REVIEW

by Corr. Member, Prof. Soren Hayrabedyan, DSc

Institute of Biology and Immunology of Reproduction "Acad. Kiril Bratanov", Bulgarian Academy of Sciences

on the dissertation thesis for the award of the Educational and Scientific Degree "Doctor" (PhD)

Institute of Microbiology "Stephan Angeloff", Department of Immunology

Professional Field: 4.3. "Biological Sciences", Doctoral Program "Immunology"

PhD Candidate: Blagovesta D. Todorova

Thesis Title: "Anti-inflammatory effect of *Crocus sativus* extract and astaxanthin in a murine model of collagenase-induced osteoarthritis"

Scientific Supervisors:

Prof. Andrey Ivanov Chobanov, PhD
Assoc. Prof. Nikolina Mikhailova Mihaylova, PhD

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1. General Presentation of the Procedure and the Doctoral Candidate

The set of materials submitted by Blagovesta D. Todorova is in full compliance with the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria and the regulations of the Bulgarian Academy of Sciences (BAS) and the Institute of Microbiology-BAS. The work includes a detailed dissertation, a thesis abstract (auto-summary), and two publications in referred journals with high impact factors and a Q1 ranking, with a total IF of 6.5.

The candidate's scientific activity includes participation in 9 scientific forums, 3 of which were international.

The candidate, Blagovesta D. Todorova, possesses an interdisciplinary background and an upward career trajectory, combining military, managerial, and biomedical competence. She holds two bachelor's degrees from the "Vasil Levski" National Military University (2012-2017), after which she obtained a master's degree in Biochemistry from Sofia University "St. Kliment Ohridski" (2018-2020). Since 2021, she has been a full-time PhD student in Immunology at the Bulgarian Academy of Sciences. Her professional experience includes successive leadership positions within the structures of the Bulgarian Army and early academic engagement as an intern and PhD student in the Department of Immunology at Institute of Microbiology-BAS. She has completed specializations crucial for her dissertation, related to working with experimental animal models, as well as cell and tissue engineering. She has very good language proficiency, along with strong communication and organizational skills. All of this provides a stable foundation for her preparation for independent scientific activity.

2. Relevance of the Topic

Osteoarthritis (OA) is a leading degenerative disease with a significant impact on public health; however, a widely recognized and accepted disease-modifying therapy is still lacking. The present thesis attempts to answer a whole host of clinically essential questions—from investigating the dynamics of innate and acquired immune populations in the knee joint and systemic tissues to evaluating two natural agents with known antioxidant and immunomodulatory properties: an alcoholic extract of *Crocus sativus* (saffron) and astaxanthin (AST).

The topic is highly relevant, and the current research is timely as it integrates a model of collagenase-induced osteoarthritis (CIOA) with a rich set of cellular and histopathological analyses, along with a thorough chemo-analytical characterization of plant extracts. In doing so, it lays a solid foundation for translation into standardized applications. The literary and methodological framework of the dissertation clearly positions the research within contemporary trends in the immunopathology of OA and the preclinical testing of biologically active products.

3. Knowledge of the Problem

The PhD candidate demonstrates a profound understanding of the problem of osteoarthritis, structuring the literature review from general pathophysiology to specific mechanisms and therapeutic directions. Key signaling axes are traced—TGF-β (Transforming Growth Factor-β) and BMP (Bone Morphogenetic Proteins) with their SMAD-dependent and SMAD-independent branches, as well as Wnt/β-catenin, NF-κB (Nuclear Factor kappa-B), and Nrf2 (Nuclear factor erythroid 2-related factor 2)—in the context of chondrocyte homeostasis, subchondral remodeling, and the inflammatory microenvironment of the joint. This synthesis is presented concisely and precisely, supported by current mechanistic relationships.

