

RAPID COMMUNICATION

Smoking may impact IL-33/ST2 signaling for better cognitive functioning in schizophrenia

REVIEW PAPER

Possibilities and limitations of X-ray diagnostics in cases of whooping cough

CASE REPORT

Implant-supported prosthetic rehabilitation of the edentulous maxilla using the OT Bridge Equator system: A case report

HBOT - an effective option for the treatment of chronic wounds in diabetes mellitus: A case report

ORIGINAL SCIENTIFIC ARTICLE

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Serum magnesium in proton pump inhibitors users and non-users

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SMOKING MAY IMPACT IL-33/ST2 SIGNALING FOR BETTER COGNITIVE FUNCTIONING IN SCHIZOPHRENIA

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ABSTRACT

We found a positive correlation between serum levels of interleukin-33 (IL-33) and the Montreal Cognitive Assessment (MoCA) score in patients with stable schizophrenia, as well as with specific sub-scores. Smoking appears to decrease serum levels of soluble IL-33 receptor, a suppressor of tumorigenicity (sST2), possibly resulting in IL-33's indirect preservation of cognitive functioning.

Keywords: Schizophrenia, IL-33, ST2, cognition, smoking.

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INTRODUCTION

The interleukin-33/suppressor of tumorigenicity (IL-33/ST2) signaling pathway is involved in the regulation of T lymphocyte activity and differentiation, dendritic cell differentiation and activation, macrophage and mast cell activation, and cytokine production (1). IL-33 has a dual function and may exert pro-inflammatory or anti-inflammatory effects in the central nervous system (CNS). Its function may depend on the primary target cells, the level of IL-33/ST2 expression, the cellular context, and the cytokine milieu (2, 3, 4). IL-33 plays a neuroprotective and reparative role in the CNS by regulating the polarization of microglia and macrophages, as well as phagocytosis (5). By binding to the ST2 receptor, IL-33 promotes the activation and proliferation of microglia, the production of various cytokines, and leads to an acute inflammatory response (6). Astrocyte and oligodendrocyte precursors express IL-33 during the first postnatal week, which coincides with crucial stages of brain development (7). These findings indicate a role for IL-33 even in the absence of inflammation. IL-33 may promote neuroinflammation and cause cognitive decline in later life (8). In recent years, there has been increasing evidence for an important immunomodulatory role of IL-33 in neurodegenerative diseases (9).

Studies in animal models suggest that IL-33 improves cognition by inhibiting the inflammatory response in the hippocampus and regulating the number of excitatory synapses (10). We have also previously published results indicating that serum IL-33 levels are elevated in exacerbation and normalized in stabilization of schizophrenia (11). Additional analysis as part of a doctoral dissertation revealed that, in patients in remission, there is a positive correlation between serum IL-33 levels and the Montreal Cognitive Assessment (MoCA) score ($r = 0.401$; $p = 0.038$), as well as the sub-scores for visuospatial/executive functions ($r = 0.437$; $p = 0.023$), serial subtraction ($r = 0.428$; $p = 0.026$), and fluency ($r = 0.392$; $p = 0.043$) (12).

Furthermore, these patients are often nicotine dependent. This dual diagnosis of addiction contributes to an already compromised somatic state and poorer overall outcomes in schizophrenia (11). Extensive research links tobacco smoking to vascular damage, which contributes to the development of hypertension and atherosclerosis and increases the risk of neurodegeneration (13).

However, for the first time, we want to emphasize the underlying immunological mechanisms that could explain the short-term benefits of smoking. There is a statistically significant, moderately negative correlation between scores on the Fagerstrom scale, which assesses nicotine dependence, and serum sST2 concentrations ($r = -0.476$; $p = 0.012$) (12). Taking this into account, we propose a new cascade, suggesting that smoking may cause IL-33 to circulate more freely by decreasing sST2 levels, thereby preserving cognitive functioning (Figure 1). When circulation is oversaturated with IL-33, it may induce its downregulation. In line with our findings, a recent study provided strong evidence that cigarette smoke

leads to an overall reduction in IL-33 expression at both the transcriptomic and protein levels (14). The increase in systemic IL-33 concentration suggests its movement from the cytosol into the circulation, indicating that higher circulation levels correspond with reduced presence in cells and tissues, and *vice versa*. The proposed mechanism may not apply to patients in the acute stages of illness, those with treatment-resistant schizophrenia, or non-smoking groups.

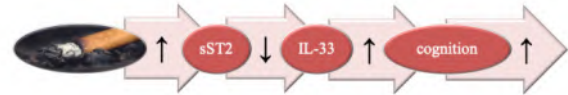


Figure 1. Hypothesis-based model of potential smoking effects on cognition *via* IL-33/ST2 signaling. Proposed new cascade: with increasing cigarette consumption, there may be a decrease in serum levels of soluble suppressor of tumorigenicity (sST2) and, consequently, an increase in the level of free IL-33, which may affect the preservation of cognitive potential.

CONCLUSIONS

It can be concluded that IL-33 could potentially be effective in delayed neurocognitive recovery, which is of great importance for patients' daily functioning and resocialization. Data regarding cognition in patients with stabilized schizophrenia revealed that IL-33 correlated with cognitive functioning, and this pattern could be further investigated in schizophrenia. It is possible that smoking could lower sST2 levels and thus impact cognition, making circulating IL-33 more available. However, observed associations should not be considered causal and may also be bidirectional. Potential confounding factors must be taken into consideration, such as duration of illness, antipsychotic treatment, and cardiometabolic comorbidities, which may affect both cytokine profiles and cognitive functioning. In this case, the effect of smoking could be rather indirect. The currently available data should not be interpreted as a clinical recommendation, and the presented hypothesis should be thoroughly tested in the future.

LIST OF ABBREVIATIONS

IL-33/ST2: Interleukin-33/Suppressor of Tumorigenicity

CNS: Central Nervous System

sST2: soluble Suppressor of Tumorigenicity

MoCA: Montreal Cognitive Assessment

STATEMENT OF HUMAN RIGHTS

These comments are based on our previous study, which was approved by the Ethics Committee of the University Clinical Centre Kragujevac (01-7015). All participants provided written consent before any study procedures. The investigation was conducted in accordance with the Declaration of Helsinki of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

All authors contributed equally to the writing of this manuscript and have approved the final version.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

SMJ, MMB, and IPJ collaborated in collecting the related data, with all authors contributing to the conceptualization and writing of the manuscript.

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INCLUSION OF SYMPOSIUM-BASED LEARNING OF RATIONAL DRUG USE FOR MEDICAL UNDERGRADUATES: EVALUATION OF ITS EFFECTIVENESS AND FEEDBACK

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ABSTRACT

Irrational prescribing continues to challenge clinical practice, and undergraduate medical students often have limited structured training in Rational Drug Use (RDU). Effective early interventions require interactive, multidisciplinary teaching approaches. This study assessed the impact of symposium-based learning (SBL) on knowledge, attitude, practice, and awareness (KAPFA) related to RDU among second-year Bachelor of Medicine and Bachelor of Surgery (MBBS) students. A quasi-interventional study was conducted among 150 students. A four-hour multidisciplinary symposium on RDU was organized, and a pre-validated questionnaire was administered before and after the session. The tool assessed knowledge (10 MCQs), attitude (5 statements), practice (5 case scenarios), feedback (7 items), and awareness (10 items). Data were analyzed using descriptive statistics and paired t-test. Knowledge scores improved significantly from 5.45 ± 1.03 to 7.02 ± 0.86 ($p < 0.001$). Attitude responses shifted toward stronger agreement across all statements. Practice scores increased from 2.34 ± 0.61 to 4.06 ± 0.57 ($p < 0.001$), indicating better application of RDU principles. Awareness showed substantial gains, with an overall increase exceeding 300% across key parameters. Improvements were consistent across all five clinical posting groups. Feedback indicated strong acceptance, with over 94% reporting the symposium as relevant and useful. SBL proved to be a highly effective educational strategy for enhancing RDU competencies in undergraduate students. Incorporating such structured and interactive methods into the curriculum may help strengthen safe and rational prescribing practices among future clinicians.

Keywords: Rational Drug Use, Symposium based learning, Rational Prescribing Competencies, Educational Intervention, Knowledge and Attitude Assessment.

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INTRODUCTION

Rational drug prescribing represents a fundamental competency expected from all medical graduates, as it need to ensure patients to receive safe, effective, and appropriate pharmacological therapy [1]. During the undergraduate programme of medical programme, particularly in the second year, students are introduced to Rational Drug Use (RDU) through clinical pharmacology sessions that involve lectures, supervised prescription writing, and prescription audits. These learning activities are intended to cultivate the ability to select suitable medications in correct doses and durations, while also considering affordability, accessibility, and patient-specific factors [2]. Although this structured exposure provides a theoretical foundation, applying these principles consistently in clinical settings remains challenging for many trainees. Despite formal teaching, irrational prescribing continues to be widely reported across healthcare settings. This persistent problem arises from several interrelated causes. Clinical environments often expose students to prescribing behaviours that do not mirror standard recommendations, creating a disconnect between classroom instruction and observed practice [3]. External pressures such as commercial influences, frequent patient turnover, limited consultation time, and system-level resource constraints may discourage careful clinical reasoning. High workload in busy outpatient and inpatient settings further contributes to suboptimal prescribing decisions. As a result, inappropriate drug use remains a universal concern, leading to consequences such as antimicrobial resistance, preventable adverse effects, and unnecessary drug interactions. Addressing these issues requires strengthening learners' conceptual understanding early in their training, as well as reinforcing rational habits before they enter more complex clinical phases. Second-year medical students occupy a crucial position in the learning continuum and at this stage, they are transitioning from preclinical learning to early clinical exposure, where they begin to witness patient care decisions made by practitioners. A recent multi-institution study found that implementing early clinical exposure (ECE) led to significantly higher self-assessed "professional practice skills" in students, with second-year participants reporting competencies comparable to those of traditional fourth-year students [4]. Moreover, students exposed early to patients report improved communication, increased motivation for self-directed learning, better integration of basic science knowledge with real-life pathology, and enhanced readiness for clinical responsibilities [5]. Enhancing their RDU competency at this juncture is important because it shapes the attitudes and practices they would carry into their clinical clerkships. Without timely reinforcement, students may adopt inappropriate prescribing patterns observed in clinical practice, which can persist into their later professional life. Therefore, improving RDU understanding during the second year can influence long-term prescribing behaviour and contribute to safer healthcare delivery. A study at a tertiary-care hospital in India showed that prescription-writing skills among second-year students were suboptimal, many prescriptions lacked complete drug and doctor information, and legibility was poor [6]. This implies that

without reinforcement, early gaps may persist into clinical practice [7]. Contemporary medical education emphasises teaching methods that align with adult learning principles. Adult learners benefit most when instruction is relevant to future practice, allows self-direction, promotes active engagement, and facilitates collaborative reasoning [8]. These principles support the use of interactive learning formats rather than passive lecture-based delivery. In this context, Symposium-Based Learning (SBL) emerges as a promising approach which insist on focused, structured discussion on a specific clinical problem and provides opportunities for analysing updates, evaluating current evidence, and exploring practical challenges associated with prescribing [9,10]. An original educational research study in pharmacology education demonstrated that interactive, small-group teaching and case-based learning approaches effectively enhanced medical students' knowledge, skills, and attitudes required for safe and rational prescribing in a clinical context [11]. Conferences and clinical meetings promote exchange of new evidence, inter-professional discussion, and real-world case evaluation factors help clinicians appraise and apply evidence rather than rely on tradition [12]. Unlike traditional didactic sessions, SBL promotes deeper conceptual understanding, peer-to-peer learning, and critical appraisal of therapeutic options, making it suitable for complex topics such as RDU. Although SBL has been used in certain educational settings, its application for teaching rational prescribing to Indian undergraduate medical students remains limited. An appropriate environment to examine SBL effectiveness, as students experience early clinical exposure demonstrates a preference for interactive and collaborative learning strategies. Studying this cohort provides insights into whether SBL can enhance the understanding and application of RDU principles at a formative stage. Through this approach, the present study aims to strengthen rational prescribing practices and contribute to ongoing efforts to improve medication safety within undergraduate medical education.

STUDY DESIGN

This quasi-interventional study was undertaken at a tertiary Medical College and Research Institute with the objective of systematically evaluating shifts in knowledge, attitude, and practices (KAP) regarding RDU among second-year MBBS students. The educational intervention consisted of a structured, expert-led symposium designed to enhance students' conceptual understanding of RDU principles, including essential drug selection, evidence-based prescribing, dose optimisation, prevention of antimicrobial resistance, and ethical considerations in pharmacotherapy. The study employed validated, pre-tested questionnaires administered both before and after the symposium to measure the extent of improvement across multiple domains of RDU awareness. Emphasis was placed on assessing cognitive gains, attitudinal refinement, and self-reported behavioural intentions related to rational prescribing. By targeting students at an early stage of clinical training, the study aims to contribute meaningful

evidence on the effectiveness of focused pedagogical strategies in strengthening responsible prescribing behaviours and promoting safe, cost-effective, and patient-centred drug use in future medical practice. The study adopted a single-group pre–post quasi-interventional design, as the educational symposium formed part of the institutional academic program, rendering the inclusion of a non-intervention control group ethically and pedagogically impractical. This approach is appropriate for evaluating short-term educational impact in authentic academic settings while acknowledging inherent limitations in causal inference.

Participants

A pre-post interventional study was conducted among 150 second-year MBBS students to evaluate the impact of a RDU symposium on knowledge, attitude, practice, and awareness related to rational drug prescribing. The sample size represented a census of all eligible second-year MBBS students during the study period, reflecting pragmatic feasibility rather than an a priori statistical estimation. The study objectives and procedures were clearly explained to the students prior to data collection, and participation was entirely voluntary. Students who provided informed consent were included, with no exclusions based on academic performance or demographic characteristics, thereby enhancing the representativeness of the sample. Confidentiality and anonymity of the responses were strictly maintained using coded questionnaires and secure data handling practices. No identifying information was collected at any stage of the survey. The data collection process was supervised by trained faculty to ensure uniform administration of the questionnaires. This methodological rigor strengthened the internal validity of the findings and supported the reliability of the students' self-reported responses. A structured, pre-validated questionnaire was administered to all participants immediately before (pre-assessment) and after (post-assessment) the symposium. The questionnaire was designed to cover five domains: Knowledge, Attitude, Practice, Feedback and Awareness. For the Knowledge, Attitude, Feedback and Practice (KAFP) sections, responses were collected individually from all 150 students to obtain a comprehensive measure of baseline understanding and post-intervention improvement across the entire cohort. For the Awareness domain, students were stratified into five equal cohorts (n=30 each) according to their clinical posting allocation— participants were stratified into five cohorts based on their exposure to clinical rotations: General Medicine (n=30), General Surgery (n=30), Pediatrics (n=30), Obstetrics and Gynecology (n=30), and Community Medicine (n=30). This stratification enabled domain-specific assessment of awareness related to rational drug use before and after symposium exposure. Such grouping ensured comparability across clinical disciplines and enhanced the interpretive rigor of pre- and post-intervention analyse. This stratification was deliberately employed to acknowledge and control for the inherent variability in clinical learning environments, including diversity in patient presentations, pharmacotherapeutic practices, prescribing behaviors, and experiential engagement with drug-related decision-making across specialties.

Such structured grouping enabled a more targeted evaluation of awareness levels and facilitated a nuanced interpretation of how learners from distinct clinical contexts assimilate and operationalize the core principles of RDU. Moreover, this approach minimized potential bias from knowledge disparities and enhanced the interpretability of interdepartmental differences in awareness levels. All collected data were anonymized, coded, and subjected to statistical analysis using paired comparisons to evaluate pre- and post-symposium changes within each domain, with particular attention to differential gains across grouped subpopulations in the Awareness section.

Questionnaire Description

A pre-validated and structured questionnaire was employed to assess the Knowledge, Attitude, Practice, Feedback and Awareness (KAPFA) domains before and after the symposium. The instrument comprised 37 core items distributed across multiple sections. Section 1 (Knowledge) included ten multiple-choice questions that evaluated students' conceptual understanding of RDU, with each correct answer scored as 1 and incorrect responses scored as 0. Section 2 (Attitude) consisted of five statements rated on a five-point "Likert scale", capturing participants' perceptions and disposition toward rational prescribing. Section 3 (Practice) presented five clinical vignettes designed to assess real-world decision-making and application of RDU principles. Section 4 (Feedback) included seven items eliciting participants' evaluation of the symposium's relevance, clarity, and perceived impact. Section 5 (Awareness) comprised ten Likert-based statements measuring students' baseline and post-symposium awareness of RDU concepts and drug-selection criteria. The questionnaire underwent a multi-step validation process. It was developed using the WHO Guide to Good Prescribing, National Essential Medicines List (NLEM), and established RDU instruments. Content validity was ensured by expert review (pharmacologist, clinician, and clinical pharmacist). A pilot study in 30 second-year MBBS students assessed clarity and feasibility. Internal consistency showed good reliability with a Cronbach's alpha of 0.82 across domains. Test–retest reliability over two weeks demonstrated good stability (ICC >0.75). Thus, the tool showed strong content validity, face validity, internal consistency, and reproducibility for assessing RDU-related KAPFA outcomes.

Scoring of Awareness Domain

Section 5 (Awareness) consisted of ten statements assessing students' awareness of Rational Drug Use (RDU) concepts, including correct drug selection, therapeutic objectives, appropriate information sources, and awareness of pharmaceutical influence. This Awareness section consisted of 10 statements, each comprising four sub-options (A–D). Each sub-option was rated independently by the students using a six-category response Likert scale: 0 = No idea, 1 = Strongly disagree, 2 = Disagree, 3 = Neutral, 4 = Agree, 5 = Strongly agree. Thus, each statement had a maximum possible score of 20 (4 options × 5 points), and the overall

maximum Awareness score was 200 (10 statements \times 20 points). Scores from all sub-options were summed to generate the composite Awareness score used for statistical analysis. This approach was adopted to capture the depth and consistency of students' awareness across multiple facets of each RDU concept. Rating all options individually allowed finer discrimination of partial knowledge, misconceptions, and overall conceptual clarity beyond single-choice responses. All ten items contributed to the cumulative Awareness score. For group-wise analysis, students were stratified according to their clinical postings, and mean Awareness scores were calculated separately for each group to allow comparison across clinical disciplines. To facilitate clearer interpretation of key RDU concepts, selected Awareness items were additionally dichotomized for descriptive reporting. Responses of Agree and Strongly agree were categorized as "Yes", indicating adequate awareness, while Neutral, Disagree, strongly disagree, and No idea were categorized as "No". This dichotomization was applied only to selected core items and was used to present proportions of students demonstrating correct awareness, without replacing the primary Likert-based composite score analysis. Thus, the Awareness results are presented using both continuous composite scores (for statistical comparison and group-wise analysis) and Yes/No frequencies (for key concept clarity), ensuring methodological rigor while improving reader interpretability.

