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Original Article

Maslinic Acid Therapeutic Effect in Rheumatoid Arthritis Through Activation of Nuclear Factor Erythroid 2 and Suppression of Nuclear Factor Kappa B as well as Apoptotic Markers

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ABSTRACT

Background: Rheumatoid Arthritis (RA) is a chronic autoimmune condition characterized by persistent joint inflammation, resulting in pain, stiffness and degradation. Despite advances in understanding RA pathophysiology, its precise mechanisms remain complex. This article explores the processes involved in RA, including the interplay of inflammatory cascades, immune responses, cytokines and apoptosis. **Materials and Methods:** This study divided 32 Wistar rats into five groups. The model group (RA) received Collagen type II (COII) injections into both knee joints. The Maslinic Acid treated (MA) model group including the COII+MA group received (i.p) daily MA treatment and COII+MA+brusatol which received both MA and brusatol (a selective Nrf2 inhibitor) daily. Controls received vehicle solutions and a control-treated group received MA. The study assessed in blood and synovial tissues Rheumatoid Factor (RF), the inflammatory (NF-κB p65, TNF-*α*, IL-6, VEGF and ICAM) and oxidative stress (ROS, MDA, SOD and GSH) pathways, nuclear factor erythroid 2-Related Factor 2 (Nrf2) and biomarkers of apoptosis and survival (Bax, cleaved caspase-3 and Bcl-2) gene expression levels. **Results:** Induction of RA by COII caused the modulation of inflammation and oxidative stress as well as Nrf2, NF-κB p65 and apoptosis, which were inhibited by MA. On the other hand, all the protective effects of MA were substantially blocked by the pharmacological inhibitor of Nrf2, brusatol. **Conclusion:** Maslinic acid provides profound protection in a rat model of RA via Nrf2 activation in association with the inhibition of oxidative stress, inflammation and apoptosis

Keywords: Apoptosis, Maslinic Acid, Rheumatoid Arthritis.

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INTRODUCTION

RA is a prevalent autoimmune disorder that afflicts approximately 1% of the population. The disease affects synovial joints, resulting in severe pain, joint deformities and a diminished quality of life.[1] The pathophysiology of RA is a complex interplay of genetic predisposition, disrupted immune responses and environmental factors, ultimately leading to chronic inflammation and joint deterioration.[2] Genetic susceptibility plays a crucial role in the development of RA, with a particular emphasis on the Human Leukocyte Antigen (HLA) genes. Specific HLA-DRB1 alleles, like the shared epitope, are linked to an elevated risk of disease onset.[3] Dysregulated immune responses characterize RA, involving innate and adaptive immunity abnormalities. The infiltration of activated immune cells, such as macrophages, dendritic cells and T lymphocytes, into the synovium, triggers a cascade of events leading to synovial inflammation.[4] RA patients often display autoantibodies like Rheumatoid Factor (RF) and Anti-Citrullinated Protein Antibodies (ACPAs). ACPAs, highly specific to RA, are associated with more severe disease outcomes. These autoantibodies contribute to joint damage by promoting

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inflammation, immune complex formation and complement activation.[4]

The activation of CD4+ T-helper cells, particularly Th1 and Th17 subsets, trigger the release of pro-inflammatory cytokines, such as IL-6 and Tumor Necrosis Factor (TNF-α) and These cytokines further encourage the recruitment of immune cells, perpetuating the inflammatory cycle.[5] In RA, persistent inflammation within the synovial membrane results in synovial hyperplasia and the formation of destructive synovial tissue proliferation known as the pannus. The pannus invades and erodes cartilage and bone, contributing to joint deformities and loss of function.[6] Resident fibroblast-like synoviocytes play a critical role in pannus formation by proliferating uncontrollably and producing enzymes that degrade the extracellular matrix. Angiogenesis is another hallmark of RA pathophysiology, as the pannus requires a blood supply to sustain its rapid growth. Angiogenesis facilitates the migration of immune cells into the synovium, further perpetuating inflammation and tissue damage.[7] Apoptosis, known as programmed cell death, assumes a dual role in the context of RA. On the one hand, excessive apoptosis of immune cells, including synovial fibroblasts and lymphocytes, contributes to tissue damage and sustains inflammation. Conversely, impaired mechanisms for clearing apoptotic cells result in the accumulation of apoptotic bodies, leading to the release of auto-antigens and the generation of auto-antibodies, which further contribute to inflammation and tissue destruction.[8] The signaling pathways regulating apoptosis in RA encompass extrinsic and intrinsic pathways. The extrinsic pathway encompasses cell surface death receptors like Fas (CD95) and TNF receptor-1, which trigger apoptosis upon ligand binding.[9] The intrinsic pathway operates through mitochondria-mediated apoptosis, involving factors such as Bcl-2 family proteins. Both the Fas (CD95) and TNF receptor-1 pathways and mitochondria-mediated apoptosis are influenced by MA.[10] The intricate interplay between inflammatory and apoptosis markers in RA constitute a complex and dynamic process that significantly contributes to the development and progression of the disease. Advances in our understanding of the roles of these markers have cleared the path for creating innovative therapeutic approaches targeting the restoration of the delicate balance between inflammation and cell death.[11] Continued research in this field promises improved outcomes and an enhanced quality of life for individuals with RA. In recent years, there has been growing interest in the potential of inhibiting the NF-κB pathway as a promising strategy for mitigating inflammatory responses. One such compound, known as MA, can be sourced from various fruits and vegetables, with olive skins being a particularly rich source.[12] Numerous studies have reported that MA exhibits remarkable anti-tumor[13,14] antioxidant and anti-inflammatory properties.[15,16] These effects can be attributed to MA's capacity to inhibit Protein Kinase-C (PKC) activity and the NF-κB pathway, while simultaneously promoting the activation of heme-oxygenase and endothelial nitric oxide synthase. This dual action results in the suppression of critical cytokines and Reactive Oxygen Species (ROS) synthesis.[17,18] Collectively, these findings strongly suggest that MA may offer a potential avenue for alleviating inflammation in RA by downregulating NF-κB and upregulation of Nrf2.