The literature review integrates joint immunology by examining innate and adaptive effector cells—synovial macrophages and monocytes (including Ly6C subtypes), neutrophils, dendritic cells, natural killer (NK) cells, B- and T-lymphocytes—and links them to tissue processes that form clinical phenotypes (pannus, angiogenesis, fibrosis, osteophytes). This builds a logical framework for the subsequent flow cytometric analyses in the experimental part. Particularly valuable is the exploration of the links between inflammatory mediators from activated macrophages and tissue consequences such as osteophytosis. This demonstrates a mature understanding of how cellular participants and cytokine/growth factors project onto the morphological phenotypes of the disease.

A strong point of the review is the examination of therapeutic approaches in OA, including natural biologically active substances. The sections on *Crocus sativus* and astaxanthin provide the necessary context for their antioxidant and anti-inflammatory potential, safety, and dosage considerations, preparing the reader for the justified selection of interventions in the experimental part of the thesis. This connection between the review of existing knowledge and the choice of therapeutic candidates lends internal coherence to the dissertation and shows that the PhD candidate does not merely cite literature but uses it constructively to design experiments and define outcomes.

Another important aspect is the comparative review of animal models of OA, in which collagenase-induced osteoarthritis (CIOA) is situated relative to alternatives such as MIA (mono-iodoacetate) and surgical

approaches (e.g., DMM). This allows for an adequate defense of the choice of CIOA as a rapid and reproducible model with clearly distinguishable phases (acute, active, chronic), suitable for interventional studies on the immune landscape. This organization demonstrates excellent control over the field and allows for a smooth transition from the conceptual level to the specific research hypotheses of the dissertation.

The literature review spans approximately 24 pages, includes 12 figures, and contains roughly 181 cited sources. This volume is accompanied by appropriate illustrative material that facilitates the transfer of theoretical insights to the experimental design and the formulation of testable hypotheses.

4. Style, Structure, and Layout

The dissertation is structured sequentially: Literature Review, Aim and Objectives, Materials and Methods, Results, Discussion, and Conclusions, with a rich and legible figurative part (histological panels, experimental schematics, flow cytometry plots) and systematic tables. The dissertation is written on 129 pages, includes 52 figures (12 in the literature review and 40 in the results), and 2 tables. A total of 243 literary sources are cited. The results are detailed over 45 pages, and the discussion covers 15 pages. The methods are described reproducibly, with sufficient operational details (antigen panels, color markers, staining conditions, statistical tests), which facilitates repeatability. The discussion argues for the interpretations without being overly speculative. The text is generally clear, with minor linguistic inaccuracies (e.g., "FACS analysis" instead of "flow cytometry"), which do not compromise comprehension.

5. Brief Summary of the Thesis

Aim: To analyze the therapeutic potential of a *Crocus sativus* extract and astaxanthin in a murine model of collagenase-induced osteoarthritis (CIOA), by monitoring immunological parameters, tissue damage, and symptomatology.

Objectives: (1) comprehensive immunological analysis of the developmental stages of OA; (2) evaluation of the impact of the saffron extract; (3) evaluation of the impact of astaxanthin.

Object and Approach: BALB/c mice (♂/♀), induction of OA, oral treatment for 30 days; endpoints—flow cytometry (FC), ELISA (Enzyme-Linked Immunosorbent Assay), histopathology, proliferation tests (MTT), apoptosis, and ex vivo osteodifferentiation.

Key Results: Saffron at a dose of 50 mg/kg body weight showed the best dose/effect ratio: it lowers TNF-α (Tumor Necrosis Factor-alpha), supports IL-4 (Interleukin 4), preserves the cartilage matrix, reduces pathological vascularization and fibrosis, decreases neutrophils, and favors non-inflammatory monocytes; it also suppresses osteoclastogenesis ex vivo. Astaxanthin exerts immunomodulation by reducing the population of $Ly6C^{hi}$ inflammatory monocytes and increasing $Ly6C^{lo}$ "patrolling" monocytes, reducing pathological vessels, and stimulating osteoblast differentiation, with more moderate clinicopathological effects compared to saffron.