Symposium Structure

A single multidisciplinary symposium functioned as the educational intervention convened for four hours and led by specialists in pharmacology, clinical pharmacy, medicine, and nursing. The programme integrated focused didactic presentations with case-based discussions to strengthen conceptual and practical understanding of RDU. Content systematically addressed core RDU principles: therapeutic efficacy, safety, cost-effectiveness, and evidence-based prescribing, while explicitly mapping the complementary responsibilities of clinicians, pharmacists, nurses, and pharmacologists in medication stewardship, reconciliation, monitoring, and pharmacovigilance. Faculty identified common antecedents of irrational drug use (diagnostic uncertainty, polypharmacy, commercial influences, and system constraints) and described measurable consequences such as adverse drug

reactions, antimicrobial resistance, and increased healthcare costs. Practical implementation strategies for resource-limited clinical environments were emphasized, including standardized treatment protocols, point-of-care decision aids, interprofessional communication pathways, and audit-and-feedback mechanisms. Subsequently, 150 students were allocated into five groups; each group generated questions and perceptions that were explored during a two-hour, faculty-moderated interactive panel to consolidate learning and identify curricular gaps.

Data collection

The structured and pre-validated questionnaire was administered to all participants immediately before and after the symposium to assess changes in knowledge, attitudes, and perceptions related to rational drug use. On both occasions, students were allotted 20 minutes to complete the questionnaire under supervised conditions to minimize external influences. They were explicitly instructed not to consult textbooks, electronic resources, class notes, or peers to ensure that responses reflected individual understanding rather than collaborative input. Completed questionnaires were collected promptly and reviewed for completeness before being coded with anonymized identifiers to maintain confidentiality and enable paired pre- and post-intervention analysis. All data were subsequently entered into a secure database, cross-checked for entry accuracy, and prepared for statistical evaluation. This standardized administration protocol ensured methodological consistency, reduced response bias, and allowed reliable comparison of learning outcomes attributable to the symposium.

Statistical analysis

Data were entered into Microsoft Excel and analysed using SPSS version 29. Descriptive statistics, such as mean and standard deviation, were used to summarise KAP scores. Pre- and post-symposium knowledge and practice scores were compared using the paired Student's t-test. Assumptions for the paired t-test, including normal distribution of difference scores, were verified prior to analysis. A p-value < 0.05 was considered statistically significant.

RESULTS

Demographic characteristics

Table 1. Demographic Characteristics of Study Participants (n = 150)

Variable	Category	n (%)
Age (years)	Mean \pm SD	21.1 \pm 0.6
Gender	Male	62 (41.3%)
	Female	88 (58.7%)
Residential Status	Hosteller	77 (51.3%)
	Day Scholar	73 (48.7%)

Variable	Category	n (%)
Type of Schooling	State Board	82 (54.7%)
	CBSE	49 (32.7%)
	ICSE	9 (6.0%)
	Others	10 (6.6%)
Access to Smartphone / Internet	Yes	150 (100%)
	No	0 (0%)
Parents in Medical Profession	Yes	38 (25.3%)
	No	112 (74.7%)
Health Worker in the Family	Yes	48 (22.9%)
	No	162 (77.1%)
Chronic Disease in Family	Yes	71 (33.8%)
	No	139 (66.2%)
Pharmacology Practical Attendance (%)	High	58 (38.6%)
	Moderate	64 (42.7%)
	Low	28 (18.7%)
Interest in Pharmacology (Self-rated)	High	58 (27.6%)
	Moderate	109 (51.9%)
	Low	43 (20.5%)
Exposure to Self-medication Practices at Home	Yes	91 (60.7%)
	No	59 (39.3%)
Participation in Academics	High	68 (45.3%)
	Moderate	55 (36.7%)
	Low	27 (18.0%)
Preferred Learning Mode	Online	21 (14.0%)
	Offline	68 (45.3%)
	Hybrid	61 (40.7%)
Participation in Non-academics	High	44 (29.3%)
	Moderate	68 (45.3%)
	Low	38 (25.4%)
Family Member in Pharmacy / Medical Store	Yes	29 (19.3%)
	No	121 (80.7%)
Awareness of Prescription Regulations	Yes	41 (19.5%)
	No	169 (80.5%)
Previous Exposure to RDU Education	Yes	15 (6.8%)
	No	135 (93.4%)
Previous Workshops / CMEs Attended	Yes	57 (38.0%)
	No	93 (62.0%)

SD = Standard deviation; CBSE = Central Board of Secondary Education; ICSE = Indian Certificate of Secondary Education; RDU = Rational Drug Use; CME = Continuing Medical Education.

The participating students' demography highlights several critical factors that may influence learning and attitudes toward RDU. For analytical purposes, pharmacology practical attendance was classified as High (>85%), Moderate (75–85%), and Low (<75%). "Type of schooling" denoted the higher secondary education board (State Board, CBSE, ICSE, or Others). All participants were from the same institution and received an identical educational intervention. The "Others" category included alternative programs. Consistent post-intervention improvements suggest that schooling background did not materially influence study outcomes. Most of the students lacked prior exposure to RDU education (93.4%), indicating that the symposium addressed a major

educational gap. A high proportion reported exposure to self-medication practices at home (60.7%), suggesting that personal and family-level behaviors may shape their understanding of drug use. Interest in pharmacology was mostly moderate to low (72.4%), reflecting the need for engaging teaching strategies to strengthen motivation in this subject. Although all participants had access to smartphones and the internet (100%), awareness of prescription regulations remained low (19.5%), stressing the importance of structured educational interventions to improve regulatory literacy among medical students.

Knowledge

The symposium led to a substantial and statistically significant improvement in participants' knowledge, demonstrating the effectiveness of the educational intervention. Post-session responses reflected greater accuracy, clearer understanding, and more consistent performance on RDU-related concepts. The mean knowledge score significantly increased from 5.45 ± 1.03 before the symposium to 7.02 ± 0.86 after the symposium, indicating a substantial improvement in participants' understanding of Rational Drug Use (RDU) concepts. The mean difference of 1.57 points reflects a meaningful gain in knowledge attributable to the educational intervention. The 95% confidence interval (1.43–1.71) does not cross zero, confirming that the improvement is statistically reliable. The paired t-test yielded a t-value of 12.34 (df = 149) with a p-value < 0.001, demonstrating a highly

significant increase in knowledge scores following the symposium. This suggests that the educational session had a strong positive impact on participants' conceptual understanding and ability to answer RDU-related MCQs correctly. Overall, the symposium was effective in enhancing knowledge, with post-intervention scores showing higher accuracy, improved comprehension, and reduced variability, as reflected by the lower SD in post-test scores.

Attitude

Students already had positive attitudes toward rational drug use, which were further strengthened by the symposium, with appreciable increase in strong agreement across all domains, reflecting enhanced understanding, commitment, and appreciation of evidence-based prescribing principle (Table 2).

Table 2. Assessment of students' attitude towards rational drug use pre and post symposium to the five statements (*). (1A-1E)

Statements	Strongly Disagree		Disagree		Neutral		Agree		Strongly agree	
	Pre-symposium	Post-symposium	Pre-symposium	Post-symposium	Pre-symposium	Post-symposium	Pre-symposium	Post-symposium	Pre-symposium	Post-symposium
1A	0	0	0	0	0	0	113 (74.5%)	96 (63.3%)	37 (24.4%)	54 (36%)
1B	1 (0.7%)	0	1 (0.7%)	1 (0.7%)	4 (2.6%)	0	121 (80%)	98 (65%)	23 (15%)	51 (34%)
1C	0	1 (0.7%)	3 (2%)	0	7 (4.6%)	0	131 (86.4%)	94 (62%)	9 (6%)	55 (36%)
1D	1 (0.7%)	0	0	0	4 (2.6%)	0	125 (82.5%)	81 (53.4%)	20 (13.2%)	69 (46%)
1E	2 (1.3%)	0	4 (2.6%)	0	0	0	120 (79%)	68 (45%)	24 (16%)	82 (54.%)

(*).**1A:** I am keen on learning about evidence-based prescribing practices and rational drug use principles.

1B: Symposium plays an important role in better understanding of rational drug use and practical challenges in implementing it.

1C: Health-care Practitioners (Doctors) should consider cost-effectiveness and affordability when making treatment recommendations for patients.

1D: Adherence to rational drug use principles can help reduce the risk of antibiotic resistance.

1E: Pharmaceutical company promotions significantly impact prescribing practices among healthcare professionals.

Across all five statements, students' attitude showed very high agreement even before the symposium, but the post-symposium responses demonstrated a clear shift from "Agree" to "Strongly Agree," indicating strengthened attitudes rather than newly formed opinions. Interest in learning evidence-based prescribing (1A) remained consistently high, while perceptions of the symposium's value in improving understanding of RDU and its practical challenges (1B) increased to almost unanimous agreement. Awareness of cost-effectiveness in prescribing (1C) and the role of RDU in preventing antibiotic resistance (1D) also improved markedly, with substantial rises in "Strongly Agree" responses. Recognition of the influence of pharmaceutical promotions (1E)

showed one of the largest positive shifts. Overall, the symposium enhanced students' depth of understanding and reinforced their commitment to rational drug use principles.

Practise based Scores:

Students showed a large, consistent improvement in practice-based case-scenario performance after the symposium, with statistical tests indicating the gain is highly significant and unlikely due to chance. In short, the symposium meaningfully improved students' practical competence in applying RDU principles.

Table 3. Assessment of Practice based scores (case-based scenarios): Pre and post symposium

		Pre-symposium score	Post-symposium score			
Mean ±Standard deviation		2.34 ± 0.61	4.06 ± 0.57			
Paired differences						
	Mean	Std deviation (SD)	95% CL	t	df	p-value
			Upper			
Pre-test	2.34	0.61	1.24 – 1.43	26.85	149	<0.001
Post-test	4.06	0.57	2.96 – 3.15	65.71	149	<0.001 (***)

***: An extremely significant difference is observed between the groups using paired T test. (MCQ pattern question. (Number of correct answer responses))

The practice-based (case-scenario) scores showed a substantial improvement following the symposium, with the mean increasing from 2.34 ± 0.61 pre-symposium to 4.06 ± 0.57 post-symposium. The paired difference analysis demonstrated a highly significant gain, with the mean difference falling within a narrow and positive 95% confidence interval (1.24–1.43), indicating a consistent improvement across participants. The very high t-value (26.85) and the p-value < 0.001 confirm that the increase in scores is statistically significant and unlikely due to chance. Overall, the symposium effectively enhanced students' ability to apply rational drug

use principles in case-based scenarios, reflecting meaningful gains in practical, application-level competence.

Feedback

The feedback scores indicate that the symposium was highly well-received, with students finding it relevant, engaging, and valuable for improving their understanding of RDU, and showing strong interest in attending similar sessions in the future.

Table 4. Feedback of the students about the symposium

Statements	Responses (N=150)	
	Yes	No
1. Were the topics discussed relevant?	142 (94%)	8 (5%)
2. Did the Programme meet your expectation?	145 (96%)	5 (3%)
3. Would you recommend such programme to your peers or juniors?	143 (94%)	7 (5%)
4. Did you find the symposium interesting?	142 (94%)	8 (5%)
5. Did the program improve your awareness and learning about rational drug use	145 (96%)	5 (3%)
6. Do you look forward to more such sessions in future	144 (95%)	6 (4%)
7. Suggested Topics for symposium:		
Diabetes mellitus	37 (24%)	
Tuberculosis	26 (17%)	
Ethical issues in clinical practice	20 (13%)	
Antimicrobial resistance	15 (10%)	
Pharmacovigilance	12 (8%)	
Cancer treatment	9 (6%)	

Yes/no response

The overall feedback from participants was overwhelmingly positive, with the majority responding “Yes” across all evaluation statements. Most students found the topics highly relevant (94%) and felt that the programme met their expectations (96%). A similarly high proportion (94%) expressed that they would recommend the symposium to peers, and 94% reported that the sessions were interesting. Furthermore, 96% believed that the symposium improved their awareness and understanding of rational drug use. Importantly, 95% of participants indicated that they look forward to attending similar sessions in the future. These results collectively demonstrate strong acceptance, high perceived value, and

clear educational impact of the symposium among the students.

RDU Awareness

The symposium led to a substantial improvement across all assessed domains, significantly enhancing students’ knowledge, awareness, and application skills related to RDU. Students demonstrated marked gains in understanding pharmaceutical influence, correct information, drug use, and decision-making.

Table 5. Pre- and Post-Symposium Awareness Scores (N = 150)

Outcome Variable	Pre-Symposia Mean \pm SD	Post-Symposia Mean \pm SD	Change (Post–Pre)	Improvement (%)
RDU Awareness	1.20 \pm 0.35	4.85 \pm 0.20	+3.65	+304%
Correct Information	1.10 \pm 0.40	4.90 \pm 0.15	+3.80	+345%
Correct Drug Selection	1.30 \pm 0.32	4.90 \pm 0.18	+3.60	+277%
Correct Drug Use	1.15 \pm 0.38	4.88 \pm 0.17	+3.73	+324%
Awareness of Pharmaceutical Influence	1.00 \pm 0.42	4.95 \pm 0.10	+3.95	+395%
Overall Awareness Score	5.75 \pm 1.9	24.45 \pm 0.8	+18.7	+325%

- Higher scores (4–5) → Strong awareness, accurate understanding, correct information, and better perception of rational drug use (RDU).
- Mid scores (3) → Uncertain or neutral awareness.
- Low scores (1–2) → Poor awareness or misconceptions.
- Lowest score (0 – No Idea) → Complete lack of awareness or no prior knowledge on the topic

The outcome measures demonstrated remarkable improvement across all domains following the symposium, with each parameter showing more than a 250% increase from pre- to post-assessment. The largest gain was seen in awareness of pharmaceutical influence (+395%), indicating a substantial shift in students’ critical understanding of industry impact on prescribing. Correct information (+345%) and correct drug use (+324%) also showed strong improvements,

reflecting enhanced conceptual clarity and application skills. RDU awareness (+304%) and correct drug selection (+277%) improved significantly, demonstrating strengthened foundational knowledge and decision-making ability. Overall, the total awareness score increased by 325%, confirming that the symposium had a profound and comprehensive educational impact across all components of rational drug use.

Table 6. Group-wise Awareness Scores Pre- and Post-Symposium (Mean \pm SD)

Clinical Posting (Departments)*	Pre-Symposia Mean \pm SD	Post-Symposia Mean \pm SD	Change (Post–Pre)	Improvement (%)
General Medicine (n=30)	1.25 \pm 0.40	4.90 \pm 0.18	+3.65	+292%
General Surgery (n=30)	1.10 \pm 0.45	4.88 \pm 0.20	+3.78	+343%
Pediatrics (n=30)	1.30 \pm 0.38	4.92 \pm 0.15	+3.62	+278%
Obstetrics and Gynecology (n=30)	1.05 \pm 0.42	4.85 \pm 0.22	+3.80	+362%
Community Medicine (n=30)	1.15 \pm 0.36	4.95 \pm 0.12	+3.80	+330%
Overall (N=150)	1.17 \pm 0.40	4.90 \pm 0.18	+3.73	+319%

*Students were equally distributed across five department clinical postings (n=30 per group)

All Clinical Posting groups demonstrated substantial improvement following the symposium, with post-symposia scores nearly quadrupling compared to baseline. The Obstetrics and Gynecology and Community Medicine groups

showed the highest proportional gains (+362% and +330%, respectively), indicating exceptional enhancement in RDU understanding among these students. General Surgery also showed a strong improvement (+343%), while General

Medicine and Pediatrics exhibited slightly lower, but still highly significant with increases of +292% and +278%. Overall, the combined improvement across all groups was

+319%, reflecting a uniformly positive educational impact of the symposium irrespective of departmental background.

Table 7. RDU Awareness: Therapeutic objectives and selection of rational drug (n=150)

Domain / Item	Pre-Symposia		Post-Symposia	
	YES (n, %)	NO (n, %)	YES (n, %)	NO (n, %)
1. Criteria for Selecting a Rational Drug				
Safety	82 (54.6%)	68 (45.4%)	145 (96.7%)	5 (3.3%)
Tolerability	78 (52.0%)	72 (48.0%)	144 (96.0%)	6 (4.0%)
Efficacy	85 (56.6%)	65 (43.4%)	147 (98.0%)	3 (2.0%)
Cost	70 (46.6%)	80 (53.4%)	142 (94.6%)	8 (5.4%)
All of the Above	76 (50.6%)	74 (49.4%)	146 (97.3%)	4 (2.7%)
2. Is Detailed History Needed for RDU?				
	79 (52.6%)	71 (47.4%)	148 (98.7%)	2 (1.3%)
3. Is Therapeutic Objective Necessary?				
	83 (55.3%)	67 (44.7%)	149 (99.3%)	1 (0.7%)
4. Therapeutic Objectives				
Treat signs & symptoms	77 (51.3%)	73 (48.7%)	145 (96.7%)	5 (3.3%)
Eradicate disease	80 (53.3%)	70 (46.7%)	147 (98.0%)	3 (2.0%)
Prevent transmission & complications	75 (50.0%)	75 (50.0%)	148 (98.7%)	2 (1.3%)

*Yes/No responses recorded from 150 students. Pre = before symposia; Post = after symposia.
Increased Yes responses post-symposia indicate improved awareness*

Across all domains, there was a striking shift from moderate baseline understanding to near-universal correctness after the symposium. Pre-symposia responses showed that only about half of the students recognized key criteria for rational drug selection, such as safety, tolerability, efficacy, and cost as essential, with “All of the above” acknowledged by just 50.6%. Post-symposia, however, over 94–99% of students answered correctly across all items, indicating an exceptional rise in conceptual clarity. Similarly, understanding the need for a detailed history and the importance of defining therapeutic objectives increased from around 52–55% pre-symposia to 98–99% post-symposia. Recognition of therapeutic goals such as treating symptoms, eradicating disease, and preventing complications also improved dramatically from 50–53% to nearly 97–99%. Overall, the symposium produced a transformative improvement in students’ foundational knowledge of rational drug use, elevating all domains to near-perfect understanding.