The primary objective of this investigation was to delve deeply into the pathophysiological processes at play in RA, encompassing the complex interactions among immune cells, cytokines, oxidative stress and apoptosis. Furthermore, the study aimed to explore the protective role of MA against the destruction of the knee joint affected by RA and elucidate its potential mechanisms.

MATERIALS AND METHODS

Study Location and Animal Subjects

This research was conducted at the Research Labs, College of Medicine, King Khalid University, Saudi Arabia. The study involved 32 Wistar rats with an average weight of 160±10 g. The study adhered to the guidelines provided by the Ethical Committee of the College of Medicine, King Khalid University (Ethical Committee No. REC # 2022-11-09), Saudi Arabia. This work complied with the ethics outlined in the Helsinki Declaration, as updated during the 64th WMA General Assembly in Fortaleza, Brazil, in October 2013, to investigate laboratory animals. The rats were housed in clean cages within a controlled animal facility, subjected to a 12-hr light/dark cycle, maintained at a steady room temperature and provided unrestricted food and water access.

Experimental Methodology

Following a two-week acclimatization phase, the 50 adult male rats were evenly divided into five groups, each consisting of ten rats. The model group (RA) involved the active immunization of rats by injecting Collagen type II (COII) into both knee joints. The COII used was of bovine origin, obtained from Sigma-Aldrich, MO, USA. Type II collagen was dissolved in 0.05 mol/L acetic acid, with a concentration of 2 mg/mL achieved through stirring at room temperature. Subsequently, this COII solution was mixed with an equal volume of complete Freund's adjuvant.[13] The MA was dissolved in a 5% Carboxy-Methyl Cellulose (CMC) solution to achieve a concluding concentration of 80 mg/kg^{-1}.[17] The treated model rats were divided into two sub-groups (10 rats/sub-group); the first sub-groups (RA+MA) was the COII+MA group consisting of matched rats that began receiving daily (i.p.) treatment with 80 mg/kg of MA while the 2nd sub-group (RA+MA+Br) received MA as the first sub-group in addition to brusatol 2 mg/kg twice per week (i.p.) (a selective Nrf2 inhibitor) (Sigma-Aldrich). This treatment commenced on day 21 and continued for nine weeks.[14] The fourth group (C), the control group, consisted of untreated rats who received vehicle solutions intraperitoneally. The fifth group of rats (C+MA) was control-treated, receiving MA at the same dose, time and method as the treated model group. This

treatment commenced on day 21 and continued for nine weeks.[14] At the end of 12 weeks, overnight fasting done for all rats every rat received an injection of a 1 mL/kg mixture of ketamine. Blood samples (2 mL) were collected from the heart into plain tubes and centrifuged at 1300x g for 3 min to collect serum, then stored at -20ºC for biochemical analysis. Subsequently, all rats were euthanized by dislocating the neck and their knee synovial fluid and synovial tissues were swiftly harvested, frozen in liquid nitrogen and preserved at -80ºC until further use.

Assessment of serum RF and VEGF synovial tissue homogenate level

Serum levels of Rheumatoid Factor (RF) were determined using Enzyme-Linked Immunosorbent Assay (ELISA) kits. The ELISA kits for RF were sourced from CUSABIO Technology LLC, TX, USA (RF) and Abcam, Cambridge, UK (TNF-α). These ELISA kits were employed in all rat groups following the manufacturers' guidelines after the 12-week study. The levels of Vascular Endothelial Growth Factor (VEGF) in synovial fluid were measured using rat VEGF Duo Set ELISA kits (R and D Systems, Minneapolis, MN, USA). Per the manufacturer's instructions, these kits employed antibodies primarily specific for VEGF-165 and VEGF-121, the two major isoforms of proangiogenic VEGF. Isolation of the Nuclear Fraction and Measurement: To assess the nuclear protein expression levels of cleaved caspase-3, Bcl-22, Bax and NF-κB p65, the nuclear and cytoplasmic fractions were extracted from the frozen synovium tissues. A commercially available kit (Cat 78833; ThermoFisher, USA) was used. Nuclear protein expression levels Nrf2 were quantified using the TransAM assay (Cat 50296, Active Motif, Tokyo, Japan). All steps were duplicated, following the manufacturer's guidelines.