6. Analysis of the Research Methodology

Model and Groups: A chemical model of OA, CIOA, was used, featuring clear phases (acute: day 0-7; active: day 7-20; chronic: day 28-30). This allows for the tracking of the transition from inflammation to tissue remodeling and the chronic phase within a short window. The study was developed using male and female animals aged 8-10 weeks; collagenase type IIA was used for induction, with described buffers and concentrations. Randomization and control groups (PBS/oil control) were correctly planned.

Extracts and Dosing: The alcoholic extract of *C. sativus* was characterized by nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC), identifying crocin-1, crocin-2, and picrocrocin. This links the chemo-analytical profile to the in vivo effects and is an important argument for the standardization of plant-based products. Astaxanthin was administered in natural and synthetic forms according to clearly described schemes.

Measurements and Panels: The flow cytometry (FC) panels covered B- and T-lymphocytes, macrophages, monocytes (Ly6C), neutrophils (Ly6G), and dendritic cells, including activation markers (CD25, CD69) and functional indicators of degranulation (CD107a). ELISA was used for pro- and anti-inflammatory cytokines (TNF-α, IL-6, IL-4). Histology included decalcification (acidic and EDTA methods), differential staining (H&E, Toluidine Blue, Safranin O, Masson's Trichrome, Von Kossa), and quantitative scoring scales (vascularization, lesions, fibrosis, osteophytes). Additionally, MTT, apoptosis, and ex vivo osteoclast/osteoblast differentiation assays (Crystal Violet, Von Kossa) were performed.

Statistics: Based on t-test/Mann-Whitney, one- and two-way ANOVA with appropriate post-hoc tests; threshold at $p<0.05$; data presented as mean ± SD.

Ethics: The relevant approvals and compliance with Directive 2010/63/EU are indicated. The methods employed are appropriate for the research objectives and allow for the generation of original data.

7. Results, Discussion, and Contribution

Validation of CIOA and Joint Pathology: Clear histological criteria for the acute, active, and chronic phases are presented, including pannus formation, loss of proteoglycans/glycosaminoglycans, angiogenesis, and fibrosis. Quantitative assessment using established scales enhances objectivity. These findings are consistent with the published validation of CIOA (H&E, Toluidine Blue, Safranin O) and with the concept of progressive subchondral bone remodeling.

Effect of *Crocus sativus* (saffron): At a dosage of 50 mg/kg body weight, the most favorable combination of immunomodulatory and histopathological effects was observed. This was expressed as decreased levels of TNF-α and a trend towards increased IL-4, reduction of pathological vascularization, lesions, and fibrosis, partially preserved cartilage architecture, reduced neutrophil presence, and a shift in the monocyte pool towards $Ly6C^{lo}$ subtypes ("patrolling" monocytes). Concurrently, suppressed osteoclastogenesis and stimulated beneficial bone remodeling were established ex vivo. The chemo-analytical profiling performed via NMR and HPLC, which identified crocin-1, crocin-2, and picrocrocin (with no detectable safranal), reinforces the causal link between the extract and the observed biological effect.

Effect of Astaxanthin: Astaxanthin leads to a decrease in Ly6C^{hi} pro-inflammatory monocytes and an increase in Ly6C^{lo} monocytes in the synovium, a reduction in neutrophils, a moderate reduction in macrophage subtypes, and decreased pathological vascularization. It stimulates osteoblast differentiation ex vivo. The clinicopathological effect is more moderate compared to saffron, but the biological effect on the immune infiltrate and remodeling is consistent.

Immunophenotyping in Systemic and Local Compartments: The analysis of immune cells was conducted simultaneously in the local compartment (synovium and surrounding joint tissue) and in systemic compartments (spleen and bone marrow). This allows for tracking how the "central" production and mobilization of cells are reflected at the site of inflammation.