DISCUSSION

Only a limited number of studies have examined symposium-based learning (SBL) in early undergraduate medical education, mostly within anatomy and physiology, where favourable learning outcomes have been reported [13,14]. Western institutions have incorporated symposiums more systematically into undergraduate curricula, with improved engagement reported through structured and choice-based symposium formats [15]. Similar educational benefits have been reported for trauma-informed care and peer-led research-oriented symposiums [16]. Collectively, these studies position symposium-based learning as a learner-centred, evidence-supported instructional strategy. The present study extends this evidence to rational drug use (RDU) education

and demonstrates that SBL can effectively enhance second-year medical students’ knowledge, attitudes, and prescribing-related competencies. The observed improvements reflect not merely short-term score increases, but the impact of a deliberately structured, outcome-aligned educational intervention designed to address core prescribing challenges [17]. Careful topic selection, multidisciplinary facilitation, and alignment with learning objectives appear to be key contributors to this educational benefit. Deficits in undergraduate prescribing competence have been consistently reported despite the formal inclusion of RDU principles in the MBBS curriculum [18]. Students frequently report insufficient confidence in rational drug selection and prescription writing. The substantial improvement observed following the symposium therefore suggests that baseline learning gaps are not fully addressed by conventional teaching formats and that structured, interactive interventions can meaningfully strengthen foundational prescribing literacy. The effectiveness of the symposium is likely attributable to its multidisciplinary and interactive design. Exposure to clinicians, pharmacists, pharmacologists, and nursing professionals enabled learners to contextualize pharmacological knowledge within real-world clinical decision-making. Such interprofessional engagement promotes relevance, reflection, and integration of theory with practice, consistent with principles of adult learning and experiential education [19]. Similar interprofessional and experiential learning models have been shown to produce short-term gains in prescribing competence and pharmacological understanding [20], supporting the observed post-intervention improvements in this study. Importantly, the largest gains were observed in domains related to pharmaceutical influence and the use of correct information sources, indicating that the symposium enhanced not

only factual knowledge but also critical appraisal and ethical awareness. This is particularly relevant to safe prescribing, where commercial influence and information asymmetry are well-recognized risk factors. Structured RDU-focused educational interventions have similarly been shown to improve both knowledge and attitudes toward safe medication use and self-medication practices [21]. Although baseline attitudinal agreement was already high, post-symposium responses shifted consistently toward stronger endorsement across all five attitudinal domains, suggesting reinforcement rather than mere affirmation of beliefs. Learning environments that emphasize clinical relevance, interaction, and reflection are known to support durable attitudinal change [22], indicating that the symposium fostered a professional mindset aligned with rational and responsible prescribing. The improvement in case-based (practice) scores further supports the educational value of the intervention. Prior studies demonstrate that structured and case-based prescribing education improves prescription quality and therapeutic reasoning beyond that achieved through standard curricula alone [23]. Training aligned with the WHO Guide to Good Prescribing has been shown to significantly improve both knowledge and prescription-writing performance [24], consistent with the gains observed in this study. High participant satisfaction and strong willingness to attend similar programmes further indicate that the symposium met learners' educational needs. Engagement and perceived relevance are key determinants of learning effectiveness and knowledge retention in pharmacology education. Students' expressed interest in additional multidisciplinary topics such as diabetes, tuberculosis, antimicrobial resistance, and cancer therapy further underscores the perceived value of this learning format [25,26]. The marked improvement across awareness domains, including pharmaceutical influence, correct information use, and correct drug selection, aligns with international recommendations for focused and problem-based prescribing education. The greater gains observed in certain disciplines reflect the clinical exposure and prescription demands inherent to those fields and are consistent with WHO-endorsed principles of targeted RDU education [27]. Overall, the findings demonstrate that symposium-based learning can serve as a powerful supplement to conventional pharmacology teaching by strengthening prescribing awareness, ethical judgment, and applied competence. While the single-institution design and short follow-up limit generalizability, the consistent improvement across all learning domains provides strong justification for broader implementation and longitudinal evaluation of SBL in undergraduate medical education.

Methodological Limitations and Risk of Bias

Despite the encouraging outcomes observed in this study, certain methodological limitations should be acknowledged. First, the study employed a single-group pre-post quasi-interventional design without a parallel control group. Although this design is commonly used for educational interventions implemented as part of routine academic programs, it limits causal inference and does not entirely exclude the influence of external factors such as maturation or concurrent

learning experiences. To minimize this risk, the pre- and post-assessments were conducted immediately before and after the symposium, thereby reducing the likelihood of confounding educational exposures. Second, the study was conducted at a single institution, which may limit the generalizability of the findings to other medical colleges with differing curricula, teaching environments, or student demographics. However, inclusion of the entire eligible second-year MBBS cohort enhanced internal validity and reduced selection bias. Third, outcomes were primarily measured using self-reported questionnaire responses, which are susceptible to response and social desirability bias. This potential bias was mitigated by ensuring anonymity, using coded questionnaires, prohibiting peer discussion during data collection, and employing a rigorously validated instrument with good internal consistency (Cronbach's alpha = 0.82). Fourth, the assessment focused on short-term learning gains immediately following the symposium. Long-term retention of knowledge and sustained changes in prescribing behaviour could not be evaluated within the scope of the present study. Future longitudinal and multi-institutional studies are warranted to assess the durability and clinical translation of these educational gains. Finally, although students were stratified by clinical posting for awareness assessment to control for variability in clinical exposure, unmeasured differences in individual learning styles and prior informal exposure to prescribing practices may have influenced baseline scores. Nonetheless, uniform improvement across all groups suggests that the symposium had a consistent educational impact.

CONCLUSION

The symposium-based learning in teaching RDU appears to be an effective supplementary strategy for strengthening rational prescribing skills among undergraduate medical students. Beyond reinforcing core pharmacological principles, this approach supports meaningful peer interaction, enhances communication abilities, builds learner confidence, and stimulates curiosity that are often difficult to achieve through routine instructional formats. SBL could be strategically integrated with existing teaching learning methods to broaden its educational impact and to address topics that require deeper conceptual engagement, such as RDU, ethics, research methodology, professionalism, and essential clinical competencies. Future research could further strengthen the evidence base by examining long-term changes in students' prescribing behaviours, comparing SBL with other active learning strategies, and conducting multi-institutional studies to validate its generalizability across diverse educational settings.

AUTHOR CONTRIBUTIONS

Each author takes full responsibility for all aspects of the work. Each contributed significantly to the conceptualization, design, data collection, analysis, and interpretation of the study. Each author authorized the final version for publication, agreed to submit it to this journal, and took part in the manuscript's writing or critical revision for significant intellectual content. The International Committee of Medical

Journal Editors (ICMJE) standards and procedures were followed in determining authorship.

ETHICAL CONSIDERATIONS

Institutional ethics committee approval was taken prior to start of the study and informed consent was taken from all the study participants after fully explaining the study purpose and procedure.

ARTIFICIAL INTELLIGENCE UTILIZATION FOR ARTICLE WRITING

Ethical principles and guidelines of use of Artificial intelligence were adhered throughout the study conduct and manuscript preparation.

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CONFLICTS OF INTEREST

None.

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Nil.

DATA AVAILABILITY STATEMENT

Data related to the research findings will be shared via google drive link by the corresponding author via email as and when required.

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ROLE OF HEAT SHOCK PROTEINS IN CLIMATE-INDUCED CARDIOVASCULAR DAMAGE

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ABSTRACT

Heat shock proteins (HSPs) are a highly conserved family of proteins that are rapidly produced in response to cellular stress, particularly elevated temperatures. They play a critical role in maintaining cellular integrity by stabilizing, repairing, and refolding damaged proteins. First identified in *Drosophila melanogaster*, HSPs are induced even by mild thermal stress and have been shown to protect a variety of tissues, including the heart and vasculature. While previous reviews have largely focused on general stress responses and cardiovascular physiology, the effects of climate-related stressors—such as heat waves, extreme temperatures, and environmental heat exposure—on cardiovascular health via HSP activation remain underexplored. This review examines the emerging evidence on HSP-mediated cardiovascular protection under climate-induced stress. We highlight how HSPs support cardiomyocyte survival, maintain calcium homeostasis, preserve electrical stability, and enhance ischemic tolerance. Their roles in protecting vascular compartments, preventing hypertrophy, atherosclerosis, obesity-related dysfunction, and limiting metabolic, oxidative, and inflammatory injury are also discussed. By integrating these findings, we illustrate the multifaceted ways in which HSPs mitigate cardiovascular risks associated with rising global temperatures. Understanding the interplay between environmental stressors and HSP biology is increasingly important in a warming world. HSPs not only act as intrinsic cellular defense mechanisms but also represent promising therapeutic targets for enhancing cardiovascular resilience. By bridging molecular mechanisms with clinical implications, this review addresses critical gaps in the literature and provides a comprehensive perspective on how HSPs can help protect cardiovascular health in the face of climate change.

Keywords: Heat shock proteins (HSPs), Climate-induced cardiovascular stress, Cardioprotection, Hypertension, Atherosclerosis.

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INTRODUCTION

The discovery in 1962 of heat-induced chromosomal puffing in the salivary glands of *Drosophila busckii* opened a new avenue of research. The proteins involved were later termed “heat shock proteins” (HSPs) after being observed in *Drosophila melanogaster* salivary glands and other tissues following a brief, non-lethal elevation of body temperature, approximately 5°C above normal (1). Even a mild thermal stress was sufficient to trigger a thermal shock response, characterized by the production of unique proteins infrequently found in adult tissues, alongside the upregulation of pre-existing or cognate HSPs. Over time, extensive evidence has demonstrated that different HSP families serve unique functions, with gene transcription being transiently activated to enhance protein production, particularly within cardiac tissue. The induction of HSP synthesis is now recognized as a key intrinsic defense mechanism that safeguards essential cellular systems against environmental and physiological stressors. The roles of the 70-kDa HSP, small HSPs, and heat shock factors (HSFs) have received considerable attention.

Multiple stress-inducing stimuli, both physical and chemical, stimulate HSPs production in a wide range of tissues, including the cardiovascular system (2). This process is initiated through mechanisms such as activation of membrane-associated receptors, alterations in membrane dynamics, or intracellular perturbations such as temperature changes and oxygen fluctuations. HSPs represent one of the most evolutionarily conserved protein families, present across both prokaryotes and eukaryotes. They exhibit strong interspecies conservation and are constitutively expressed at basal levels in nearly all cell types. Across organisms as diverse as corals, desert ants, plants, microbes, and mammals, stress exposure leads to HSP overexpression. Because of their universality, HSP induction has even been employed as a broad biomarker for environmental stress and pollution. At the molecular level, the transcription of the HSP gene is regulated by the binding of heat shock elements in the promoter regions to activated factors. Typically, HSFs exist as inactive monomers in the cytoplasm but undergo activation, trimerization, and nuclear translocation under stress conditions (3). This activation often involves Ras-dependent hyperphosphorylation mediated by mitogen-activated protein kinases (4). The HSF family regulates HSP transcription either independently or cooperatively, acting through gene activation or repression. In humans, six HSFs are encoded, with HSF1 and HSF2 being the most prominent in vertebrates. Functionally, HSPs exert cytoprotective effects by serving as molecular chaperones, facilitating protein folding, intracellular trafficking, and the repair of misfolded or denatured proteins. In this role, they transiently interact with diverse client proteins until their folding or stabilization is complete.

Among the HSPs implicated in atherosclerosis, HSP27, HSP60, HSP70, and HSP90 have been most extensively examined (5). HSP27 functions as an intracellular chaperone, with its activity regulated by cycles of phosphorylation and dephosphorylation within large protein complexes that form

an ATP-independent network. Beyond this, HSP27 contributes to the preservation of RNA stability, facilitates antioxidant defense mechanisms, and exerts anti-apoptotic effects. Its release into the extracellular space may result from tissue injury or through secretory pathways such as lysosomes and exosomes. In the extracellular environment, HSP27 interacts with multiple Membrane receptors on endothelial and immunological cells, such as CD91, CD40, CD36, CD14, and scavenger receptor A (SR-A), and Toll-like receptors (TLR2, TLR3, TLR4) (6).

Ischemic heart disease, in both its acute and chronic forms, continues to be a leading cause of death in Western countries, despite the availability of various pharmacological treatments such as Calcium channel blockers, vasodilators, angiotensin-converting enzyme inhibitors, and adrenergic receptor antagonists. Beyond external interventions, the heart also possesses intrinsic protective mechanisms. Experimental studies in mice have shown that enhanced synthesis of HSPs improves tolerance to ischemic injury. These proteins are produced within both cardiac and vascular tissues, although the types generated in each compartment differ slightly. In adult mice under non-stress conditions, several HSPs, including Hsp27, Hsc70, Hsp70, and Hsp84, are continuously expressed across multiple organs, including the heart.

In contrast, unstressed rat hearts exhibit high levels of $\alpha\beta$ -crystallin with comparatively lower amounts of Hsp27. Hsp27 is expressed in endothelium cells, smooth muscle cells, and cardiac myocytes in both rat and human hearts, whereas $\alpha\beta$ -crystallin is restricted to cardiomyocytes. While limited investigations have examined the roles of heat shock factors (HSFs) in cardiac tissue, the presence of HSF1, HSF2, and HSF4 has been confirmed. Although the steady expression of HSPs in the adult heart is well recognized, their regulatory mechanisms and expression profiles during embryonic, neonatal, and developmental stages remain insufficiently understood. Only recently have studies clarified the expression of Hsp70 and Hsc70 in the immature ovine heart during perinatal and juvenile stages. Despite extensive research on HSPs in cardiovascular physiology, early reviews primarily focused on general stress responses, ischemic tolerance, and cardiac pathology, often overlooking the specific effects of climate-induced stressors such as heat waves, temperature extremes, and environmental perturbations on heart and vascular health. Moreover, the integration of molecular mechanisms, metabolic interactions, and clinical outcomes under these conditions remains limited. This review aims to bridge these gaps by providing a comprehensive synthesis of HSP-mediated cardio protection under climate stress, highlighting mechanistic insights, environmental triggers, and potential therapeutic strategies relevant to rising global temperatures. The major heat shock protein subtypes involved in climate-induced cardiovascular stress, their key cardiovascular effects, and relevant environmental triggers are summarized in Table 1.

Table 1. Heat Shock Protein Subtypes, Cardiovascular Effects, and Climate/Stress Triggers

HSP Subtype	Major Cardiovascular Effects	Climate / Stress Triggers Relevant to CVS
HSP27 (HspB1)	Stabilizes cytoskeleton; protects endothelial integrity; reduces apoptosis; modulates inflammation; protective role in atherosclerosis and vascular remodeling	Heat waves, shear stress, oxidative stress, ischemia–reperfusion, metabolic stress (obesity, diabetes)
αB-crystallin (HspB5)	Preserves cardiomyocyte structure; supports contractile function; enhances ischemic tolerance; protects myofilaments	Thermal stress, hypoxia, ischemia, mechanical strain
HSP60	Mitochondrial protein folding; immune activation when extracellular; implicated in hypertension, atherosclerosis, and metabolic cardiovascular risk	Oxidative stress, inflammation, metabolic stress, chronic heat exposure
HSP70 (HspA1A/Hsp72)	Strong cardioprotection; improves ischemic tolerance; maintains calcium homeostasis; reduces arrhythmias; modulates immune responses in hypertension and atherosclerosis	Acute and chronic heat stress, heat waves, hypoxia, ischemia–reperfusion, oxidative stress
HSC70 (HspA8)	Constitutive chaperone; supports protein quality control; extracellular form protects against inflammatory hypertrophy	Basal cellular stress, inflammation, metabolic stress
HSP90	Stabilizes signaling proteins (eNOS, kinases); regulates vascular tone; involved in endothelial function and atherosclerosis	Heat stress, oxidative stress, inflammation, disturbed shear stress
HSP20 (HspB6)	Enhances myocardial contractility; reduces apoptosis; limits infarct size; promotes autophagy	Ischemia–reperfusion, hypoxia, thermal stress
HSP22 (HspB8)	Regulates cardiac hypertrophy; supports mitochondrial function; involved in aging-related cardiac remodeling	Chronic stress, pressure overload, metabolic stress
HO-1 (HSP32)	Antioxidant and anti-inflammatory effects; protects vascular endothelium; improves ischemic tolerance	Heat stress, hypoxia, oxidative stress, environmental toxins
Heat Shock Factors (HSF1/HSF2)	Master regulators of HSP expression; coordinate cardioprotective stress responses	Heat waves, oxidative stress, ischemia, metabolic and inflammatory stress

HSPs IN HYPERTENSION

Hypertension remains a leading risk factor for cardiovascular morbidity and mortality worldwide, with complex etiologies involving genetic, environmental, and immunological components. Emerging evidence has implicated immune-mediated mechanisms in the initiation and maintenance of elevated blood pressure, highlighting the role of endogenous antigens such as HSPs and reactive lipid modifications, including isoketals, in disease pathogenesis. Among HSPs, Heat Shock Protein 70 (HSP70) has garnered particular attention due to its immunogenic potential and consistent association with both experimental and human hypertension. HSPs are highly conserved molecular chaperones that maintain cellular proteostasis under physiological and stress conditions, including oxidative stress, ischemia, and mechanical strain (7). HSP70, in particular, is upregulated in response to cellular stress and functions to refold misfolded proteins, prevent aggregation, and facilitate protein degradation. While these protective roles are well-established, HSP70 can also serve as an autoantigen, capable of eliciting adaptive immune responses under certain pathological conditions.

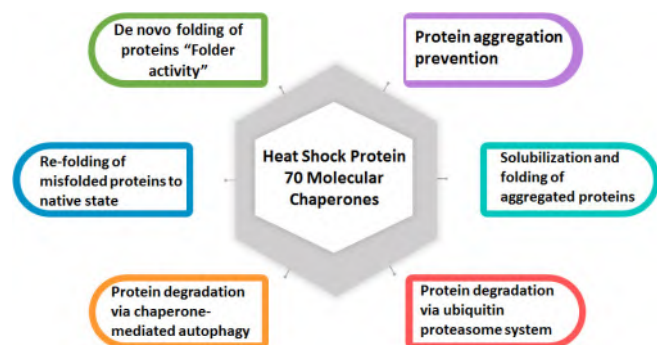
**Figure 1.** Molecular Mechanisms of Heat Shock Protein

Figure 1. illustrates the cellular signaling pathways involved in heat shock protein induction during stress conditions. Environmental and physiological stressors activate heat shock factors (HSFs), leading to their trimerization and nuclear translocation. Binding of HSFs to heat shock elements (HSEs) in gene promoters induces transcription of HSPs, which function as molecular chaperones to stabilize proteins, prevent aggregation, and maintain cellular homeostasis in cardiovascular tissues.

