal of Molecular Sciences*. 25(14):7559. PMID: 39062803. PubMed: <https://pubmed.ncbi.nlm.nih.gov/39062803/>
22. Lai, J. H., Wu, D. W., Huang, C. Y., et al. (2025). Induction of LY6E regulates interleukin-1 β production, potentially contributing to the immunopathogenesis of systemic lupus erythematosus. **Cell Communication and Signaling*, 23*(1), 146. [PubMed](<https://pubmed.ncbi.nlm.nih.gov/40114200/>)
23. Lai, Y., Zhuang, L., Zhu, J., et al. (2024). Novel approach to alleviate lupus nephritis: Targeting the NLRP3 inflammasome in CD8⁺CD69⁺CD103⁺ TRM cells. *Journal of Translational Medicine*, 22, 1139. doi: 10.1186/s12967-024-05951-9. PMID: 39716284.
24. Lan, W., Lu, Q., Jiang, Z., Ma, W., Hou, L., Li, D., Rui, X., & Tang, F. (2026). From pathogenesis to precision medicine in systemic lupus erythematosus: Emerging biomarkers and targeted interventions. *iScience*, 29(5), 115738. <https://doi.org/10.1016/j.isci.2026.115738>. PMID: 42095089.
25. Langelotto MD, et al. (2025). Plant-derived extracellular vesicles as delivery system and natural bioactive source. *Drug Delivery and Translational Research*. PMID: 39196501. PubMed: <https://pubmed.ncbi.nlm.nih.gov/39196501/>.
26. Ma, F., Ou, Y., Liu, Y., & Ge, S. (2026). Clinical value of combined serum galectin-9 and urine MCP-1 testing for assessment of systemic lupus erythematosus disease activity and early diagnosis of lupus nephritis. *American Journal of Translational Research*. PMID: 41868890.
27. Machine Learning-Based Identification of Novel Exosome-Derived Metabolic Biomarkers for the Diagnosis of Systemic Lupus Erythematosus and Differentiation of Renal Involvement. 2025. PMID: 40019633. PubMed: <https://pubmed.ncbi.nlm.nih.gov/40019633/>
28. Maeda, K., Abdi, R., & Tsokos, G. C. (2024). The role of podocytes in lupus pathology. *Current Rheumatology Reports*, 27(1), 10. PMID: 39731699.
29. Matusiewicz, A., Paś, A., Wiktorzak, S., & Olesińska, M. (2026). Redefining difficult-to-treat systemic lupus erythematosus: Biomarkers of molecular refractoriness beyond clinical failure. *International Journal of Molecular Sciences*, 27(9), 4026. <https://doi.org/10.3390/ijms27094026>. PMID: 42123602.
30. Mauro, D., et al. (2023). UBE2L3 regulates TLR7-induced B cell autoreactivity in systemic lupus erythematosus. *Journal of Autoimmunity*, 103023. PMID: 37001433.
31. Mavragani, C. P., Kirou, K. A., Seshan, S. V., & Crow, M. K. (2023). Type I interferon and neutrophil transcripts in lupus nephritis renal biopsies: Clinical and histopathological associations. *Rheumatology*, 62(7), 2534–2538.
32. Tailliar, M., Schanstra, J. P., Dierckx, T., Breuil, B., Hanouna, G., Charles, N., Bascands, J.-L., Dussol, B., Vazi, A., Chiche, L., Siwy, J., Faguer, S., Daniel, L., Daugas, E., & Jourde-Chiche, N. (2021). Urinary peptides as potential non-invasive biomarkers for lupus nephritis: Results of the Peptidu-LUP study. *Journal of Clinical Medicine*, 10(8), 1690. PMID: 33920017.
33. Tang C, Zhang S, Teymur A, et al. (2023). V-Set Immunoglobulin Domain-Containing Protein 4 as a novel serum biomarker of lupus nephritis and renal pathology activity. *Arthritis & Rheumatology*, 75(9), 1573–1585. PMID: 37163449. PubMed: <https://pubmed.ncbi.nlm.nih.gov/37163449/>
34. Tang, C., Zhang, S., Teymur, A., et al. (2023). V-set immunoglobulin domain-containing protein 4 as a novel serum biomarker of lupus nephritis and renal pathology activity. **Arthritis & Rheumatology*, 75*(9), 1573–1585. PubMed: <https://pubmed.ncbi.nlm.nih.gov/37163449/>.