- **B-cells (CD19) and T-cells (CD3, CD4, CD8):** During the course of osteoarthritis, an increase in antibody-producing, antigen-presenting, and cytokine-secreting B-cells (CD19) was noted, as well as in effector T-cells (CD3), including the CD4 helper and CD8 cytotoxic sub-populations. Activation was confirmed with markers such as CD25 (IL-2Rα) and CD69 (early activation).
- **NK cells (CD107a):** NK cells—key effectors of innate immunity—showed phenotype dynamics: in the early phase, a "cytotoxic" profile dominated (more degranulation, measured by CD107a, and higher perforin/granzyme activity), whereas in the chronic stage, a more "effector/modulatory" profile was observed. This shift corresponds to published immune cell profiles in OA and provides a bridge between the temporal dynamics and the observed tissue changes.
- **Neutrophils (Ly6G):** The observed changes were expected in CIOA: an early and transient dominance of neutrophils in the synovium with NET-associated and protease/ROS-mediated damage; a typical peak in the acute/active phase, followed by a decline during resolution. The established effect of saffron was a consistent reduction in the neutrophil load and the "enzymatic" stress on the matrix, correlating with a reduction in TNF-α and angiogenesis and with better histopathological indices. With astaxanthin, the reduction was more moderate, with a trend towards an earlier "subsidence" of the neutrophil peak but with a weaker structural contribution than that of saffron.
- **Monocytes (CD11b; Ly6C) and Macrophages (F4/80):** In CIOA, the bone marrow recruits Ly6C^{hi} monocytes to the synovium during the active phase; they fuel M1-like programs (secreting TNF-α). With resolution, the pool shifts towards Ly6C^{lo} and M2-like remodeling phenotypes. Saffron induced a clear shift from Ly6C^{hi} to Ly6C^{lo}, a reduction of the F4/80^{+} pro-inflammatory component, and suppressed osteoclastogenesis *ex vivo*; this coincides with the limitation of fibrosis/lesions and cartilage preservation. Astaxanthin caused a stable decrease in the Ly6C^{hi} / Ly6C^{lo} ratio with a moderate reduction in macrophage activation; it more pronouncedly directed towards bone remodeling and osteoblast differentiation but with a more modest disease-modifying effect compared to saffron.
- **Dendritic Cells:** In CIOA, activated DCs sustain T-cell priming and maintenance in the synovium and lymphoid organs; persistent co-stimulation occurs in chronic OA. Saffron mitigates DC activation (functionally: lower co-stimulation and cytokine response), which reduces the maintenance of persistent T-cell activation. Astaxanthin has a similar but more moderate effect on DC activation, contributing to reduced activation of the adaptive response but without a strong reprogramming effect.

- **Summary:** Both agents demonstrate immunomodulation towards a reduced acute inflammatory response, leading to enhanced resolution. Saffron consistently shows a stronger disease-modifying footprint at the level of the neutrophil–monocyte-macrophage axis and the DC/T/B/NK cell network, which also manifests in better histopathological outcomes. Astaxanthin supports the remodeling/osteoblastic direction with a more moderate global anti-inflammatory effect.

The discussion is coherent and effectively links the immune landscape (Ly6C^{hi} / Ly6C^{lo} monocytes, M1/M2 macrophages, NK sub-populations, T-cell balance) with the tissue consequences (angiogenesis, lesions, fibrosis, chondrons) and with the two tested agents. For *Crocus sativus* (50 mg/kg), the doctoral candidate correctly identifies convergent effects: reduced TNF-α, a trend for increased IL-4, reduction of neutrophils, and a shift towards Ly6C^{lo} monocytes. For astaxanthin (AST), the discussion argues for a moderate but consistent immunomodulatory profile (with reduced Ly6C Ly6C^{hi} and increased Ly6C^{lo} monocytes; trends in macrophages and neutrophils), consistent with the presented graphs and the conclusions on tissue remodeling. The text builds upon recent publications (from 2022/2023) and expands them with local (synovium/bone marrow) analyses. The interpretation of NK cells and macrophages is in sync with her own data and the cited context. The citations are numerous in the discussion section (~56), including to the two related papers from the procedure and to current literature from 2023-2024.