In experimental models of hypertension, HSP70 accumulation has been consistently observed in renal tissues, whereas levels of other HSP family members, such as HSP60 and HSP90, remain relatively unchanged. This selective renal deposition is of particular interest because the kidneys play a pivotal role in long-term blood pressure regulation via sodium handling, renin-angiotensin-aldosterone system (RAAS) modulation, and local vascular responses (8). Accumulated renal HSP70 may thus serve as a target for circulating immune cells, initiating a cascade of inflammatory events that exacerbate renal dysfunction and hypertension.

HSP for vascular compartment protection

In reaction to environmental stress, all cell types within the vascular wall commence the synthesis of heat shock proteins (HSPs). The production of these vascular HSPs can be induced by stimuli like circulating hormones, reactive oxygen species (ROS), and sodium arsenite (9). Nitric oxide (NO) is thought to contribute to Hsp70 induction in blood vessels, as the suppression of NO synthase (NOS) by N^ω-nitro-L-arginine (L-NNA) diminishes Hsp70 gene transcription (10). The exact signaling route by which nitric oxide (NO) induces Hsp70 production is not fully elucidated. However, it may involve enhanced calcium influx or reactive oxygen species (ROS) produced during heat shock, potentially activating both constitutive and inducible nitric oxide synthase (NOS) pathways. In the aorta, akin to cardiomyocytes, two Hsp32 isozymes: heme oxygenase-1 (HO-1) and HO-2—are expressed constitutively. HO-1 is involved in heme degradation, transforming it into biliverdin, iron, and carbon monoxide (11). The transcription in the aorta can be swiftly accelerated in reaction to acute physical stress, elevated temperatures, hypoxia, hemin, hydrogen peroxide (H₂O₂), heavy metals, or reperfusion after myocardial ischemia. Research indicates that previous whole-body heat shock elevates Hsp70 levels safeguard certain essential processes in rat coronary artery endothelial cells during ischemia-reperfusion. In these hearts, vasodilation mediated by endothelial cells in response to 5-hydroxytryptamine stays entirely operational after a 4-hour ischemia interval accompanied by intracoronary cardioplegia. Moreover, in endothelial cells, heat shock-induced phosphorylation of Hsp27 seems to protect certain intracellular structures from external stresses. Upon metabolic suppression of these cells, due to glucose depletion and rotenone exposure, cytoskeletal elements, such as F-actin, undergo fast degradation.

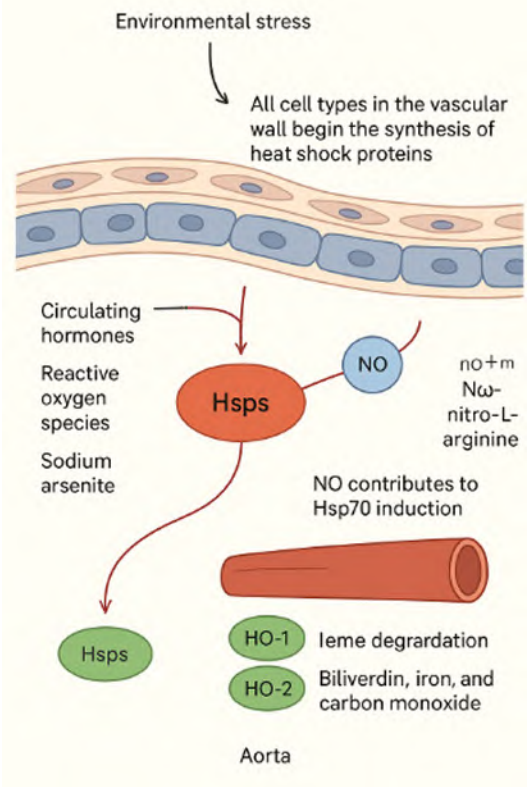


Figure 2. HSP for vascular protection

Figure 2 depicts the protective roles of heat shock proteins within the vascular compartment under stress conditions. HSPs are induced in endothelial cells and vascular smooth muscle cells in response to oxidative stress, nitric oxide signaling, and thermal injury. Their actions preserve endothelial function, stabilize the cytoskeleton, reduce oxidative damage, and maintain vasodilatory responses, thereby contributing to vascular integrity and resistance to ischemia-reperfusion injury.

HSP protection of cultured cardiomyocytes

Extensive research has explored how cardiac cells respond to stress *in vitro*. Various cell models have been utilized, including freshly isolated neonatal and adult cardiomyocytes, as well as myogenic cell lines such as C2C12 and H9c2. The cellular environment appears to influence stress responses, as extracellular matrix components like collagen can reduce both basal and thermally induced synthesis of several heat shock proteins (HSPs) in cultured cardiomyocytes. Evidence indicates that early activation of the HSP gene transcription enhances cell survival under severe or potentially lethal stress. The expression of hsp70 and hsp90 can be stimulated by heat or metabolic stress, while hsp70 and HO-1 levels can also be elevated by the antioxidant ebselen (12). Hypoxic conditions have been shown to increase HSP levels, particularly HSP70, which correlates with improved resistance to lethal heat stress. Achieving similar HSP70 levels through hyperthermic pretreatment does not always confer equivalent protection, suggesting that HSP-mediated cytoprotection is context-dependent. The function of heat shock

proteins (HSPs) has been examined in relation to exposure to bacterial lipopolysaccharide (LPS), particularly within the H9c2 cell line. Originating from embryonic cardiac tissue, H9c2 cells retain several cardiac-specific characteristics, making them a valuable *in vitro* model for studying cardiomyocyte stress adaptation and HSP-related protective mechanisms (13).

HSP in Calcium homeostasis

Induction of heat shock proteins (HSPs) plays a protective role in cardiomyocytes by supporting calcium homeostasis during ischemic stress. Dysregulated calcium levels are a key feature of ischemia-reperfusion injury, causing impaired contraction, mitochondrial dysfunction, and cell death. Reported that hearts preconditioned with heat exhibit reduced mitochondrial calcium accumulation during ischemia, suggesting that HSPs help prevent harmful calcium overload. Subsequent studies have confirmed this effect, providing strong evidence that HSPs modulate intracellular calcium under stress conditions. Rabbit papillary muscles collected 24 hours after heat pretreatment maintained normal calcium handling during reoxygenation following hypoxia. Basal diastolic intracellular calcium levels were similar in heat-pretreated and control cardiomyocytes, indicating maintained baseline calcium regulation (14). Furthermore, myofilament calcium sensitivity remained unaltered at healthy extracellular calcium levels, ensuring proper excitation-contraction coupling. Even under elevated extracellular calcium, contractile function was not compromised, emphasizing the stabilizing effect of HSPs on calcium-dependent mechanisms. Heat-pretreated cardiomyocytes recovered diastolic cell length more rapidly and completely than untreated cells, reflecting faster restoration of relaxation dynamics and reduced risk of diastolic dysfunction. Overall, these observations highlight the critical role of HSPs in maintaining calcium balance, protecting heart cells from ischemic damage, and preserving cardiac mechanical function under stress.

HSP in Electrical stability

Preconditioning with heat stress, or the intentional induction of certain heat shock proteins (HSPs) such as Hsp70, prior to ischemic events, has been shown to provide protective effects against arrhythmias that occur after ischemia. Studies using intact rats subjected to temporary coronary occlusion and isolated rat hearts undergoing brief ischemic episodes have reported a significant reduction in post-ischemic arrhythmias and ventricular fibrillation following heat pretreatment (15). This cardioprotective effect highlights the role of stress-induced molecular adaptations in maintaining myocardial electrical stability. Research demonstrates a biphasic decline in ventricular fibrillation in heat-preconditioned rats after myocardial infarction (16), with peak protection occurring roughly 30 minutes after pretreatment. The decrease in arrhythmic incidents is accompanied by a notable reduction in infarct size compared to untreated controls.

These results indicate that the induction of HSPs can alleviate both structural and electrophysiological damage caused by ischemia. At a mechanistic level, Hsp70 and associated chaperones help maintain protein structure, preserve mitochondrial function, and inhibit apoptotic pathways (17), thereby preventing the cellular dysfunction that promotes arrhythmias. Improved perfusion in surviving myocardial tissue may contribute indirectly to more uniform electrical conduction and reduced repolarization heterogeneity, further mitigating rhythm disturbances. Thus, heat shock-mediated preconditioning is a vital endogenous mechanism that enhances myocardial resilience, offering insights into therapeutic approaches to limit ischemia-reperfusion-induced arrhythmias and reduce infarct size in cardiovascular disease.

Mediators of Ischemic Tolerance

A distinct method for triggering the heart's intrinsic protective mechanisms, independent of HSPs, is referred to as ischemic ("classic") preconditioning. Unlike HSP-mediated cardioprotection, which manifests around 24 hours after treatment, protection from ischemic preconditioning arises within hours of the ischemic event. This preconditioning rapidly stimulates the production of new HSPs and promotes the nuclear translocation of existing ones. However, the precise role of HSPs in the immediate cardioprotective effects of ischemic preconditioning remains uncertain. During this process, small heat shock proteins such as B-crystallin and Hsp27 α shift to the myofilament and cytoskeletal compartments (18). Reactive oxygen species (ROS) appear essential for activation, as interventions with allopurinol or catalase inhibit HSF1 activation. The activity of manganese superoxide dismutase (Mn-SOD) follows a biphasic pattern that closely mirrors the cardioprotective timeline. Upregulation of Mn-SOD has been shown to enhance activity of endogenous catalase, glutathione peroxidase, and glutathione reductase after ischemia preconditioning (19). Enhanced tolerance to ischemia correlates with elevated tissue Hsp70 levels and diminished incidence of arrhythmias. Ischemic preconditioning does not provide late-stage protection in the rat heart, even with increased levels of Hsp70, suggesting it operates through mechanisms separate from classical Hsp-mediated cardio protection (20). Cardiac ischemia/reperfusion injury induces higher expression of HSP70 and HSP90 mRNA, with HSP70 levels substantially exceeding HSP90. This upregulation likely results from HSF1 activation triggered by ROS accumulation. Elevated HSP70 and HSP72 provide protective effects during ischemia/reperfusion events (21). Repeated endurance exercise, which induces HSP72, has been shown to reduce Myocardial infarct dimensions and cardiac apoptosis. Furthermore, cardiac-specific overexpression of HSP20 protects against ischemia/reperfusion injury by improving contractile performance, reducing myocyte apoptosis, and markedly decreasing infarct size. The protective effects of HSP20 appear to involve the activation of autophagy, a key process in mitigating ischemia/reperfusion damage.

Cardiac Senescence

The efficacy of HSPs in safeguarding and enhancing heart function during and after ischemic events is significantly influenced by tissue age. Advances in healthcare have extended human life expectancy, resulting in a larger elderly population. Both aged humans and animals demonstrate increased vulnerability to myocardial ischemia. Since Hsp-mediated stress protection may be particularly important for older individuals, numerous studies have investigated its potential benefits in this age group. The capacity to induce heat shock protein production in response to stress diminishes with age (22). Similarly, cultured hepatocytes from older animals exhibit reduced Hsp synthesis. In senescent human fibroblasts and rat splenic cells, Hsp70 synthesis in response to heat shock is significantly diminished, corresponding with a substantial drop in HSF1 binding to heat shock elements within the Hsp70 gene promoter. Caloric restriction can completely reverse the age-associated reduction in Hsp70 gene transcription. The regulation of stress-induced Hsp production in older organisms is intricate and cannot be attributed solely to decreased HSF1 binding at the promoter regions of hsp genes (23). In the aging cardiovascular system, stress-induced Hsp production is also impaired. Hsp70 expression in the arterial walls of older animals in response to acute hypertension is considerably lower than in their younger counterparts. Despite this decline, the complete activation of hsp genes remains feasible under specific conditions, suggesting that different HSF1 activation pathways may function variably in adult versus aged tissues.

Cardiac hypertrophy

Pathological myocardial hypertrophy is acknowledged as a significant factor in sudden cardiac mortality, myocardial infarction, and heart failure. Hearts undergoing hypertrophy are more susceptible to ischemic injury. During post-ischemic reperfusion in hypertrophied hearts, common observations include chronic arrhythmias, persistent low cardiac output, and elevated release of intracellular enzymes. Investigating the capacity of hypertrophied cardiac tissue to upregulate heat shock proteins (HSPs) is valuable, as these proteins may help mitigate the decreased ischemic tolerance seen in such hearts. In fully compensated hypertrophy, basal HSP levels in the myocardium generally remain unchanged. In rat cardiomyocytes studied 2–4 days after thoracic aortic constriction, transient overexpression of Hsp70 and Hsp60 has been observed (24). Stress-induced transcription of hsp genes appears to be preserved, at least during the compensatory phase of hypertrophy. Young spontaneously hypertensive rats (SHRs) preconditioned with heat exhibited nearly a threefold increase in cardiac Hsp70 compared with age-matched normotensive controls (25); this heightened heat shock response in juveniles is largely due to impaired thermoregulation rather than an intrinsic enhancement in HSP synthesis.

In contrast, hsp gene expression in animals subjected to abdominal aortic constriction is comparatively modest versus SHRs (26). In hypertrophied hearts from these models, heat exposure caused a sevenfold rise in Hsp70 mRNA, whereas non-hypertrophied controls only showed a threefold increase. The capacity of hypertrophied hearts to produce key HSPs such as Hsp70 declines with age and is influenced by the type of hypertrophy. Nonetheless, heat preconditioning can reduce ischemia-reperfusion injury in hypertrophied hearts, likely via induction of HSPs other than Hsp70. Experimental Overexpression of a dominant-negative Hsp70 or targeted silencing of histone deacetylase 2 (HDAC2) using siRNA has been shown to attenuate cardiac hypertrophy, highlighting a functional link between Hsp70 and epigenetic regulators in remodeling processes (27). In rat models, hypertrophy induced by isoproterenol or aortic constriction is associated with reduced HDAC2 activity, suggesting that Hsp70 may modulate hypertrophy by stabilizing HDAC2 and affecting downstream transcription (28). Class II HDACs—including HDAC4, HDAC6, HDAC7, and HDAC9 seem to inhibit hypertrophy through repression of transcription mediated by MEF2, GATA, and NFAT, thereby maintaining cardiomyocyte homeostasis under stress (29). Hsp22 expression increases in ventricular hypertrophy in both cultured cardiomyocytes and intact mouse hearts (30). Elevated Hsp22 correlates with the emergence of spontaneous hypertrophy and the reactivation of the fetal gene program, indicating that Hsp22 may act as a molecular regulator of abnormal cardiac growth.

HSP in atherosclerosis

Numerous members of the HSPs families have been detected in atherosclerotic plaques within human blood vessels. The physiological and pathological implications of their abundant presence in these plaques are not yet fully understood. The prevailing hypothesis suggests that their accumulation reflects the stressful microenvironment experienced by cells in developing plaques. Berberian and colleagues were among the first to report elevated levels of the inducible Hsp70 protein within the core of atherosclerotic plaques (31). This increase is particularly associated with infiltrating macrophages and is concentrated near the edges of necrotic regions within the vessel wall. In addition to Hsp70, higher levels of Hsp60, Hsp90, and Hsp27 have also been observed. The initial trigger for enhanced Hsp production in atherosclerotic tissue remains debated. Oxidized low-density lipoproteins (Ox-LDL), known for their cytotoxicity and role in atherosclerosis, have been demonstrated to induce Hsp70 production in cultured human endothelial cells (32). Hsp70 production is significantly lower in endothelial cells derived from umbilical veins compared to these cultured cells. Studies indicate that the necrosis of smooth muscle cells caused by circulating toxins can be mitigated by administering exogenous Hsp70. Moreover, pre-exposure to heat stress inhibits its smooth muscle cell proliferation after mechanical injury by inducing Hsp synthesis. Variations in shear stress, a tangential component of blood flow, activate genes associated with atherogenesis in endothelial regions susceptible to

lesion formation. The small Hsp27 protein, present in vascular endothelial cells, undergoes phosphorylation in response to changes in shear stress, even though its overall expression levels remain unchanged (33).

By modulating TLR signaling, HSP27 enhances NF- κ B activation, leading to the production of both pro- and anti-inflammatory cytokines, particularly interleukin-10 (IL-10) (34). Evidence indicates that HSP27 exerts protective effects against atherosclerotic progression. Its interaction with estrogen receptors may account for the cardioprotective influence of estrogens observed in atherosclerosis. Evidence indicates that HSP27 exerts protective effects against atherosclerotic progression. Estrogen signaling through estrogen receptors promotes HSP27 upregulation, which enhances endothelial cell survival, suppresses inflammatory signaling, and contributes to plaque stabilization, thereby providing a mechanistic basis for the cardioprotective effects of estrogens in atherosclerosis. HSP60 also contributes to disease mechanisms, with multiple epitopes displaying cross-reactivity between bacterial HSP60 and host T and B cells. HSP70 is expressed within atherosclerotic plaques and is elevated in advanced lesions. This protein has been reported to suppress NF- κ B activity, indicating potential anti-inflammatory roles (35); however, findings remain inconsistent, making its precise contribution uncertain. Circulating HSP70 levels have been associated with both increased and decreased disease severity. Administration of HSP70 stimulates pro-inflammatory IL-6 production while simultaneously enhancing regulatory T cell (Treg) responses, reflecting a dual role in immune modulation.

HSP in obesity induced CVS complications

Dysfunctional adipose tissue induces chronic systemic inflammation and metabolic irregularities, provoking both inflammatory and metabolic stress. Metabolic stress encompasses difficulties associated with obesity-related conditions, including insulin resistance, type 2 diabetes, metabolic syndrome, hormonal imbalances, tissue hypoxia, cellular edema, and increased production of reactive oxygen species. This condition triggers a physiological stress response marked by increased production and secretion of heat shock proteins (HSPs). Upon release into circulation, HSPs provoke the secretion of inflammatory mediators, hence intensifying chronic tissue inflammation and facilitating the advancement of metabolic and cardiovascular illnesses. A multitude of studies have examined the biomarker potential of HSP27, emphasizing its metabolic functions and association with cardiac events. Alterations in HSP27 expression have been identified in multiple obesity-related metabolic diseases, with diminished expression reported in the adipose tissue of women who have gestational diabetes and obesity (36).