Measurements of synovial tissue homogenate TNF-α and ICAM levels

Synovial tissue homogenate Tumor Necrosis Factor-Alpha (TNF-α) level were determined using Enzyme-Linked Immunosorbent Assay (ELISA) kits. The ELISA kits TNF-*α* was sourced from Abcam, Cambridge, UK. These ELISA kits were employed in all rat groups following the manufacturers' guidelines after the six-week study. While the serum levels of interleukin-intracellular Cell-Adhesive Molecule (ICAM) was quantified using ELISA kits (Cat MBS267983, My BioSource, CA, USA and Cat ab100713, Abcam, UK).

Isolation of the Nuclear Fraction and Measurement

The nuclear and cytoplasmic fractions of the frozen left retinae were prepared to form n=10 retinas/group. Nuclear levels (activation) of NF-*κ*B p65 were measured by the TransAm assay (Cat 40596, Active Motif, Tokyo, Japan). (The nuclear levels (activation) of Nrf2 were measured using the TransAM assay (Cat 50296 Active Motif, Tokyo, Japan). All procedures were performed in duplicate and following the manufacturers' instructions.

Real-Time Polymerase Chain Reaction (qPCR)

To quantify the mRNA levels of specific apoptotic markers, the qPCR method was employed. Primer sequences designed for amplifying Bcl-2, Bax and cleaved caspase-3 were purchased from ThermoFisher. The primer pairs used were as follows: Bax (Acc. No. NM_017059): F: ATGGAGCTGCAGAGGATGATT and R: TGAAGTTGCCATCAGCAAACA. For Bcl-2 (U34964.1): F: TGGGATGCCTTTGTGGAACT and R: TCTTCAGAGACTGCCAGGAGAAA. Forcleaved caspase-3 (Cat #PA5-114687): F: ATCGCTTCCTAGTTCCGCTG and R: TGGTCCTAATCGGTTGCACC. For RNA isolation from SYN tissues was performed using the HeneJet isolation kit (Cat K0731, ThermoFisher). Subsequently, first-strand cDNA was synthesized using the Verso synthesis kit (Cat AB1453 A, ThermoFisher), following the instructions provided by each kit. The mRNA amplification reaction was conducted in the BioRad CFX96 system (USA) using Ssofast Evergreen supermix master mix, following the manufacturer's instructions. The reaction mixture (10 µL) consisted of the following components: template cDNA (1 µL/500 ng), forward primer for each target (0.2 µL/200 nm/reaction), reverse primer for the target (0.2 µL/200 nm/reaction), master mix (5 µL) and nuclease-free water (3.6 µL). The amplification process involved the following steps: One cycle of heating for 30 sec at 95ºC. Thirty-four cycles of denaturation for 30 sec at 95ºC. Annealing was conducted for 34 cycles, each for 30 sec at 60ºC. Finally, a single melting step was performed, lasting 1 sec at 95ºC. Following this, the $2^{-\Delta\Delta Ct}$ method was utilized to standardize the relative expression levels of Bcl-2 and Bax to the mRNA levels of the reference gene.

Western Blotting

We utilized the Quantity-Pro ABC assay kit to measure total protein content in cytoplasmic and nuclear fractions. The samples were diluted to 2 µg/µL, boiled for 5 min and loaded at 40 µg/mL concentration onto SDS-PAGE. After transferring to nitrocellulose membranes, they were blocked in 5% skimmed milk/TBST and probed with specific primary monoclonal antibodies against: Nrf2 and NF-B p65 and β-actin. Membranes were washed, exposed to secondary antibodies and detected with an ECL Pierce kit. Gel scans were analyzed with C-Di Git blot scanner. Both Nrf2 and NF-B p65 were normalized to β-actin.

Statistical Analysis

Analyses and graphs were produced using the GraphPad prism analysis software (version 8). The Kolmogorov-Smirnov test was used to determine the degree of normality. The data were compared using the two-way ANOVA followed by Tukey's *t*-test as a *post hoc* test. *p*<Analyses and graphs were produced using the GraphPad Prism analysis software (version 8). The Kolmogorov-Smirnov±SD).

RESULTS

Synovial tissue RF and Synovial fluid VEGF levels

Induction of RA by Collagen type II (COII) injections into the left knee of rats resulted in a significant elevation in Serum Rheumatoid Factor (SRF) levels when compared to the standard control and control MA groups. Daily intraperitoneal (i.p.) administration of MA for six weeks led to a substantial reduction in SRF levels compared to the RA-induced group, with no significant difference observed compared to the control groups (Figure 1A). On the other hand, administration of brusatol concomitantly with MA cancelled the effect of MA in diabetic rats as shown in group DM+MA+Br (Figure 1A). Synovial fluid levels of VEGF showed insignificant changes in MA-treated control rats compared with the control non-treated group. While RA rats showed a significant increase in synovial fluid VEGF levels compared with the control groups. Treatment with MA in RA-induced rats produced a significant reduction in the synovial fluid VEGF compared with the RA-induced rats but still significantly higher level compared with control groups (Figure 1A). Again, concomitant administration of both MA and brusatol nullified the effect of MA on the VEGF in diabetic rats (Figure 1B).

Synovial fluid ROS, MDA, SOD and GSH levels

Synovial fluid levels of the oxidative stress agent MDA and the antioxidants SOD and GSH were assessed. Daily administration of MA in the control group significantly increased the levels of SOD and GSH in the synovial fluid compared to the untreated control group. RA induction in rats resulted in a significant increase in MDA levels in the synovial fluid, accompanied by a significant reduction in endogenous antioxidants, SOD and GSH compared to the control groups. Conversely, administering MA for six weeks

in rats significantly reduced the MDA levels in synovial fluid while significantly increasing both SOD and GSH levels compared to the RA-induced group. These changes were insignificantly different from the untreated control group. In All the parameters of oxidative stress and antioxidants, administration of brusatol combined with Ma nullified the effects of MA in diabetic rats (Figures 2 A, B, C, D).