35. Urinary markers differentially associate with kidney inflammatory activity and chronicity measures in patients with lupus nephritis. (2023). PubMed: <https://pubmed.ncbi.nlm.nih.gov/36717181/>
36. Urinary markers differentially associate with kidney inflammatory activity and chronicity measures in patients with lupus nephritis. 2023. PMID: 36717181. PubMed: <https://pubmed.ncbi.nlm.nih.gov/36717181/>
37. Urinary neutrophil gelatinase-associated lipocalin (uNGAL) and kidney injury molecule-1 (uKIM-1) as markers of active lupus nephritis. Ibrahim WHM, Sabry AA, Abdelmoneim AR, et al. (2024). *Clinical Rheumatology*, 43(1), 167–174. PMID: 37516706. PubMed: <https://pubmed.ncbi.nlm.nih.gov/37516706/>
38. Urinary soluble CD163 is a promising biomarker for the diagnosis and evaluation of lupus nephritis. 2022. PMID: 35911758. PubMed: <https://pubmed.ncbi.nlm.nih.gov/35911758/>
39. Urinary soluble CD163 is useful to predict lupus nephritis activity and to monitor standard-of-care therapy response. (2025). **Rheumatology**. PubMed: <https://pubmed.ncbi.nlm.nih.gov/40824081/>
40. Yang, S., et al. (2025). Edible ginseng-derived exosomes as drug delivery vehicles reduce the dose and improve the anti-cancer effect of CDDP. *Biochemical and Biophysical Research Communications*, 758, 151658. PMID: 40121965.
41. Yi, T., Zhang, W., Hua, Y., et al. (2024). Rutin alleviates lupus nephritis by inhibiting T cell oxidative stress through PPAR γ . *Chemico-Biological Interactions*, 394, 110972. doi: 10.1016/j.cbi.2024.110972. PMID: 38555047.

42. Young, N. A., Schwarz, E., Zeno, B. M., Bruckner, S., Mesa, R. A., Jablonski, K., Wu, L.-C., Roberson, E. D. O., & Jarjour, W. N. (2024). Inhibition of miRNA associated with a disease-specific signature and secreted via extracellular vesicles of systemic lupus erythematosus patients suppresses target organ inflammation in a humanized mouse model. *Frontiers in Immunology*, 14*, 1090177. PubMed: <https://pubmed.ncbi.nlm.nih.gov/38939646/>
43. Yung, S., Yap, D. Y., & Chan, T. M. (2020). A review of advances in the understanding of lupus nephritis pathogenesis as a basis for emerging therapies. *F1000Research*, 9, F1000 Faculty.
44. Zeng, Y., Xu, W., Chao, P., Xiao, Y., & Yang, T. (2025). Neutrophil extracellular traps as a potential marker of systemic lupus erythematosus activity. *International Immunopharmacology*. doi: 10.1016/j.intimp.2024.113840.
45. Zhang H, Zheng X, Huang S, et al. (2025). Urinary extracellular vesicle RNAs as novel biomarkers for diagnosis and prognosis of lupus nephritis. *Clinical Kidney Journal*. PMID: 41104259. PubMed: <https://pubmed.ncbi.nlm.nih.gov/41104259/>
46. Zhang Q, et al. (2024). Renal remodeling by CXCL10-CXCR3 axis-recruited mesenchymal stem cells and subsequent IL411 secretion in lupus nephritis. *Signal Transduction and Targeted Therapy*. PMID: 39557841. PubMed: <https://pubmed.ncbi.nlm.nih.gov/39557841/>
47. Zhang S, Xu RY, Kang LL. (2024). Biomarkers for systemic lupus erythematosus: A scoping review. *Immunity, Inflammation and Disease*. 12(10):e70022. PMID: 39364719. PubMed: <https://pubmed.ncbi.nlm.nih.gov/39364719/>
48. Zhang T, et al. (2025). Urine proteins reveal distinct coagulation and complement cascades underlying acute versus chronic lupus nephritis. *Journal of Clinical Investigation*. 135(19):e186143. PMID: 41031884. PubMed: <https://pubmed.ncbi.nlm.nih.gov/41031884/>
49. Zhang T, Zhao L, Xu M, Jiang P, Zhang K. (2024). Moringin alleviates DSS-induced ulcerative colitis in mice by regulating Nrf2/NF-κB pathway and PI3K/AKT/mTOR pathway. *International Immunopharmacology*. 134:112241. PMID: 38761782. PubMed: <https://pubmed.ncbi.nlm.nih.gov/38761782/>,
50. Zhang X, et al. (2018). Composite urinary biomarkers to predict pathological tubulointerstitial lesions in lupus nephritis. *Lupus*.
51. Zhang, L., Zhao, L., Du, K., Chen, J., Ding, H., Petersen, F., Ye, S., Lin, Z., & Yu, X. (2024). Serum levels of CXCL5 are decreased and correlate with circulating platelet counts in systemic lupus erythematosus. *International Journal of Rheumatic Diseases*, 27*(3), e15089. PubMed: <https://pubmed.ncbi.nlm.nih.gov/38439196/>
52. Zhang, S., Xu, R., & Kang, L. (2024). Biomarkers for systemic lupus erythematosus: A scoping review. *Immunity, Inflammation and Disease*, 12(10), e70022. PMID: 39364719.
53. Zhang, T., et al. (2025). Urine proteins reveal distinct coagulation and complement cascades underlying acute versus chronic lupus nephritis. *Journal of Clinical Investigation*, 135(19), e186143. PMID: 41031884.
54. Zhao, L., Zhang, Q., Feng, Z., Zhang, J., & He, F. (2022). Serum amyloid A-to-albumin ratio as a potential biomarker to predict the activity, severity, and poor prognosis of systemic lupus erythematosus. *Journal of Clinical Laboratory Analysis*, 36(3), e24282. PMID: 35141936.