Oliva et al. reported that HSP27 expression was reduced in the adipose tissue of 12 women with insulin-treated gestational diabetes mellitus relative to 12 women with normal

glucose tolerance (37). Their study evaluated various heat shock proteins, including HSP27, HSP70, α HSP, and HSP60, in conjunction with clinical parameters such as body mass index (BMI) and lipid profiles. One year following ablation therapy, only the basal HSP27 levels in the blood showed an increase, while higher circulating HSP27 concentrations were linked to an elevated risk of recurrent atrial fibrillation. The study found no correlation between BMI and HSP27 levels; however, patients with atrial fibrillation had a significantly higher BMI than the control group. Obesity remains a critical contributor to the development of coronary artery disease (CAD). Supporting this, Abaspour et al. reported a relationship between HSP27 mRNA copy number in peripheral blood mononuclear cells and CAD severity. However, BMI and hip circumference did not significantly differ among groups based on HSP27 expression (38). The involvement of HSP40 in obesity is still debated, as it is activated under various stress conditions, including hypoxia, inflammation, and mechanical tissue injury. Both serum and adipose tissue HSP40 levels are elevated in individuals with obesity compared to those of normal weight (39). Higher expression is observed in obese patients with insulin resistance compared to their insulin-sensitive counterparts. Furthermore, Sell et al. identified a correlation between HSP60 levels and the advancement of obesity during bariatric surgery in a group of 53 obese women (40).

Kuka et al. investigated the correlation between plasma HSP60 concentrations and multiple health indicators, including hypertension, oxidative stress, lipid profiles, and cardiometabolic risk factors such as abdominal obesity, metabolic syndrome, and diabetes mellitus, in a cohort of 129 hypertensive and 39 normotensive women (41). Their findings indicated a correlation between blood pressure and HSP60 levels in subjects with hypertension, but in the normotensive group, HSP60 levels were correlated with total glutathione. Increased anti-HSP60 levels were associated with elevated coronary artery calcium scores, suggesting subclinical atherosclerosis; however, factors such as diabetes, hypertension, obesity, and dyslipidemia did not seem to affect these antibody concentrations. The research suggested that serum anti-HSP60 may function as an independent biomarker for the early identification of atherosclerosis in asymptomatic obese persons.

CONCLUSION

Heat shock proteins (HSPs) play a multifaceted role in cardiovascular protection under diverse stress conditions. Evidence indicates their involvement in hypertension regulation, maintenance of vascular integrity, and protection of cultured cardiomyocytes. HSPs contribute to calcium homeostasis and electrical stability, thereby preserving myocardial function and preventing arrhythmias. They act as key mediators of ischemic tolerance, modulating adaptive responses to hypoxic injury. HSPs influence cardiac senescence and hypertrophy, mitigating age- and stress-related cardiac remodeling. Their role extends to metabolic and inflammatory

contexts, including atherosclerosis and obesity-induced cardiovascular complications, highlighting their systemic relevance. This review stresses the therapeutic potential of HSP modulation in preventing and managing climate- and stress-induced cardiovascular damage. By integrating molecular, cellular, and clinical evidence, it provides a comprehensive framework for future research, emphasizing HSPs as critical targets for cardioprotection and translational interventions in cardiovascular medicine.

CONFLICT OF INTEREST

Conflict of interest declared none.

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None.

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SERUM MAGNESIUM IN PROTON PUMP INHIBITORS USERS AND NON-USERS

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ABSTRACT

Proton pump inhibitors (PPIs) are widely prescribed for acid-related gastrointestinal disorders and are generally considered safe. However, prolonged PPI use has been associated with electrolyte imbalances, particularly hypomagnesemia, which can result in severe complications such as arrhythmias, seizures, and tetany. Despite growing global concern, evidence on this association remains limited and inconclusive in Indian hospital populations, where PPI prescription rates are notably high. This study evaluated the relationship between prior PPI use and serum magnesium levels among hospitalized patients. A prospective case-control study was conducted on 370 inpatients (185 PPI users and 185 non-users) admitted to the Department of General Medicine at a tertiary care centre. Patients who had received PPI therapy for at least six months were included as cases, while those without prior PPI exposure served as controls. Serum magnesium levels were estimated using the modified methylthymol blue method, and demographic and clinical data were analyzed using SPSS version 24. The mean serum magnesium concentration was significantly lower in PPI users compared to controls (1.77 ± 0.48 mg/dL vs. 2.02 ± 0.43 mg/dL; $p < 0.001$). Hypomagnesemia (< 1.8 mg/dL) was observed in 54.6% of PPI users versus 25.9% of controls. Logistic regression analysis indicated that PPI use was independently associated with hypomagnesemia (odds ratio = 2.475; 95% CI: 1.605–3.816). These findings suggest that long-term PPI therapy is significantly associated with reduced serum magnesium levels. Therefore, regular monitoring of serum magnesium is recommended in chronic PPI users to prevent potential adverse clinical outcomes.

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INTRODUCTION

Proton pump inhibitors (PPIs) constitute one of the most potent and extensively prescribed classes of acid-suppressive pharmacologic agents, capable of attenuating both basal and stimulated gastric acid secretion by approximately 80–95% (1). Their mechanism of action involves the covalent binding to cysteine residues on the H^+/K^+ -ATPase enzyme located in the secretory canaliculi of gastric parietal cells, thereby irreversibly inhibiting the terminal step of gastric acid production (2). This pharmacodynamic property renders PPIs indispensable in the management of acid-related gastrointestinal disorders such as gastroesophageal reflux disease (GERD), peptic ulcer disease, Zollinger–Ellison syndrome, and stress-induced mucosal injury. Owing to their robust efficacy, favorable tolerability profile, and ease of use, PPIs rank among the top three most frequently dispensed medications globally. In India, hospital-based studies indicate elevated prescription rates among inpatients, often extending beyond evidence-based indications. However, despite their established therapeutic benefits and perceived safety, accumulating evidence has linked prolonged PPI therapy with a spectrum of adverse health outcomes, including increased risks of osteoporotic fractures, vitamin B₁₂ deficiency, chronic kidney disease, enteric infections, and electrolyte imbalances, most notably hypomagnesemia.

Magnesium, the second most abundant intracellular cation, is an essential cofactor in over 300 enzymatic reactions, playing pivotal roles in nucleic acid and protein synthesis, energy metabolism, neuromuscular excitability, and regulation of transmembrane ion fluxes. Deficiency in serum magnesium can precipitate a wide range of clinical manifestations, encompassing nonspecific symptoms such as fatigue, nausea, and muscle cramps to severe neurological and cardiovascular complications, including paresthesias, seizures, cardiac arrhythmias, hypotension, and, in extreme cases, sudden cardiac death. The mechanism of PPI-induced hypomagnesemia is thought to involve reduced intestinal absorption and alterations in magnesium transporters due to changes in intraluminal pH. Several case reports and observational studies have described this association, although findings remain inconclusive. The U.S. FDA had issued warnings recommending serum magnesium monitoring in long-term PPI users, especially those receiving concomitant medications such as diuretics or digoxin that further predispose them to hypomagnesemia. Given the widespread and often prolonged use of PPIs in Indian hospital settings, it is clinically important to assess their impact on serum magnesium. This study was therefore undertaken to evaluate the association between prior PPI use and serum magnesium levels in hospitalized patients. PPIs are commonly used for peptic ulcers, GERD, Zollinger–Ellison syndrome, erosive gastritis, and NSAID-induced ulcers. The most frequently prescribed agents include omeprazole, pantoprazole, lansoprazole, rabeprazole, esomeprazole, and dexlansoprazole. By irreversibly inhibiting the gastric H^+/K^+ -ATPase pump, PPIs effectively suppress both basal and stimulated gastric acid secretion, making them more potent than H₂ receptor antagonists (4). Although

generally considered safe, prolonged use of PPIs has been associated with adverse outcomes such as gastrointestinal and respiratory infections, osteoporosis, vitamin B₁₂, iron, calcium deficiencies, and chronic kidney disease (3,5). In 2011, the FDA issued a safety update cautioning that long-term PPI therapy may lead to hypomagnesemia, particularly in patients receiving diuretics or digoxin (6). Several case reports and clinical studies have described symptomatic hypomagnesemia during long-term PPI use, with patients presenting with seizures, arrhythmias, hypocalcemia, and hypokalemia (7–9). Magnesium levels typically normalize after discontinuation of PPI therapy, but fall again upon rechallenge, irrespective of PPI type. A meta-analysis by Srinutta et al. confirmed that chronic and high-dose PPI use increases the risk of hypomagnesemia (10,11). In elderly Japanese patients, long-term PPI use was associated with significantly lower serum magnesium levels. A South Indian cross-sectional study reported that nearly 90% of inpatients were prescribed PPIs, highlighting high usage and potential for drug interactions. Taken together, existing literature supports a possible association between PPIs and hypomagnesemia, but findings remain inconclusive, warranting further evaluation in hospital-based populations. The primary objective of this study was to compare serum magnesium levels between patients with and without prior PPI use over the past six months or more, while secondary objectives included assessing age and gender-related differences in magnesium levels and estimating the risk of hypomagnesemia associated with PPI therapy.

METHODS

Study population

We prospectively recruited a total of 372 hospitalized patients between May 23 and Dec 23 in the Department of General Medicine at Apollo Institute of Medical Sciences & Research (AIMSR), Hyderabad, India. Enrollment was consecutive, and patients were stratified into two groups according to prior exposure to PPIs.

Inclusion Criteria

The study included adults aged 18 to 75 years who were hospitalized under the Department of General Medicine at the Apollo Institute of Medical Sciences & Research (AIMSR), Hyderabad, India. Participants were consecutively enrolled between May 2023 and December 2023. The case group comprised patients with documented proton pump inhibitor (PPI) use for at least five days per week over a minimum duration of six months, while the control group included patients with no prior PPI exposure. Only individuals who provided written informed consent were included in the study.

Exclusion criteria

Patients with a history of malignancy, renal impairment, or chronic diarrhea, those receiving magnesium supplementation at the time of evaluation, and individuals who were

unable or unwilling to provide informed consent were excluded from the study.

Sample size

The sample size was determined a priori based on conventional power analysis methods. Considering a prevalence of PPI exposure of approximately 46% among the general hospitalized population, and anticipating an odds ratio (OR) of 1.8 for the primary outcome (hypomagnesemia), the calculation was performed using a two-tailed α level of 0.05 and a statistical power of 80% ($\beta = 0.20$) to detect a significant association between PPI use and reduced serum magnesium levels. Based on these assumptions, the minimum required sample size was estimated to be 186 participants per group, resulting in a total of 372 subjects. This estimation ensured adequate power to detect clinically meaningful differences between PPI users and non-users. To maintain methodological rigor, demographic, clinical, and laboratory data were collected prospectively at the time of enrollment. Medication histories were meticulously reviewed to verify prior PPI exposure and ensure accurate classification into case and control groups.

Data Collection

Comprehensive demographic and clinical data were systematically collected for all enrolled participants using standardized case record forms to ensure consistency and reliability. Patients in the case group had documented outpatient PPI use for at least six months prior to admission. During hospitalization, the mean duration of continued PPI therapy was 19 days, with most patients clustered within 2–3 weeks of treatment and a maximum recorded exposure of seven weeks. The recorded demographic variables included age, sex, and relevant lifestyle factors. Clinical parameters encompassed the presence of comorbid conditions, including but not limited to diabetes mellitus, systemic hypertension, dyslipidemia, chronic kidney disease, and other long-standing systemic disorders that could influence disease progression or therapeutic response. Detailed documentation was also maintained for the admitting clinical diagnosis, baseline laboratory investigations, and concurrent pharmacological therapies to minimize potential confounding effects. In participants with a history of proton pump inhibitor (PPI) use, specific details regarding the prescribed PPI agent, dosage regimen, cumulative duration of therapy, indication for use, and route of administration were meticulously recorded. Where applicable, adherence patterns and any prior modifications to PPI therapy were also assessed to facilitate a more nuanced evaluation of exposure-outcome relationships.

MEASUREMENTS

Assessment of PPI Exposure

Patients were categorized based on documented PPI exposure over the preceding six months or longer, encompassing agents such as omeprazole, pantoprazole, lansoprazole, and esomeprazole. In participants with a history of proton

pump inhibitor (PPI) use, a comprehensive exposure assessment was undertaken through systematic review of medical records, prescription charts, and institutional electronic pharmacy databases. Detailed information was extracted on the specific PPI agent prescribed, indication for therapy, daily dosage, frequency of administration, route of administration, and cumulative duration of use prior to inclusion in the study. For individuals who received more than one course of PPI therapy, cumulative exposure was quantified by summing the total duration of all treatment episodes. To ensure data accuracy, medication histories were cross-verified between patient charts and electronic dispensing records, and discrepancies were resolved by consulting treating physicians or pharmacy logs. The formulation and route of administration—oral or intravenous were recorded for each participant. Oral therapy represented the predominant route of administration, consistent with outpatient and maintenance therapy, whereas intravenous PPI formulations were typically reserved for inpatient use, particularly in cases of acute gastrointestinal bleeding, stress ulcer prophylaxis, or when oral intake was clinically contraindicated. To minimize pharmacologic confounding, concurrent or prior exposure to histamine-2 receptor antagonists (H₂RAs) was systematically evaluated. The timing of the most recent H₂RA prescription was categorized into three exposure windows: recent use (91–180 days before enrollment), remote use (181–365 days before enrollment), or no prior exposure. This temporal classification facilitated differentiation between residual pharmacodynamic effects of H₂RA therapy and true non-exposure, thereby improving the specificity of PPI-related outcome assessment. Venous blood samples (2 mL) were obtained from each participant at the time of admission, prior to initiation of inpatient therapy, for the measurement of serum magnesium concentration and for routine biochemical investigations. Serum magnesium levels were compared between patients with prior PPI exposure and those without, to determine whether sustained acid-suppressive therapy contributes to hypomagnesemia. Secondary analyses examined demographic influences, specifically age and gender-related variations in serum magnesium, and quantified the risk of hypomagnesemia attributable to PPI therapy through adjusted regression modeling. This stratified analytical approach was intended to discern both the direct and confounding effects of prolonged PPI use on electrolyte homeostasis.

Estimation of Serum Magnesium

Serum samples were collected and analyzed by the Department of Biochemistry, the central laboratory of Apollo Institute of Medical Sciences & Research, following standardized protocols to ensure consistency and reliability. Serum magnesium concentration was determined using the **modified methylthymol blue (MTB) colorimetric method**, a validated and widely adopted technique for the quantitative assessment of serum electrolytes (12). The analyses were performed on a **Beckman Coulter AU5800 automated clinical chemistry analyzer** (Beckman Coulter Inc., Brea, CA, USA), which operates on the principle of complex formation between magnesium ions and methylthymol blue, producing

a blue-colored chelate measurable spectrophotometrically at **520 nm**. To ensure analytical precision and accuracy, **internal quality control sera** at both normal and pathological levels were included in each analytical batch, and the analyzer was **calibrated daily** using manufacturer-supplied traceable standards. All measurements were performed in **duplicate**, and the mean of the two readings was used for statistical analyses. Strict adherence to **standard operating procedures** and quality assurance protocols minimized both pre-analytical and analytical variability. For this study, **hypomagnesemia** was defined as a serum magnesium concentration of **<1.8 mg/dL**, in accordance with the institutional reference interval (1.8–2.2 mg/dL). All biochemical estimations were conducted by trained laboratory personnel **blinded to participants' clinical data** to reduce potential measurement bias.

Sensitivity Analysis for Hypomagnesemia Specificity

To evaluate the robustness, specificity, and internal validity of the observed association between proton pump inhibitor (PPI) use and hypomagnesemia, we conducted a comparative sensitivity analysis using prescriptions for histamine H₂ receptor antagonists (H₂RAs) as a reference drug class. H₂RAs were selected because they share similar therapeutic indications with PPIs, but have no established mechanistic or causal relationship with magnesium depletion. By examining serum magnesium levels among H₂RA users, we aimed to ascertain whether the observed effect was specific to PPI exposure or merely reflected a nonspecific association related to acid-suppressive therapy or confounding comorbidities. All statistical models were adjusted for potential confounders exhibiting standardized differences greater than 0.10, including the number of medications dispensed in the year preceding the index date (a validated measure for comorbidity), systemic corticosteroid use within the previous year, any documented history of diabetes mellitus or heart failure during the three years prior to the index date, and the presence of systemic malignancy in the preceding year.

Assessment of covariables

Assessment of covariables in the present study was performed according to the standardized protocols similar to those described by Hoffmans et al.¹³ Diabetes mellitus was defined as a fasting plasma glucose concentration ≥ 126 mg/dL, a nonfasting plasma glucose concentration ≥ 200 mg/dL (if fasting samples were unavailable), or the use of glucose-lowering medication, including oral hypoglycemic agents or insulin. Hypertension was defined as an average systolic blood pressure ≥ 140 mm Hg and/or an average diastolic blood pressure ≥ 90 mm Hg, or the use of antihypertensive medication. Cardiovascular disease, including coronary heart disease and stroke, was ascertained through medical history, clinical examinations, and verification via medical records or linkage with hospital discharge data, and adjudicated by qualified physicians. Chronic liver disease and systemic malignancy were determined based on self-reported physician diagnoses, hospital discharge summaries, and

registry data. Chronic kidney disease was defined using estimated glomerular filtration rate (eGFR) values calculated with the CKD-EPI creatinine equation, with eGFR < 60 mL/min/1.73 m² indicating reduced kidney function.

Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 24.0 (IBM Corp., Armonk, NY, USA). Continuous variables were initially assessed for normality using the Shapiro–Wilk test. Those variables following a normal distribution were summarized as mean \pm standard deviation (SD), while non-normally distributed variables were presented as median with interquartile range (IQR). Intergroup comparisons for normally distributed continuous variables were conducted using the unpaired Student's t-test. In contrast, non-parametric alternatives such as the Mann–Whitney U test were considered for skewed data. Categorical variables were expressed as absolute frequencies and percentages, with differences between groups evaluated using the chi-square (χ^2) test or Fisher's exact test when expected cell counts were less than five. To examine the independent association between PPI use and the occurrence of hypomagnesemia, multivariate logistic regression analysis was performed, adjusting for potential confounding factors including age, sex, comorbidities, and concurrent medication use. The results of the logistic regression were presented as odds ratios (OR) along with 95% confidence intervals (CI) to quantify the strength and precision of associations. All statistical tests were two-tailed, and a p-value of < 0.05 was considered indicative of statistical significance. Model goodness-of-fit was assessed using the Hosmer–Lemeshow test, and multicollinearity among independent variables was evaluated through variance inflation factors (VIFs) to ensure the robustness of the regression model.