Synovial fluid levels of inflammatory markers

Rats with induced RA exhibited significantly elevated TNF-α and ICAM levels in their synovial fluid compared to the control groups. However, daily treatment with MA in RA-induced rats for six weeks significantly reduced these inflammatory markers compared to the RA group. The changes were not statistically significant compared to the control groups (Figures 2A, B, C). It was clear that administration of brusatol with MA cancelled the results of the MA in diabetic rats when administrated alone (Figures 3 A, B).

Synovial tissue homogenate protein levels of Nrf2 and NF-κB p65

The induction of RA in rats significantly increased the protein expression of NF-κB p65 while resulting in a significant reduction in the protein expression of Nrf2 compared to the control groups. Daily administration of MA in RA-induced rats effectively reduced the protein expression of NF-κB p65 and increased the protein expression of Nrf2 compared to the RA non-treated rats (Figure 3A, B). On the other hand, rats in DM+MA+Br group showed elimination of the effect of MA when given alone on both

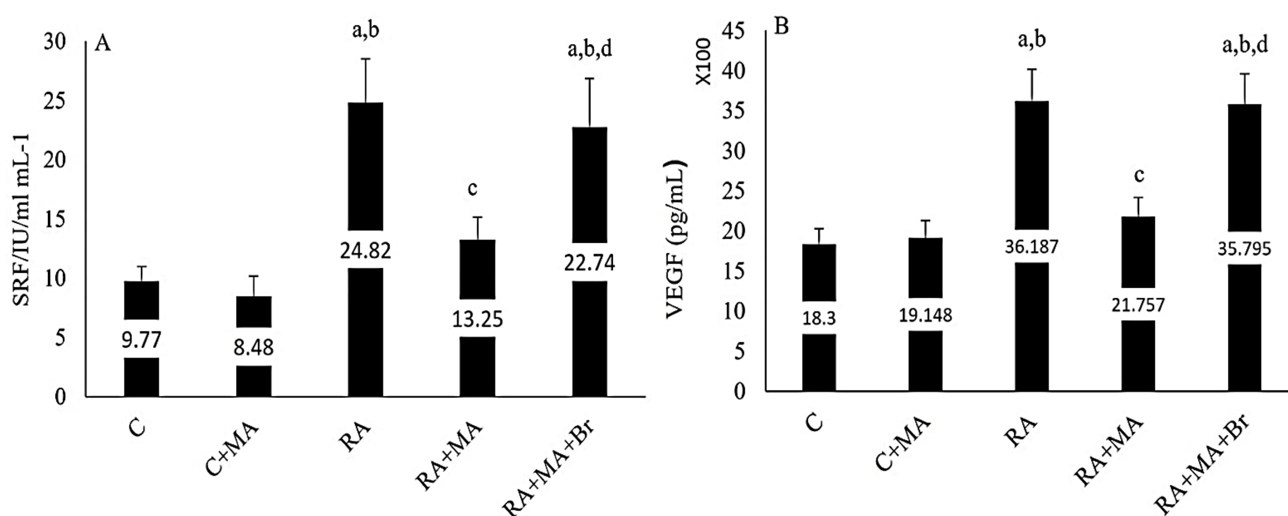

Figure 1: Serum level of RF (A) and synovial tissue homogenate levels of VEGF (B) in all experimental groups of rats (*n*=10) after week 12. Values are expressed as Mean±SD. Values were considered significantly different at $p<0.05$. [a] significantly different as compared to control rats. [b] significantly different as compared to control MA treated group. [c]: significantly different compared to the RA group. [d] significantly different compared to RA treated group.

SYN tissue homogenate expression protein levels of Nrf2 and NF-κB p65 in diabetic rats (Figure 4 A, B).

Synovial tissue homogenate mRNA expression protein levels of Cleaved caspase-3, Bax and Bcl-2

Induction of RA in rats led to a significant increase in the Synovial tissue homogenate mRNA levels of Bax and cleaved caspase-3, along with a significant reduction in the mRNA levels of Bcl-2 when compared to the RA rat group. Conversely, the administration of MA in RA-induced rats resulted in a significant reduction in the synovial tissue homogenate mRNA levels of Bax and cleaved caspase-3, coupled with a significant elevation in the mRNA levels of Bcl-2 compared to the RA rat groups. Again, administration of both MA and brusatol in Diabetic rats