8. Main Scientific Contributions

- A dynamic map of immune populations in CIOA across three compartments (synovium, bone marrow, spleen) has been created, integrating activation markers and functional indicators (CD107a), which contributes to the understanding of the cellular pathogenesis.
- A dose-response evaluation for saffron extract treatment was performed, and an optimal dose of 50 mg/kg was identified with a multi-endpoint effect (cytokines, histology, immune cells, apoptosis, osteo-differentiation).
- A comparative framework between saffron and astaxanthin was constructed, identifying biomarkers of response (TNF-α/IL-4; Ly6C; CD107a; angiogenesis/fibrosis/osteophytes) useful for future preclinical screening.
- Chemo-analytical data (NMR/HPLC) were linked to in vivo effects, which is a prerequisite for standardization and inter-batch reproducibility.

9. Evaluation of Publications and the Personal Contribution of the Doctoral Candidate

The dissertation is supported by two refereed and peer-reviewed articles in MDPI *Life*, each with an IF of 3.2 and Q1 ranking: (1) "Collagenase-Induced Mouse Model of Osteoarthritis: A Thorough Flow Cytometry Analysis" (2022), which establishes and details the immune map of CIOA; and (2) "*Crocus sativus* Extract as a Biological Agent for Disease-Modifying Therapy of Collagenase-Induced Mouse Model of Osteoarthritis" (2023), which integrates chemo-analytical profiling of the extract (NMR/HPLC) with in vivo effects and dose-response analysis. Both publications are directly related to the thesis topic and demonstrate the ability to generate and publish original data. The dissertation exceeds the published work

through expanded analyses (e.g., NK kinetics and bone marrow aspects, additional apoptosis assessment, and quantitative histological evaluation of pathological changes (morphometry)). The doctoral candidate's contribution is clearly expressed through her leading role as the first author, as well as her participation in 9 scientific forums. These results indicate a high level of scientific activity, international recognition, and demonstrate the candidate's readiness for independent research work.

10. Thesis Abstract (Auto-summary)

The abstract accurately reflects the structure, content, and main contributions of the dissertation. It is 93 pages long and is illustrated with 56 figures that detail the key results and approaches. The bibliography contains 243 literary sources (from the dissertation), underscoring the thorough theoretical foundation of the research. The presentation is clear and comprehensive, compliant with academic requirements.

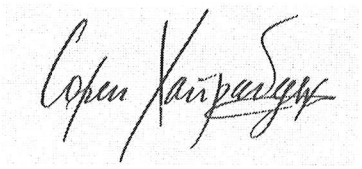
Conclusion

The presented dissertation is scientifically mature, methodologically precise, and application-oriented, with significant original contributions to the immunopathology of CIOA and the preclinical evaluation of natural agents, supported by the chemo-analytical standardization of *Crocus sativus*. The results are consistent across multiple independent endpoints, and the integration of immunoprofiling, histopathology, and functional ex vivo data is particularly strong.

On this basis, I formulate a **positive overall assessment** and support the awarding of the Educational and Scientific Degree "Doctor" to Blagovesta D. Todorova, as I consider that the requirements for awarding this degree according to the Law on the Development of the Academic Staff in the Republic of Bulgaria and the regulations of BAS are fully met. The work contains original scientific contributions and demonstrates the PhD candidate's profound knowledge and skills.

I believe that Blagovesta D. Todorova deserves to be awarded the Educational and Scientific Degree "Doctor" in the professional field of "Biological Sciences"!

Date: 11.09.2025

Reviewer: 

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