Ethical Considerations

The study protocol was reviewed and approved by the Institutional Ethics Committee of Apollo Institute of Medical Sciences & Research (AIMSR), Hyderabad, India (Approval No: EC/NEW/INST/1527/2023/09/142). The study was conducted in accordance with the Declaration of Helsinki and applicable Good Clinical Practice (GCP) guidelines. Written informed consent was obtained from all participants prior to enrollment, and patient confidentiality was maintained throughout the study.

RESULTS

Baseline Characteristics

The study included 370 participants: 185 PPI users (cases) and 185 non-users (controls). The mean age was comparable between groups (cases: 50.4 ± 13.2 years; controls: 47.9 ± 15.0 years; $p = 0.10$). Gender distribution was also similar (male: 56.2% vs. 57.3%; $p = 0.83$) (Table 1).

Table 1. Characteristics and inpatient duration of proton pump inhibitor (PPI) use among cases (n = 185)

Variable	Value (%)
Patients under active treatment with PPIs, n	185/370 (50.0%)
Specific PPI used, n	
– Pantoprazole	112/185 (60.5%)
– Lansoprazole	38/185 (20.5%)
– Omeprazole	24/185 (13.0%)
– Esomeprazole	11/185 (6.0%)
Treatment duration in hospital*	
– Mean ± SD (days)	19.03 ± 6.17
– Median (days)	17
– Mode (days)	14
– Range (days)	14 – 49
Daily dose, mg† (mean ± SD)	
– Pantoprazole	40.2 ± 8.6
– Lansoprazole	28.7 ± 6.4
– Omeprazole	27.5 ± 7.9
– Esomeprazole	22.0 ± 5.3
Route of administration, n†	
– Oral	166/185 (89.7%)
– Intravenous	19/185 (10.3%)

Among the 185 patients receiving PPIs, pantoprazole was the most frequently prescribed agent, followed by lansoprazole, omeprazole, and esomeprazole. The majority of patients were treated orally, with intravenous use reserved for a minority. The average inpatient duration of therapy was approximately 19 days, with most patients clustered around 2–3 weeks of treatment and a maximum observed duration of seven weeks. Mean daily doses for each PPI were within standard therapeutic ranges, indicating that hypomagnesemia in this cohort was not attributable to excessive dosing but rather to the chronicity and continued use of therapy (Figure 1).

Sensitivity Analysis for Hypomagnesemia Specificity

Table 2. Association between timing of recent H₂RA use and case–control status

Timing of Most Recent H ₂ RA Prescription (a)	Case Patients (n = 185)	Control Patients (n = 185)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI) (b)
≤ 90 days (Current use)	15 (8.1%)	20 (10.8%)	0.72 (0.35–1.49)	0.84 (0.38–1.83)
91–180 days (Recent use)	8 (4.3%)	10 (5.4%)	0.79 (0.31–1.99)	0.91 (0.33–2.52)
181–365 days (Remote use)	6 (3.2%)	9 (4.9%)	0.64 (0.23–1.80)	0.76 (0.25–2.27)
No H ₂ RA exposure	156 (84.3%)	146 (78.9%)	-	-

Hypomagnesemia was defined as serum magnesium < 1.8 mg/dL (institutional reference range 1.8–2.2 mg/dL). (a) Includes Pantoprazole, Lansoprazole, Omeprazole, Esomeprazole.

Adjusted model controlled for age, sex, diabetes mellitus, heart failure, systemic malignancy, and concomitant corticosteroid use. PPI use was defined as therapy for ≥ 5 days per week for ≥ 6 months prior to hospitalization.

In the sensitivity analysis using histamine H₂ receptor antagonist (H₂RA) exposure as a comparator, no significant association was observed between H₂RA use and hypomagne-

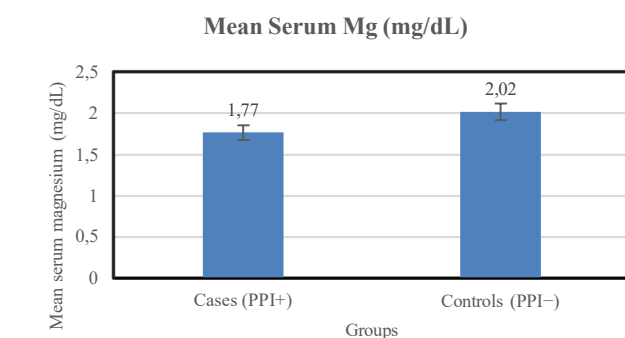


Figure 1. Mean serum magnesium values by PPI users

semia across any exposure window. The adjusted odds ratios for current, recent, and remote H₂RA use were 0.84 (95% CI: 0.38–1.83), 0.91 (95% CI: 0.33–2.52), and 0.76 (95% CI:

0.25–2.27), respectively, all of which crossed unity, indicating a lack of statistically significant effect. These findings suggest that the association between proton pump inhibitor (PPI) therapy and reduced serum magnesium levels is not a class effect of acid-suppressive drugs in general. The absence of a similar relationship with H₂RA exposure reinforces the specificity and internal validity of the observed link between

chronic PPI use and hypomagnesemia, supporting a plausible mechanistic basis unique to PPIs rather than confounding by indication or comorbid conditions.

Association of Demographics and Magnesium Levels with PPI Use

Table 3. Characteristics of patients enrolled as cases and controls

Variable	Category / Statistic	Cases (PPI+) (n = 185)	Controls (PPI-) (n = 185)	χ^2 / t value	p-value
Demographics					
Age (years), mean \pm SD		53.8 \pm 13.4	52.6 \pm 12.9	0.89 (t)	0.37
Age group	18–30 yr	14 (7.6%)	29 (15.7%)	5.92	0.052
	31–50 yr	75 (40.5%)	68 (36.8%)	—	—
	\geq 51 yr	96 (51.9%)	88 (47.6%)	—	—
Sex	Male	104 (56.2%)	106 (57.3%)	0.04	0.84
	Female	81 (43.8%)	79 (42.7%)	—	—
Comorbidities					
Diabetes mellitus	Yes	68 (36.8%)	61 (33.0%)	0.50	0.47
Hypertension	Yes	74 (40.0%)	70 (37.8%)	0.17	0.68
Cardiovascular disease	Yes	28 (15.1%)	23 (12.4%)	0.52	0.47
Chronic liver disease	Yes	11 (5.9%)	9 (4.9%)	0.14	0.71
Chronic kidney disease	Yes	8 (4.3%)	7 (3.8%)	0.07	0.79
Systemic malignancy	Yes	5 (2.7%)	4 (2.2%)	0.10	0.76
Clinical Characteristics					
Type of admission	Medical	121 (65.4%)	118 (63.8%)	0.08	0.78
	Surgical	36 (19.5%)	41 (22.2%)	0.35	0.55
ICU admission	Yes	14 (7.6%)	10 (5.4%)	0.64	0.42
QTc-prolonging drugs	Yes	27 (14.6%)	21 (11.4%)	0.83	0.36
Systemic corticosteroid use	Yes	19 (10.3%)	14 (7.6%)	0.67	0.41
Number of concomitant medications (median (IQR)) (a)	7 (5–10)	5 (3–8)	3.45 (t)	0.001*	
Biochemical Parameters					
Serum magnesium (mg/dL), mean \pm SD		1.77 \pm 0.48	2.02 \pm 0.43	5.63 (t)	<0.001*
Serum magnesium category	< 1.8 mg/dL	101 (54.6%)	48 (25.9%)	32.00	<0.001*
	1.8–2.2 mg/dL	52 (28.1%)	91 (49.2%)	—	—
	> 2.2 mg/dL	32 (17.3%)	46 (24.9%)	—	—

(a) Number of distinct drugs is a surrogate marker for comorbidity.

Hypomagnesemia defined as serum magnesium < 1.8 mg/dL.

Significance threshold: $p < 0.05$; statistically significant results marked with *

Serum magnesium estimated by the modified methylthymol blue colorimetric method

Baseline demographic and clinical characteristics were comparable between the PPI user group (n = 185) and the non-user control group (n = 185). The mean age of participants did not differ significantly between groups (53.8 \pm 13.4 vs. 52.6 \pm 12.9 years; $p = 0.37$), and the sex distribution was nearly identical (males: 56.2% vs. 57.3%; $p = 0.84$). Similarly, the prevalence of major comorbid conditions, including diabetes mellitus, hypertension, cardiovascular disease, chronic liver disease, chronic kidney disease, and systemic

malignancy, did not show statistically significant differences between cases and controls ($p > 0.05$ for all). However, PPI users had a significantly higher number of concomitant medications compared with non-users (median (IQR): 7 (5–10) vs. 5 (3–8); $p = 0.001$), suggesting a greater overall drug burden among PPI-treated patients. Other clinical parameters, such as type of admission, ICU stay, corticosteroid exposure, and use of QTc-prolonging agents, were not significantly different between groups. (Table 3) The close similarity in

demographic and comorbidity profiles between PPI users and controls strengthens the internal validity of the observed association between PPI therapy and hypomagnesemia by minimizing potential confounding factors. The significantly higher polypharmacy rate among PPI users may partially contribute to altered electrolyte balance, but does not independently explain the magnitude of magnesium depletion observed. Thus, the findings reinforce that reduced serum magnesium levels are more likely attributable to chronic PPI exposure rather than differences in underlying disease burden or demographic characteristics. No significant association was observed between PPI use and age category ($p = 0.05$) or gender ($p = 0.83$). In contrast, hypomagnesemia (<1.8 mg/dL) was significantly more common in PPI users (54.6%) compared with controls (25.9%; $p < 0.001$) (Table 3).

Relationship of PPI Use to Serum Magnesium

Use of a PPI was associated with a markedly lower serum magnesium concentration relative to non-use. Participants receiving PPIs demonstrated a mean serum magnesium level of 1.77 ± 0.48 mg/dL, whereas controls exhibited substantially higher levels (2.02 ± 0.43 mg/dL; $t = 5.63$, $p < 0.001$), indicating a clear negative shift in magnesium homeostasis attributable to PPI exposure. When evaluated categorically, this pattern became more pronounced. Over half of the PPI (54.6%) presented with hypomagnesemia (< 1.8 mg/dL), a prevalence more than double that of individuals not exposed to PPIs (25.9%), and this association was highly significant ($\chi^2 = 32.00$, $p < 0.001$). (Table 2) Correspondingly, normal and high-normal magnesium values (≥ 1.8 mg/dL) were more frequently observed among controls, suggesting that PPI administration not only reduces mean magnesium levels but

also shifts the overall population distribution toward deficiency. These findings are consistent with the biological plausibility that PPIs impair intestinal magnesium absorption through reduced active transport in the gut, a mechanism that has been proposed in prior clinical and experimental research. The magnitude of reduction observed in this cohort reflects a clinically meaningful disturbance in electrolyte balance, reinforcing concerns regarding long-term PPI therapy. Taken together, the data strongly support a robust association between PPI exposure and diminished serum magnesium levels, stressing the need for routine monitoring—particularly in patients receiving prolonged therapy or those with additional risk factors for electrolyte depletion.

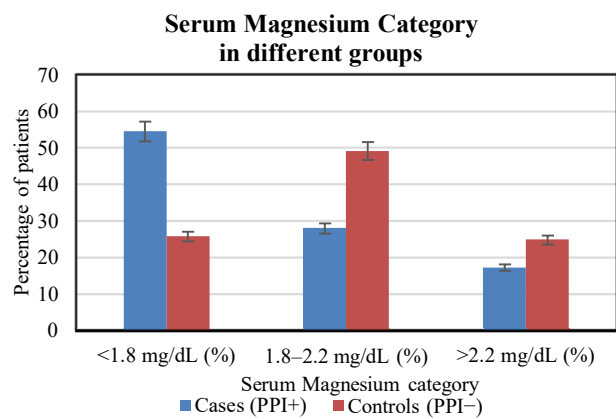


Figure 2. Serum Magnesium category in different groups

Regression analysis of serum magnesium levels

Table 4. Regression analysis and distribution of serum magnesium levels in cases (PPI users) and controls.

Variable	Category	Unadjusted OR	Adjusted OR	95% CI (Lower–Upper)	p-value	Serum Magnesium Levels (%)	Total (%)
Age	—	1.002	1.000	0.985–1.015	0.986	—	—
Gender	Male	1 (ref)	1 (ref)	—	—	—	—
	Female	1.82	1.94	0.773–1.845	0.424	—	—
Group	Control	1 (ref)	1 (ref)	—	—	<1.8 = 25.9 1.8–2.2 = 49.2 >2.2 = 24.9	100
	Cases	2.145	2.475	1.605–3.816	<0.001*	<1.8 = 54.6 1.8–2.2 = 28.1 >2.2 = 17.3	100
Overall Total	—	—	—	—	—	<1.8 = 40.3 1.8–2.2 = 38.6 >2.2 = 21.1	100

“Unadjusted OR” represents crude odds ratios before accounting for confounders. “Adjusted OR” includes adjustments for variables like age, sex, comorbidities, and concurrent medication use. The p-value marked with * (<0.001 *) indicates statistical significance.

Regression analysis (Table 4) demonstrated that PPI use was a significant independent predictor of hypomagnesemia, with cases showing 2.475-fold higher odds of reduced serum magnesium compared to controls (95% CI: 1.605–3.816, $p < 0.001$). Neither age nor gender showed a significant

association with magnesium status ($p > 0.05$). Distribution analysis further highlighted this relationship, as more than half of PPI users (54.6%) exhibited serum magnesium <1.8 mg/dL, in contrast to only 25.9% of non-users, while normal magnesium levels (1.8–2.2 mg/dL) were more frequently

observed in controls (49.2% vs. 28.1%). These findings indicated a robust association between chronic PPI use and hypomagnesemia, whereas demographic variables such as age and gender were not contributory.

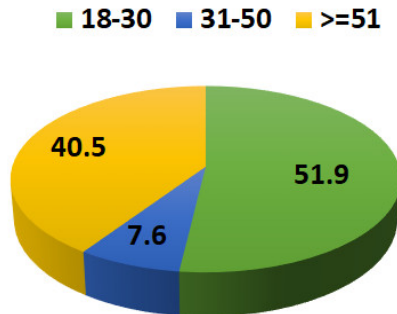


Figure 3. Frequency Distribution (%) of ages in cases

Fig 3 PPIs were most commonly used in the age group ≥ 51 (51.9%), followed by 30 -50 (40.5%) and 18-31 years (7.6%).

DISCUSSION

In the present study, we investigated the association between chronic proton pump inhibitor (PPI) therapy and alterations in serum magnesium concentrations. Among the 185 patients receiving PPIs, pantoprazole emerged as the most commonly prescribed agent, followed sequentially by lansoprazole, omeprazole, and esomeprazole. The administered daily doses for all PPI formulations were within standard therapeutic limits, stressing that the observed hypomagnesemia was unlikely to result from suprathreshold exposure, and it appears to be a consequence of prolonged and continuous PPI administration. Emerging clinical evidence consistently supports this duration-dependent relationship. Several observational and mechanistic studies have demonstrated that hypomagnesemia is significantly associated with PPI use extending beyond six months, even when dosing remains within recommended ranges (14). Furthermore, systematic reviews have reported that long-term PPI exposure can precipitate not only isolated magnesium depletion, but also secondary electrolyte imbalances, such as hypocalcemia and hypokalemia, reinforcing the concept that chronic therapy rather than excessive dosage is the principal determinant of these metabolic derangements (15). Baseline comparisons between PPI users and non-users revealed no significant differences in age distribution, sex, comorbid conditions, or type of hospital admission, and the concomitant use of QTc-prolonging medications was similarly balanced across groups. However, serum magnesium levels showed a striking disparity: more than half of PPI users (54.6%) demonstrated hypomagnesemia (< 1.8 mg/dL) compared with only 25.9% of non-users ($p < 0.001$). This finding aligns with recent research of Seah et al., who reported that nearly half of patients with severe hypomagnesemia had PPI use implicated as a contributing cause, with higher risk in those on high doses, with renal impairment, diabetes, or low BMI (16). Similarly, a study from Pakistan found hypomagnesemia in 51.5% of

chronic PPI users, with longer duration, hypertension, and diabetes being independent predictors (17). Conversely, normal magnesium levels (1.8 - 2.2 mg/dL) were more prevalent among controls (49.2% vs. 28.1%). The mean serum magnesium concentration in cases (1.77 ± 0.49 mg/dL) was significantly lower than that of controls (2.02 ± 0.43 mg/dL, $p < 0.001$). Regression analysis further confirmed that PPI use was an independent predictor of hypomagnesemia, conferring a 2.475-fold increased risk compared with controls (95% CI: 1.605–3.816, $p < 0.001$). Neither age nor gender demonstrated a significant association with serum magnesium status ($p > 0.05$), indicating that the observed effect was specifically attributable to PPI exposure rather than demographic variables. These findings align with prior evidence that PPI-induced hypomagnesemia can occur within a relatively short period, as early as two weeks. However, cases have also been documented after several years of therapy. A reported cross-sectional study found that hypomagnesemia was associated with PPI use irrespective of the type of PPI, and demographic factors such as gender did not significantly modify serum magnesium levels after adjustment for other risk factors (18). Analysis of a Japanese adverse-drug-event database indicated that although male sex and age under 60 were risk factors, age and sex did not consistently show a strong effect across all PPI users, reinforcing the idea that hypomagnesemia risk is driven more by drug exposure than by demographic profile alone (19). Moreover, hypomagnesemia had been shown to resolve following PPI withdrawal and to recur upon re-challenge, regardless of the PPI type. Clinically, severe hypomagnesemia may manifest with convulsions, arrhythmias such as bradycardia, hypotension, or even fatal outcomes. Indeed, in patients recovering from acute kidney injury, prolonged renal magnesium loss has led to generalized seizures due to very low serum magnesium levels (20). Similarly, combined hypomagnesemia and hypokalemia have been shown to produce QT prolongation and torsades de pointes in the setting of drug-induced electrolyte disturbances, which, if not promptly treated, may culminate in fatal arrhythmias (21). Although oral magnesium supplementation is commonly employed, it does not consistently restore serum magnesium concentrations in patients affected by hypomagnesemia associated with PPI therapy. A recent case report demonstrated that even with oral replacement, hypomagnesemia recurred as long as the PPI was continued—levels normalized only after the PPI was withdrawn (22). This stresses the importance of PPI withdrawal as the cornerstone of management. For individuals requiring continued acid suppression, alternative strategies such as substitution with H₂ receptor antagonists may offer partial benefit. Observational data in hemodialysis patients showed that users of H₂ receptor antagonists have higher mean serum magnesium levels compared with PPI users, suggesting that switching to H₂ antagonists may mitigate risk (23). Our study results provided strong evidence that PPI use is significantly associated with hypomagnesemia, independent of age, sex, or comorbid burden, reinforcing the importance of routine monitoring of serum magnesium in patients on long-term therapy.