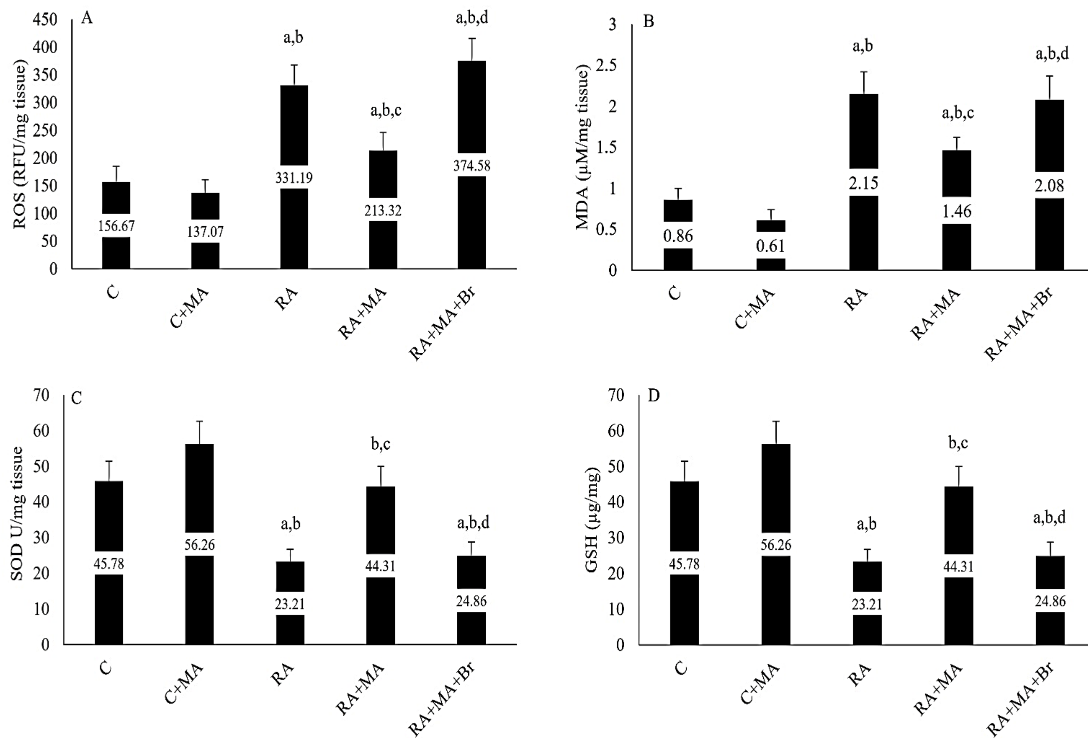

Figure 2: Synovial tissue homogenate levels of oxidative stress markers [ROS (A), MDA (B), SOD (C) and GSH (D)] in all experimental groups of rats (n=10) after week 12. Values are expressed as Mean±SD. Values were considered significantly different at p<0.05. [a] significantly different as compared to control rats. [b] significantly different as compared to control MA treated group. [c]: significantly different compared to the RA group. [d] significantly different compared to RA treated group.

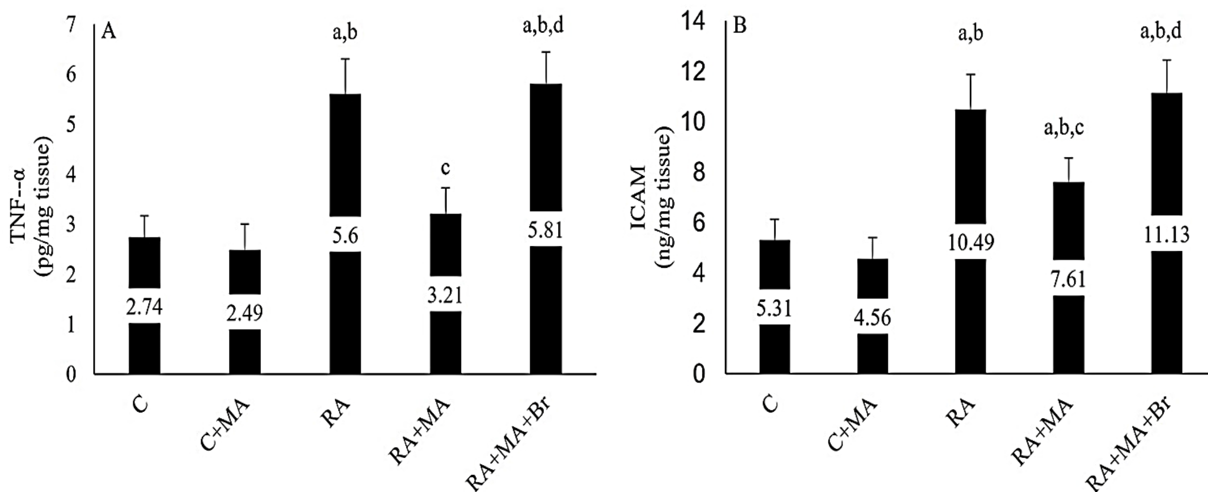

Figure 3: Synovial tissue homogenate levels of inflammatory markers [TNF-α (A) and ICAM (B)] in all experimental groups of rats (n=10) after week 12. Values are expressed as Mean±SD. Values were considered significantly different at p<0.05. [a] significantly different as compared to control rats. [b] significantly different as compared to control MA treated group. [c]: significantly different compared to the RA group. [d] significantly different compared to RA treated group.

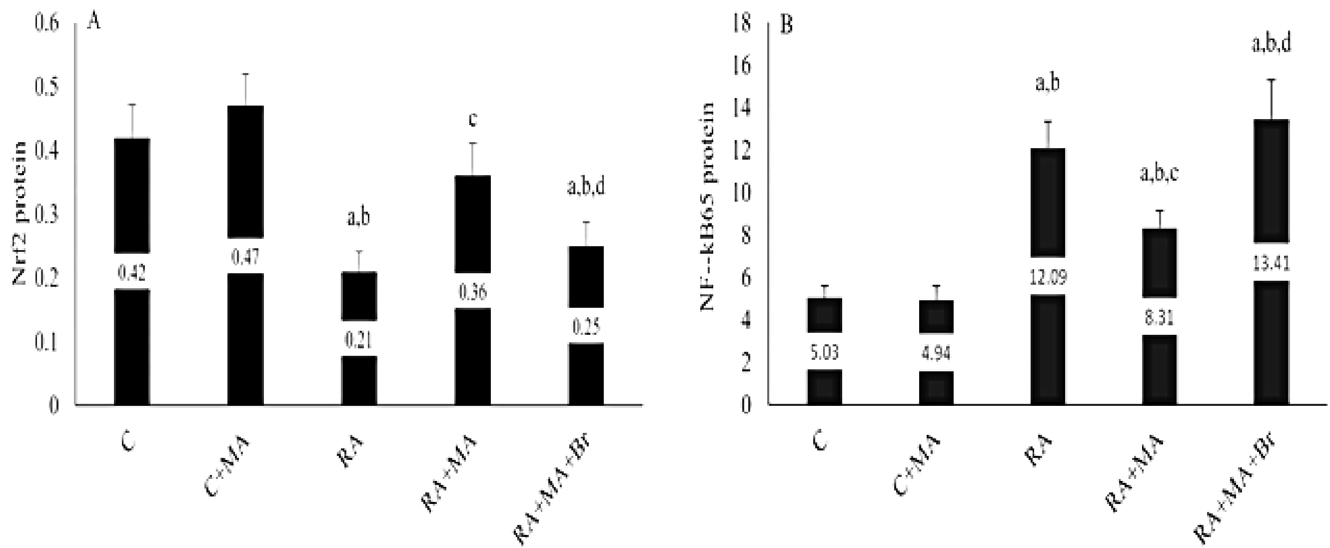

Figure 4: Synovial tissue homogenate nuclear protein levels of [Nrf2(A), NF-κB p65 (B)] in all experimental groups of rats (n=10) after week 12. Values are expressed as Mean±SD. Values were considered significantly different at p<0.05. [a] significantly different as compared to control rats. [b] significantly different as compared to control MA treated group. [c]: significantly different compared to the RA group. [d] significantly different compared to RA treated group.

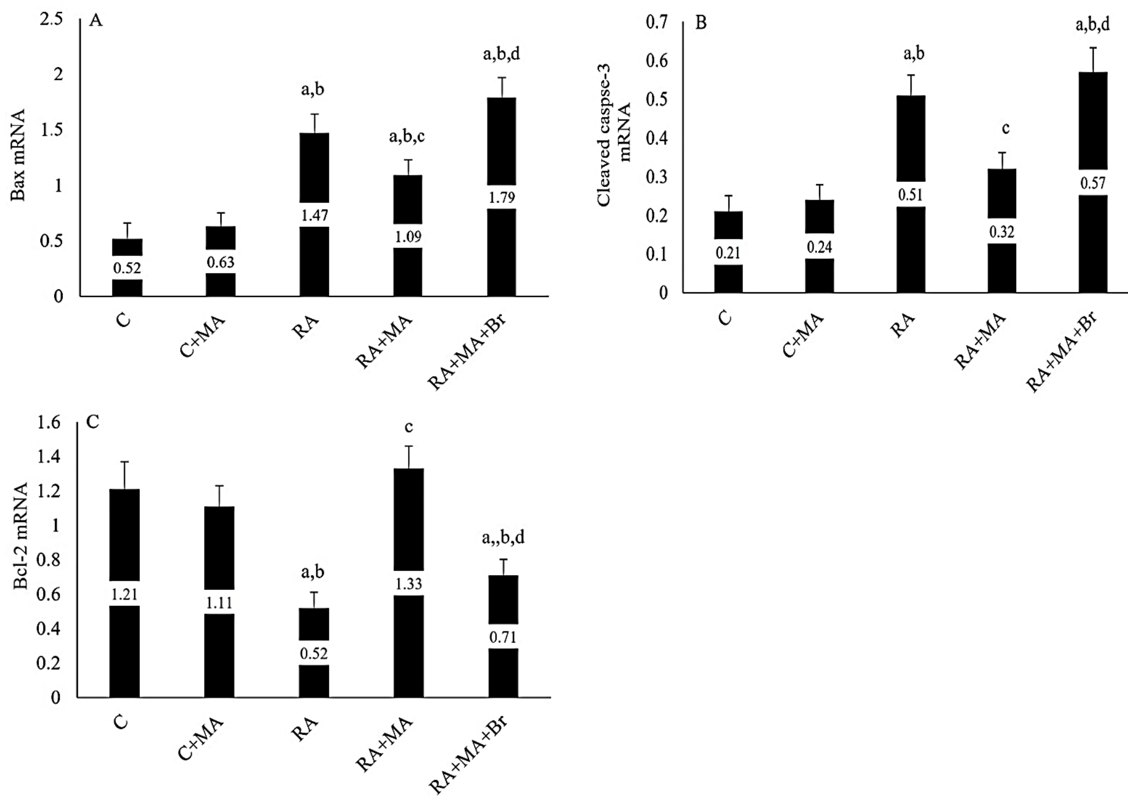

Figure 5: Synovial tissue homogenate mRNA expression levels of apoptotic markers Bax (A), Cleaved caspase-3 (B) and Bcl-2 (C)] in all experimental groups of rats (n=10) after week 12. Values are expressed as Mean±SD. Values were considered significantly different at p<0.05. [a] significantly different as compared to control rats. [b] significantly different as compared to control MA treated group. [c]: significantly different compared to the RA group. [d] significantly different compared to RA treated group.

cancelled the effects of MA administration alone on the mRNA levels of Bax and cleaved caspase-3 and Bcl-2 (Figure 5 A, B, C).