CONCLUSION

Proton pump inhibitors (PPIs) constitute a widely prescribed class of medications with an established safety profile when administered for short durations. However, prolonged use has been increasingly associated with hypomagnesemia, a clinically relevant adverse effect. Although interindividual variation among specific PPI formulations appears minimal, sustained therapy necessitates careful monitoring. It is imperative for clinicians to periodically assess serum magnesium concentrations in patients undergoing chronic PPI treatment. In such cases, therapeutic strategies may include dosage adjustment, dietary counseling to enhance magnesium intake, or supplementation as warranted. Continuous clinical vigilance is essential to prevent, detect, and effectively manage hypomagnesemia, particularly in patients predisposed to electrolyte imbalances.

AUTHOR CONTRIBUTIONS

All authors accept full responsibility for every aspect of the work. Each made substantial contributions to the study's conception and design, data acquisition, or analysis and interpretation. All authors participated in drafting the manuscript or critically revising it for important intellectual content, approved the final version for publication, and consented to submission to this journal. Authorship was determined in accordance with the criteria and guidelines of the International Committee of Medical Journal Editors (ICMJE).

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The author reports no financial or any other conflicts of interest in this paper.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used for writing or editing the manuscript, and that none of the images were modified or generated using AI.

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TOPICAL *HELICHRYSUM ITALICUM* ESSENTIAL OIL-BASED OINTMENT HASTENS EXCISIONAL WOUND HEALING BY ALLEVIATING INFLAMMATION AND OXIDATIVE STRESS IN DIABETIC RATS

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ABSTRACT

This study aimed to develop novel topical semi-solid formulation based on Helichrysum italicum (H. italicum) essential oil and to assess its wound-healing capacity through macroscopic and biochemical examination of markers of inflammation and oxidative stress. Methods: The wound-healing effect was evaluated on an excision wound model. The study was carried out on male Wistar albino rats with induced diabetes type 1. Diabetic rats were randomly divided into four groups: control (CTRL), 1% silver sulfadiazine (SSD), ointment base (OINT), and HIEO OINT (H. italicum essential oil-based ointment). The investigated formulations were applied once daily, for three weeks. The formulation containing H. italicum essential oil has shown no changes in color, smell, consistency, or homogeneity during the storage period at room temperature for six months. Three-week administration of HIEO ointment led to a significant reduction in wound size and the percentage of wound contraction was the highest in HIOE point group. A significant increase in wound contraction percentage was observed from day 7. The treatment with HIEO ointment significantly reduced tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6), and enhanced IL-10 levels. Additionally, a three-week administration of HIEO ointment elevated antioxidative enzymes detected in wound tissue. Our findings showed that topical application of HIEO ointment showed immense potential in augmenting skin wound regeneration in diabetes rats by upregulating the antioxidant status as well as modulating cytokines.

Keywords: *Helichrysum italicum, essential oil, wound, inflammation, oxidative stress.*

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INTRODUCTION

Wound healing is the body's natural response to tissue injury. It represents a complex and dynamic biological process consisting of integrated cellular and biochemical cascades, which lead to the re-establishment of the structural and functional integrity of the damaged tissue (1). The healing process consists of four temporally and spatially overlapping phases: hemostasis, inflammation, proliferation, and remodeling (2, 3). Different factors are associated with a compromised capacity to promote the regeneration of damaged tissue and, consequently delayed wound healing. Factors affecting the healing process can be categorized into local and systemic. Local factors are factors that directly affect the characteristics wound, while systemic factors represent the individual's health condition, which affects the ability to heal. There is an association of several factors and systemic factors act through local effects that affect wound healing (4).

One of the major contributors to delayed and consequently chronic wound healing problems is diabetes mellitus (DM). Prolonged wound healing in patients with DM occurs due to a delayed, incomplete and uncoordinated healing process, which permeates all phases, from inflammation to remodeling (5). As a result, people with diabetes can develop a variety of complications, including chronic wounds that do not heal, such as foot ulcers (6).

Considering the huge impact of chronic diabetic wounds on the economy and society, there is an urgent need to develop new therapeutic strategies that can contribute to proper wound healing and exhibit as few side effects as possible. Although various therapeutic protocols are available for wound healing, including antibiotics, antiseptics, and anti-inflammatory agents, medicinal herbs and natural products have been the main source of wound healing agents in recent years. The significant increase in the use of medicinal plants is the result of greater safety compared to synthetic drugs, but also more affordable prices (7).

Helichrysum italicum (*H. italicum*) is a perennial, aromatic plant belonging to the *Asteraceae* family. *H. italicum*, commonly known as "immortelle," is renowned for its therapeutic properties and has been traditionally used in folk medicine for its wound-healing abilities (8, 9). The essential oil derived from *H. italicum* contains a complex mixture of bioactive molecules, including monoterpenes and sesquiterpenes, which have demonstrated potent anti-inflammatory, antioxidant, and antimicrobial actions (10, 11). These properties make *H. italicum* essential oil a promising ingredient for topical preparations developed to enhance wound healing efficacy, particularly in diabetic conditions where inflammation and oxidative stress are heightened.

Therefore, this investigation was designed to develop and characterize *H. italicum* essential oil-based topical semi-solid dosage form and evaluate wound-healing properties by performing a biochemical analysis in diabetic animal model.

MATERIALS AND METHODS

H. italicum essential oil obtaining

H. italicum essential oil was purchased from "Alekhpharm", Belgrade, Serbia. This commercial essential oil was obtained by hydrodistillation from flowering parts of *H. italicum*.

Composition and preparation of semi-solid product: ointment

For the formulation of the ointment, as a semi-solid pharmaceutical dosage form, cholesterol, lanolin, paraffinum liquidum, and vaselinum album were used to form an ointment base. The *H. italicum* essential oil-based ointment was prepared by incorporating 0.5% (w/w) essential oil *H. italicum* into the ointment base by levigation method (12). The formulation is presented in Table 1.

Table 1. Ointment base and *H. italicum* essential oil –based ointment formulations.

Ingredient	Ointment Base (%)	Ointment HIEO (%)
HIEO	/	0.50
Cholesterol	5.00	5.00
Lanolin	15.00	15.00
Parafinum liquidum	15.00	15.00
Vaselinum album	65.00	ad 65.00

HIEO – *H. italicum* essential oil

Assessment of physical properties of ointment containing *H. italicum* essential oil

Organoleptic properties

Organoleptic properties, which include examination of the appearance, odour, color, consistency, homogeneity and spreadability of the prepared ointment with incorporated *H. italicum* essential oil, were assessed by visual observation. Homogeneity was tested by pressing a small amount of the formulation between the thumb and forefinger (13). Spreadability was assessed by applying preparation in circular motions on the skin and visually monitoring the trace that remains on the skin.

Assessment of long-term stability of the H. italicum essential oil-based ointment

The long-term stability of the *H. italicum* essential oil-based ointment was assessed by determination of organoleptic characteristics and spreadability during the storage period. The ointment was stored at room temperature ($22^{\circ} \pm 2^{\circ}\text{C}$) for 6 months and the sampling was conducted after 6 months of ointment storage.

Wound healing effects of ointment with *H. italicum* essential oil in diabetic rats

Ethical statement

This investigation was conducted in the Laboratory for pharmaceutical technology and Center for experimental and preclinical investigations of the Faculty of Medical Sciences, University of Kragujevac, Serbia. The part of study involving animals was performed in accordance with the the European Directive for Protection of the Vertebrate Animals used for Experimental and Other Scientific Purposes 86/609/EES and the principles of Good Laboratory Practice. Ethical committee for the welfare of laboratory animals of the Faculty of medical sciences, University of Kragujevac was approved the experimental protocol (Number 01-6292).

Animals

Male *Wistar albino* rats (280–350 g) were obtained from the Military Medical Academy, Belgrade, Serbia and housed in clean cages under adjusted conditions (12:12 h light–dark cycle, at a temperature of 22 ± 2 °C). The rats had ad libitum access to water and food.

Diabetes mellitus induction

Diabetes mellitus type 1 was induced with a single injection of streptozotocin in a dose of 50 mg/kg. Streptozotocin was dissolved in 1 ml of 0.05 M freshly prepared citrate buffer solution (pH 4.5) and administrated by intraperitoneal injection after a 12 h starvation (14). Seventy-two hours after streptozotocin administration diabetes was confirmed by measuring tail-veinous blood glucose level using a portable glucometer. Rats with blood glucose levels >11.1 mmol/l were included in the investigation.

Excision wound model

One week after confirmation of diabetes type 1, excision wounds were created (15). First of all, the rats were anesthetized by intraperitoneal injection of the mixture of ketamine and xylazine in a dose of 5 mg/kg and 10 mg/kg respectively. The dorsal of animals were shaved, disinfected with 70% ethanol and wounds were created by scissors and a scalpel. The dimension of the wound was 2x2 cm.

Groups and treatment

Immediately after the creation of excision wound, animals were photographed and placed in individual cages. Animals were randomly divided into the following groups ($n = 8$ per group):

1. Negative control group - animals without treatment (CTRL);
2. Positive control group - animals treated with a commercially available ointment 1% silver sulfadiazine (SSD);
3. Animals treated with an ointment base (OINT);

4. Animals treated with a 0.5% *H. italicum* essential oil based ointment (HIOINT).

The formulations were applied once daily in an amount of 0.5 g by sterile cotton for three weeks (16). At the end of the experimental protocol, the rats were anesthetized with ketamine and xylazine mixture and sacrificed by decapitation.

Wound contraction

The evolution of the wounds was followed every one week by measuring the areas in the treated animals (in cm²). The percentage of wound contraction was calculated for each animal on various days by using following formula (17):

$$\% \text{ Wound contraction} = \frac{[(\text{Initial WA} - \text{Specific day WA}) / \text{Initial WA}] * 100}{\text{where is: WA} - \text{wound area}}$$

Tissue collection

After sacrifice, samples of the rat's skin were isolated and stored at -80°C for further biochemical analysis. Wound tissues were taken to estimate the oxidative status and markers of inflammation.

Markers of inflammation

A sample of skin tissue (100 mg) was homogenized with 0.5 mL of cell lysis buffer. The obtained homogenates were centrifuged at 5000 rpm, for 10 minutes, at a temperature of 4°C. The obtained supernatants were stored at a temperature of -70°C.

Determination of the concentration of inflammation markers TNF- α , IL-6, and IL-10 in the supernatant obtained from skin tissue was carried out via ELISA kit according to the instructions.

Markers of oxidative stress

The wound tissue sample was homogenized in cold PBS (phosphate-buffered saline) (1:10). The obtained homogenates were centrifuged at $1200 \times g$ for 10 minutes at a temperature of 4°C and the obtained supernatants were stored at a temperature of -70°C.

The parameters of the tissue redox state were measured from obtained supernatants according to previously described protocol (18). The supernatant was used for the determination of the pro-oxidative parameter index of lipid peroxidation (measured as TBARS). Additionally, we determined the parameters of the antioxidative defense system such as level of reduced glutathione (GSH), and the activity of antioxidant enzymes: catalase (CAT), and superoxide dismutase (SOD).

Statistical analysis

IBM SPSS Statistics 20.0 Desktop for Windows was used for statistical analysis. All data were expressed as mean \pm

standard deviation. The distribution of data was assessed by the Shapiro–Wilk test. For the normal distribution, the data were assessed via a one-way ANOVA followed by Tukey’s multiple comparison post hoc tests. When the distribution was different from than normal comparison between groups was assessed by the Kruskal–Wallis test. A p-value < 0.05 was considered statistically significant.

RESULTS

Organoleptic characteristics

Physico-chemical characterization of the ointment with incorporated *H. italicum* essential oil was carried out by organoleptic testing and spreadability testing. Organoleptic examination revealed that the formulation with *H. italicum* essential oil has a characteristic appearance of homogeneous ointment, without lumps, yellow color and intense odor. The preparation has a fine, uniform, semi-solid consistency (Figure 1). In terms of spreadability, it was observed that the examined ointment spreads well on the skin. During the storage period at room temperature for six months, no changes in color, smell, consistency, or homogeneity of the ointment were observed (Table 2).

Table 2. Organoleptic characteristics and spreadability of ointment with *H. italicum* essential oil after 3 and 180 days of preparation

Parameters	3 days	180 days
Colour	Yellow	Yellow
Odour	Characteristic odor of the essential oil	Characteristic odor of the essential oil
Consistency	Semi-solid	Semi-solid
Homogeneity	Homogeneous ointment	Homogeneous ointment
Spreadability	Easily	Easily

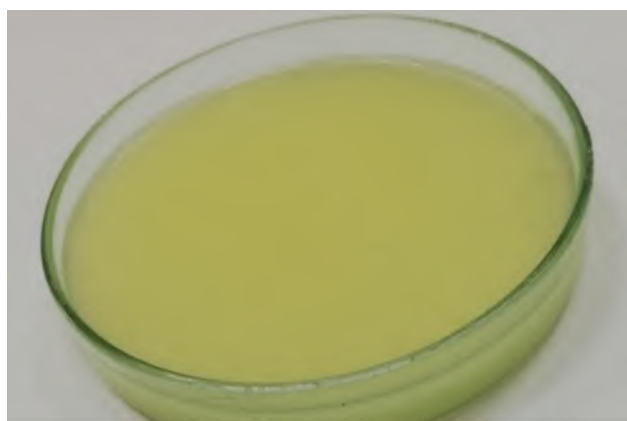


Figure 1. Photograph of *H. italicum* essential oil – based ointment

Wound healing effects

Wound contraction

The progress of wound-healing contraction, examined over the three weeks is shown in Figure 2. The results have shown significant differences in the wound closure rate between the group treated with *H. italicum* ointment and the other examined groups. Therefore, these data indicated that *H. italicum* essential oil formulated in the form of ointment possesses wound healing activity. The results of wound contraction, represented in Figure 2 point out that topical application of *H. italicum* ointment resulted in a significant increase in the percent of wound contraction compared to the control group, on all key days of the duration of the experiment. Additionally, three-week administration of *H. italicum* ointment resulted in a significant increase in wound contraction compared to the group treated with an ointment base. On the other side, the wound contraction in the group was slightly higher compared to the SSD group, but not significantly.

Markers of inflammation

The concentrations of pro-inflammatory markers TNF- α and IL-6, as well as anti-inflammatory IL-10, in wound tissue homogenate are shown in Figure 3. The highest concentration of the pro-inflammatory mediator TNF- α was detected in the control group. Topical administration of ointments has caused a significant reduction in the concentration of this parameter in all observed groups compared to the control group (Figure 3A).

At the same time, the three-week application of *H. italicum* essential oil-based ointment significantly reduced the concentration of IL-6 compared to the control and ointment groups. On the other side, the concentration of IL-6 was almost identical compared to the SSD group (Figure 3B).

Additionally, the statistically highest concentration of the anti-inflammatory marker, IL-10, was observed in the group of animals treated with the *H. italicum* essential oil-based ointment (Figure 3C).

Markers of oxidative stress

The results of the oxidative status in the wound tissue homogenate created by the excision method are shown in Figure 4. Topical treatment with *H. italicum* essential oil-based ointment significantly increased the activity of SOD relative to all examined groups. On the other hand, the activity of CAT was remarkably increased in the SSD group, comparing the values of this enzyme in groups treated with vehicle or *H. italicum* ointment. Neither treatment made a difference in the level of GSH and TBARS.

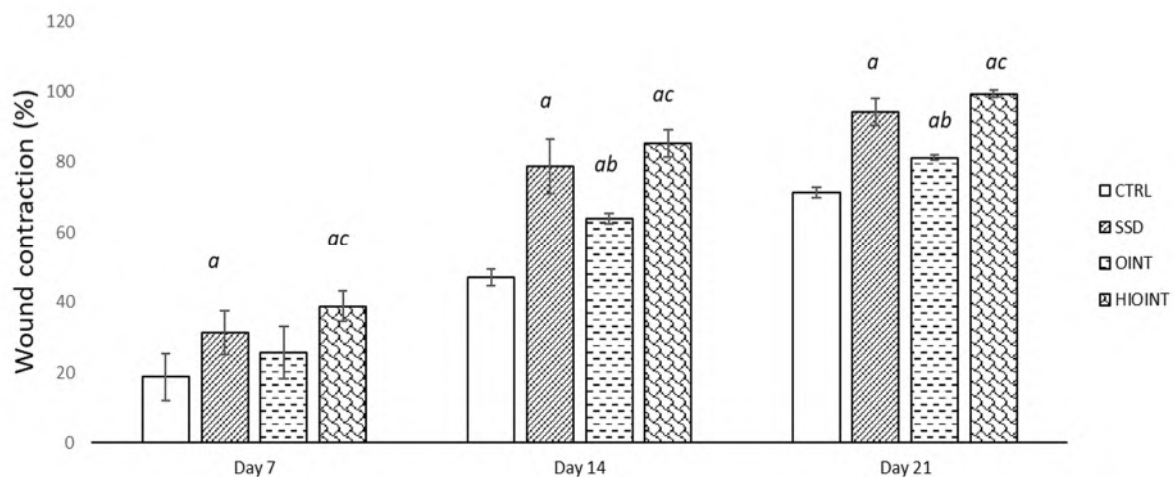


Figure 2. Effects of applied ointments on the wound contraction.