DISCUSSION

In this study, the induction of RA in the experimental groups was confirmed by the presence of elevated levels of serum rheumatoid factors and biochemical alterations in the knee synovial fluids of the experimental rat groups. This research highlights the significant protective effects of intraperitoneal (i.p.) administration of Methyl Anthranilate (MA) in mitigating RA in rats, thereby reducing the development of pathological changes and deformities. These protective effects are primarily referred to the anti-oxidative, anti-inflammatory and anti-apoptotic effects of MA. Furthermore, changes in the inflammatory cytokine, Vascular Endothelial Growth Factor (VEGF) expression, intrinsic cell death markers and inhibition of NF-κB p65 confirmed these protective effects. Despite the considerable progress in our understanding of the pathophysiology of RA, the precise mechanisms involved remain intricate and multifaceted. However, noteworthy observations indicate that MA enhances the levels of GSH and SOD, concurrently activating Nrf2 in both the control and RA groups. So, activating the Nrf2/antioxidant pathway is pivotal in the defense against RA-related ailments. Typically, Nrf2 is confined to the cytoplasm and undergoes cytoplasmic proteasomal ubiquitination due to its interaction with keap-1.[19] Yet, when exposed to stress, Reactive Oxygen Species (ROS) trigger phosphorylation, redox alterations and keap-1 dissociation, enabling Nrf2 to move into the nucleus and initiate transcription.[12]

In the context of this study, treatment with MA led to a significant increase in the levels of SOD and GSH in rats with RA. The induction of RA triggers the inflammatory response and oxidative stress in affected joints, activating various mechanisms that generate ROS, such as NADPH oxidase and protein kinase C. ROS levels can further escalate due to the suppression of Nrf2 transactivation via acetylation and methylation.[13] However, treatment with MA not only stimulated Nrf2 activity but also increased GSH and SOD levels. This was accompanied by a reduction in ROS generation and MDA levels in the affected knee joints of control rats, underscoring the potent independent antioxidant effect of MA. Severe inflammation can exacerbate the body's oxidative stress response and trigger tissue apoptosis (Nezu, Suzuki *et al.* 2017). A close and mutually reinforcing relationship exists between oxidative damage and inflammation.[14] NF-κB acts as the primary transcription factor responsible for inflammation in cells, promoting the production of Reactive Oxygen Species (ROS) by increasing the expression of inflammatory cytokines and mediators like TNF-α and IL-6. This activation primarily occurs through the Toll-Like Receptor-type 4 (TLR4).[15] Our findings align with this, as we observed a significant increase in TNF-α, IL-6, ICAM levels and hyper-activated NF-κB in untreated rats with RA. In RA joints, inflammation is marked by heightened immune cell infiltration, elevated white blood cell presence and complement system activation.[15] The present study, showed a notable elevation in the nuclear activities of NF-κB and the levels of ICAM, TNF-α and IL-6, accompanied by the oxidative stress response, which suggests that inflammation plays an integral role in the progression of RA. However, MA significantly reversed these effects in the experimental rats' synovium, cartilaginous plates and bone homogenates without affecting these markers in the knees of control rats. Consistent with our findings, the anti-inflammatory properties of MA have been well-documented in various disorders, often aligning with its antioxidant effects.[15] MA also prevents osteoclast formation by inhibiting NF-κB and immune cell infiltration.[16] They exhibited protective effects against cardiomyopathy induced by diabetes mellitus by inhibiting NADPH oxidase and NF-κB.[17] In addition, MA effectively prevented inflammation and oxidative damage induced by ischemia/reperfusion in endothelial cells and pericytes by reducing ROS production and the expression of adhesive molecules (E-selectin, ICAM and VCAM) and inhibiting NF-KB.[17] Moreover, MA has been shown to mitigate Lipopolysaccharide (LPS)-induced inflammation in macrophages and cortical astrocytes by reducing ROS, NO generation and suppressing 2F-kB, as well as the production of TNF-α and IL-6.[18]

Interestingly, the inhibitory effects of MA on ROS, inflammatory cytokines and cell apoptosis markers in the synovial tissue of rats were prevented after suppressing Nrf2 by brusatol. This indicated that MA protects the rheumatoid-affected joint through elevation of Nrf2 with concomitant suppression of NF-κB with subsequent inhibition of oxidative stress levels and inflammatory cascades.

The interplay between oxidative stress and inflammatory cytokines typically triggers cellular apoptosis, ultimately damaging organs. In cellular contexts, the caspase family of enzymes is the most recognized initiator of cell death.[20] Caspase-8 and caspase-9 are the key initiator caspases responsible for initiating the cleavage and activation of executioner caspases, specifically caspases-3 and caspase-9.[21] Cellular apoptosis typically involves extrinsic and intrinsic cell death pathways.[22] Activating death receptors by the inflammatory factor TNF-α prompts extrinsic and intrinsic cell death by elevating caspase levels.[21] In contrast, intracellular signaling, like ROS, can initiate intrinsic cell apoptosis by increasing p53/Bax, lowering Bcl-2 levels and activating Bax, resulting in mitochondrial damage and the release of cytochrome-C.[21] In the context of RA, intrinsic cell death emerges as the predominant modality of cell apoptosis, characterized by increased expression of Bax and caspase-3 and reduced levels of Bcl-2.[21] Our research extends the validation of this phenomenon in the untreated rats' knee joints affected by RA. It highlights the significant capacity of MA to suppress the transcription of Bax and reduce cleaved caspase-3 protein levels while simultaneously elevating Bcl-2 levels. These results strongly endorse the

anti-apoptotic impact of MA, which can be attributed to its anti-inflammatory and antioxidant qualities. Intriguingly, we also noted a stimulatory effect of MA on the transcription of Bcl-2 in the knee joints of control rats. This may be attributed to the concurrent activation of Nrf2, indicating that, aside from its antioxidant impact, MA is a potent anti-apoptotic factor by enhancing the cellular availability of Bcl-22.[23] In line with our findings, MA's anti-apoptotic effects have been associated with its antioxidant and anti-inflammatory properties. These attributes effectively inhibit both extrinsic and intrinsic apoptosis in various tissues and cell types *in vivo* and *in vitro* settings.[16,21] Nonetheless, it is vital to acknowledge the limitations of this study. First, the potential therapeutic value of MA might be enhanced if it were administered directly into the affected joints in addition to its systemic application. Secondly, our findings are primarily observational and further validation is warranted, potentially using transgenic animals or the inhibition/knockdown of Nrf2 to substantiate our results. Furthermore, it is plausible that MA activates Nrf2 by directly stimulating SIRT1. Diving into this mechanism in future investigations may illuminate the precise mode of action. Additionally, studies on a human scale are essential, especially given that MA has exhibited no toxic effects even at high doses. Conclusion: Our data remains intriguing and demonstrates the protective impact of MA against the degeneration of knee joints induced by Collagen type II antigen injection. This protection is attributed to its anti-inflammatory, antioxidant and anti-apoptotic effects. Encouragingly, further pre-clinical and translational research is needed to substantiate these effects, which could offer a promising novel therapy for RA patients.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ACPA: Anti-Citrullinated Protein Antibodies; **Bax:** Bcl-2-associated X protein; **Bcl-2:** B-cell lymphoma 2; **C:** Control Group; **CD4:** Cluster of Differentiation 4; **CMC:** Carboxy-Methyl Cellulose; **COII:** Collagen Type II; **ELISA:** Enzyme-Linked Immunosorbent Assay; **GSH:** Glutathione; **HLA:** Human Leukocyte Antigen; **i.p:** Intraperitoneal; **ICAM:** Intercellular Adhesion Molecule; **IL:** Interleukin: **MA:** Maslinic Acid; **MA:** Methyl Anthranilate; **MDA:** Malondialdehyde; **NF-κB:** Nuclear Factor Kappa-light-chain-enhancer of activated B cells; **Nrf2:** Nuclear Factor Erythroid 2-related Factor 2; **PKC:** Protein Kinase C; **qPCR:** Quantitative Polymerase Chain Reaction; **RA:** Rheumatoid Arthritis; **RF:** Rheumatoid Factor; **ROS:** Reactive Oxygen Species; **SDS-PAGE:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; **SOD:** Superoxide Dismutase; **SRF:** Serum Rheumatoid Factor; **SYN:** Synovial; **Th:** T-helper; **TNF-α:** Tumor Necrosis Factor Alpha; **VEGF:** Vascular Endothelial Growth Factor.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical clearance and approval of the study protocols obtained from the Ethical Committee of the College of Medicine, King Khalid University (Ethical Committee No. REC # 2022-11-09), Saudi Arabia.

AUTHOR CONTRIBUTIONS

Ahmed M. Morsy: Study design, Manuscript drafting, Saeed M. Alqahtani: Study design, Manuscript revision, Salah O. Bashir: Study design, Manuscript drafting and revision, Hind Zafra: Manuscript drafting, Implementation of experiments, Amal F. Dawood: Data collection and analysis, Deema K. Sabir: Data collection and analysis, funding acquisition, Mohamed D. Morsy: Study design, Funding acquisition, Manuscript drafting, Seham A. Abdelaziz: Implementation of experiments, Mahmoud M. Morsy: Implementation of experiments, Dalia F. El Agamy: Implementation of experiments, Manuscript drafting.

SUMMARY

This study explores the effects of Maslinic Acid (MA) on Rheumatoid Arthritis (RA) in a rat model, where RA was induced by Collagen type II (COII) injections. Rats were divided into groups receiving different treatments, including MA and brusatol, a selective Nrf2 inhibitor. The research focused on assessing inflammatory, oxidative stress and apoptosis pathways through various biomarkers like NF-κB p65, TNF-α, ROS and Bax. MA treatment significantly reduced inflammation, oxidative

stress and apoptosis, while its effects were blocked when brusatol was administered. The findings suggest that MA may slow RA progression by targeting the Nrf2 pathway.

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