Values are expressed as mean \pm standard deviation (n = 8). ^ap < 0.05 compared to CTRL group;

^bp < 0.05 compared to SSD group; ^cp < 0.05 compared to OINT group;

^dp < 0.05 compared to HIOINT group. CTRL – control; SSD – silver sulfadiazine;

OINT – ointment base;

HIOINT – *H. italicum* ointment;

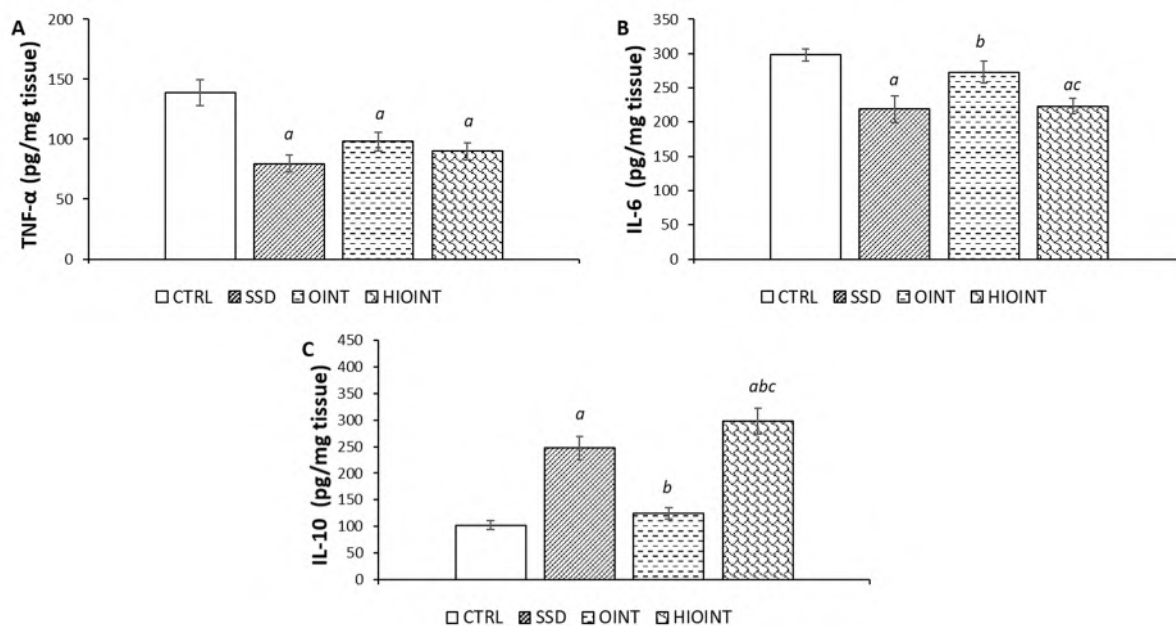


Figure 3. Effects of applied ointments on the markers inflammation:

A) TNF- α ; B) IL-6; C) IL-10. Values are expressed as mean \pm standard deviation (n = 8).

^ap < 0.05 compared to CTRL group; ^bp < 0.05 compared to SSD group; ^cp < 0.05 compared to OINT group;

^dp < 0.05 compared to HIOINT group. CTRL – control; SSD – silver sulfadiazine;

OINT – ointment base; HIOINT – *H. italicum* ointment;

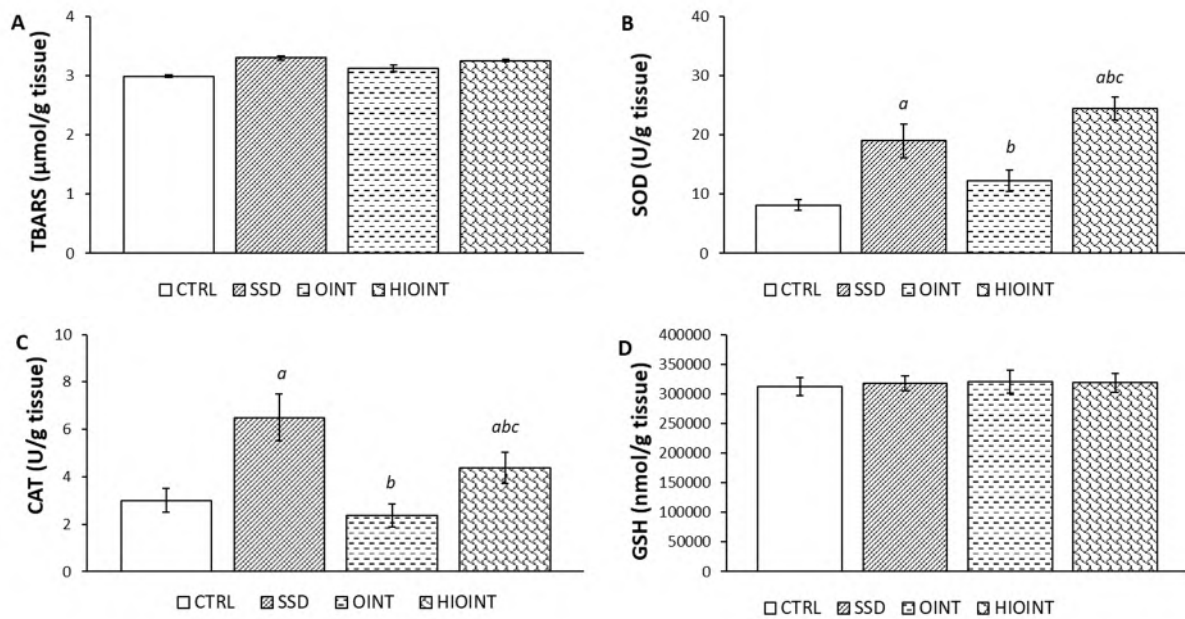


Figure 4. Effects of applied ointments on the oxidative stress markers in wound tissue:

A) TBARS; B) SOD; C) CAT; D) GSH. Values are expressed as mean \pm standard deviation ($n = 8$).

^a $p < 0.05$ compared to CTRL group; ^b $p < 0.05$ compared to SSD group; ^c $p < 0.05$ compared to OINT group;

^d $p < 0.05$ compared to HIOINT group. CTRL – control; SSD – silver sulfadiazine;

OINT – ointment base; HIOINT – *H. italicum* ointment;

DISCUSSION

Over the years, the use of synthetic preparations has been in drastic decline, primarily due to their close association with the occurrence of serious side effects. As a result, their unsafe use has enabled preparations based on natural products to regain their popularity and gain great interest from the scientific public in the prevention and treatment of various conditions. Although the wound-healing activity of immortelle extracts and their ingredients is well known, there is almost no data on the effects of different topical preparations based on immortelle essential oil. Accordingly, the first part of this investigation was to formulate semi-solid topical preparation containing *H. italicum* essential oil and to determine its stability during six months of storage on the room conditions.

Literature data suggest that a concentration of immortelle essential oil of 0.5% is optimal and suitable for topical application and that its increase is not recommended due to the possibility of skin irritation (19). Accordingly, immortelle essential oil was incorporated in a concentration of 0.5% into the ointment base to obtain immortelle-based ointment. In order to assess the physical stability of the prepared semi-solid formulation, the ointment sample was stored for six months at a room temperature of $22 \pm 0.1^\circ\text{C}$, the parameters of interest were monitored in the following time points: at the beginning and end of the storage period.

Within the organoleptic characteristics, changes in color, smell, consistency and homogeneity were monitored over a period of six months. The results of the six-month follow-up indicate that there were no changes in the organoleptic properties of the tested formulation. From the aspect of physical stability, the absence of change in color, smell, consistency and homogeneity is an indicator of the preliminary stability of the investigated immortelle ointment.

Determining spreadability, as an index of ease of application, is one of the necessary methods before placing the preparation on the market, considering that it is necessary for semi-solid products for local application to be easily applied to the surface of the skin. Spreadability greatly affects the effectiveness of topical therapy and is an important characteristic responsible for the delivery of the active substance to the target site, ease of application and most importantly, the needs of the user (20). So, we monitored the spreadability of our preparations over time, which remained unchanged, which is an additional confirmation of the stability of semi-solid preparation based on immortelle. Bearing in mind the percentage of immortelle essential oil in the mentioned formulation, it can be concluded that the ability to spread is mainly influenced by the substrates used for the production of ointments.

After confirmation of the stability of *H. italicum* essential oil-based ointment, we investigated the effects of developed ointment on the wound-healing process in diabetic rats with special emphasis on the markers of inflammation and oxidative stress in wound tissue.

The most important factors in the wound-healing process are the contraction and epithelization of the wound (21), therefore monitoring these parameters is of key importance in assessing the wound-healing potential of a different formulation. The results of our research showed that the percentage of wound contraction was significantly higher in the group of animals treated with preparation based on immortelle essential oil. The impressive effect of the immortelle ointment was observed from the first moment, more precisely after seven days of application and was recorded until the last day of the experimental period. Therefore, the given results indicate a shorter re-epithelialization time in the groups treated with essential oil compared to the other groups. The potential for wound healing may be attributed to the presence of terpenes known for their astringent and antimicrobial properties, which contribute to enhanced wound contraction and promote epithelialization.

The inflammation phase plays an important role in the healing process, which is necessary for proper wound healing. The pro-inflammatory cytokines IL-6 and TNF- α are involved in cell differentiation and proliferation, coordinating granulation tissue synthesis, angiogenesis, reepithelialization, and collagen remodeling mechanisms (22). Moreover, these cytokines enhance the migration and proliferation of leukocytes in the wound, enhancing the removal of necrotic tissue and the phagocytosis of antigens (23). IL-10 is another interleukin involved in the inflammatory response in wounds, which acts as an anti-inflammatory mediator, by inhibiting the synthesis of pro-inflammatory cytokines and plays a role in angiogenesis (24). However, an imbalance between inflammatory cytokines can lead to a chronic inflammatory process, which results in the disruption and delay of the healing process (25). The results of cytokine quantification in the tissue homogenate isolated from the wound area, previously caused by the excision method, showed that the anti-inflammatory activity of topical formulation based on immortelle essential oil in skin wounds is a consequence of the reduction of IL-6 and TNF- α levels. In addition, treatment with preparation based on immortelle increased the activity of IL-10, which reduces the expression of pro-inflammatory cytokines, as well as scar formation. Therefore, immortelle ointment achieved anti-inflammatory potential similar to the standard, silver sulfadiazine. Considering the concentration of inflammation markers detected in the tissue sampled at the end of the experimental period, it can be noticed that immortelle essential oil achieved the given anti-inflammatory activity in the remodeling phase. Our results are consistent with literature data, which shows the anti-inflammatory activity of immortelle essential oil. Previous research has shown that the inflammatory phase restores homeostasis, and that in the proliferative phase, fibroblasts and other cells from the connective tissue infiltrate the wound site and secrete

cytokines, attract keratinocytes, and lead to re-epithelialization (26). Preventing prolonged inflammation by suppressing the production of inflammatory cytokines is a desirable target for wound healing products, whereas excessive inflammation leads to the development of chronic wounds and scarring (27). Modulation of cytokine production by topical formulations based on immortelle essential oil indicates anti-inflammatory activity, which supports the previously described results of the essential oil itself and its dominant ingredients.

Disruption of the wound healing process is also associated with oxidative stress, which occurs due to an imbalance in the generation of reactive oxygen species (ROS) and the endogenous antioxidant defense mechanism. Essential oxidants, such as ROS, are generated in various physiological processes. Their effects on the wound healing process are concentration dependent, generally showing positive effects as secondary transporters at low concentrations. However, at high concentrations they damage DNA, proteins and lipids in cells causing toxicity. In diabetic patients, oxidative stress caused by hyperglycemia adversely affects the wound healing process and accordingly, herbal products with antioxidant activity may be useful agents for re-establishing anatomical and cellular tissue continuity (28). Numerous studies have shown that at the site of skin wounds, the concentration of ROS increases and the production of antioxidants decreases, which leads to delayed wound healing (29). In the inflammatory phase, neutrophils and macrophages produce large amounts of ROS, which directly attack pathogens, but also damage healthy surrounding tissues. Inhibition of ROS production triggers angiogenesis and fibroblast proliferation, stimulating skin wound closure. The results of a three-weeks administration of topical formulation indicate that rats treated with immortelle essential oil formulation showed a significant increase in CAT and SOD activity. The increase in the activity of antioxidant protection enzymes, achieved by immortelle formulation, indicate that the reduction of oxidative stress is one of the mechanisms responsible for the achieved effects in the wound healing process.

The limitations of our investigation include the fact that biochemical analysis of the measurement of oxidative stress and inflammation parameters was conducted only on the last day of the treatment protocol. To provide comprehensive and accurate findings regarding the formulation's impact on specific phases of the healing process, these examinations should be performed at multiple time points throughout the 21-day protocol. Evaluating different time points would illuminate the varying effects of *H. italicum* ointment on each stage of wound healing.

CONCLUSION

The results of the six-month follow-up indicate that there were no changes in the organoleptic properties of the tested formulation, what demonstrated the stability of immortelle ointment during six months storage under room temperature conditions. Additionally, the our investigation reveal that

immortelle ointment treatment accelerated the wound healing by modulating the expression of cytokines TNF- α , IL-6 and IL-10 as well as elevating the secretion of antioxidative markers.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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COMPARATIVE STUDIES OF CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF *JUNIPERUS COMMUNIS* L. ESSENTIAL OIL FROM DIFFERENT LOCALITIES IN THE REPUBLIC OF SERBIA

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ABSTRACT

This work aims to determine the chemical composition of Juniperus communis (J. communis) essential oil from different localities in the Republic of Serbia and examine its antimicrobial and antioxidative effects together with chemometric analysis and principal component analysis. Essential oils were extracted via hydro distillation from the fruits of J. communis gathered from four different habitats in Serbia. Extraction yields ranged from 1.56 % in the sample from Bavanište to 1.98% in the sample from Mačkat. A total of 23 compounds in the four essential oil samples were identified by Gas Chromatography-Mass Spectrometer (GC-MS). The dominant compounds were α -pinene (32.68-51.10 %), β -phellandrene (6.43-24.77 %), and β -pinene (9.84-14.09 %). Compared with the other ecological factors, precipitation showed a strongly positive correlation (0.871) with essential oil yields. The four samples could be classified into two clusters based on the variance in their components. All the essential oils samples showed bioactivities. Among them, the essential oil from the Mačkat sample showed the best ABTS radical scavenging activity (IC₅₀=237.74 μ g/ml), DPPH radical scavenging activity (IC₅₀=308.83 μ g/ml) and antimicrobial activities. Consequently, the essential oil extracted from Mačkat has the potential for commercial viability in the food, cosmetic, or medical fields.

Keywords: *Juniperus communis*, Essential oil, Chemical composition, Antioxidant activity, Antimicrobial activity.

INTRODUCTION

Essential oils are natural volatile mixtures of complex compounds that are the result of the secondary metabolism of aromatic plants. Thanks to their different chemical composition, essential oils have several biological activities such as digestive (1), anti-inflammatory (2), antimicrobial, antiviral (3), antioxidant (4), hepatoprotective (5), and anticancer (6). Given that there is a huge increase in the resistance of microorganisms to the use of synthetic antimicrobial agents, the attention of the scientific community has been increasingly focused in recent years on the results of studies that examine the effect of various active metabolites of plant origin, among which essential oils and their components stand out the most.

Juniperus communis L. (*J. communis*), popularly known as juniper, pine, or spruce, is an evergreen shrub or lower tree from the *Cupressaceae* family. This perennial plant is distributed throughout the Northern Hemisphere, in the mountainous areas of Europe (Alps, Pyrenees, Dinarides, Carpathians), Central Asia, North America, and less often in North Africa. In Serbia, it occurs naturally mostly in the mountains of Kosovo and Metohija (Šara, Mokra Gora, Kopaonik, Rogozna) and Southwestern Serbia (Tara, Zlatibor, Golija, Pešter) (7), which especially applies to areas of degraded forests and abandoned agricultural areas. This plant species has a long history of use in folk medicine as a diuretic, anti-inflammatory, antiseptic, stomachic, anti-rheumatic, and anti-diabetic, for kidney and bladder inflammation (8-11). It is most important bioactive ingredient is essential oil, which has a wide range of applications (food, cosmetic, pharmaceutical industry, veterinary) and high commercial value. The characteristic composition of the essential oil obtained by steam distillation from ripe, unfermented berries of *J. communis* mostly includes terpene hydrocarbons (monoterpenes up to 85 %): α -pinene (20-50 %), myrcene (1-35 %), sabinene (<20 %), limonene (2-12 %), β -pinene (1-12 %), caryophyllene (<7 %), terpinene-4-ol (0.5-10 %), then sesquiterpenes (up to 27%) and their oxidized derivatives (up to 4 %) (7). The wide distribution of this species, due to the different effects of environmental factors, leads to variability in terms of the chemical composition and biological activity of its metabolites (12). Variations in chemical and structural type are exactly what makes essential oils functionally versatile and thus more interesting to the scientific public. Variability of the chemical composition of *J. communis* essential oil is also confirmed by a wide range of values in the requirements of the European Pharmacopoeia (European Pharmacopoeia 8). Among the activities of juniper essential oil tested so far, the greatest application potential is reflected in its antimicrobial activity against a wide range of bacteria, the effect of which is determined by the chemical nature of the metabolites, its concentration, and the taxonomic properties of microorganisms (13-14). Considering the potentially toxic and carcinogenic effects of synthetic antioxidants in humans and animals their replacement with natural antioxidants is beginning to be justified. Depending on the presence of active components, the essential oil of juniper berries shows positive effects in

slowing down the lipid peroxidation of foods of animal origin (15), as well as antiradical activity against the DPPH radicals (16). However, scientific reports emphasizing the potential of juniper essential oil for health purposes, as well as in food production and storage, are rare.

This work aims to determine the chemical composition of *J. communis* oil from different localities in the Republic of Serbia and examine its antimicrobial and antioxidative effects together with chemometric analysis and principal component analysis (PCA), and thus contribute to the results of previous research, considering the qualitative and quantitative differences of *J. communis* essential oils of different geographical origins. The essential oil compositions of the four samples were analyzed using Gas Chromatography-Mass Spectrometer (GC-MS). The samples were then classified by PCA and hierarchical cluster analysis (HCA) to determine their relationships. Furthermore, with this work, we want to clarify in more detail whether the biological potential of *J. communis* essential oil is mainly the result of the activity of the components present in the highest concentrations or whether the investigated antimicrobial and antioxidative effects arise from the synergism of all present molecules.

MATERIAL AND METHODS

Plant material

Drug samples were collected from wild juniper bushes from four different locations in the Republic of Serbia (Kopaonik (JC1), Mačkat (JC2),), Takovo (JC3), and Bavanište (JC4)) in October 2019. Table 1 displays the meteorological data for the four habitats from which samples were collected. After collection, the drug was cleaned from the leaves, then properly dried and stored under conditions that do not allow spoilage and contamination (dry environment with low humidity levels; temperature between 10-20°C; dark container; sealed container). The fruits are crushed in an electric mill (IKA® A11 basic) and thus prepared for steam distillation. The preparation of the plant material as well as the isolation of the essential oil were carried out in the laboratory of the Faculty of Medical Sciences of the University of Kragujevac.

Isolation of essential oil

One hundred grams of chopped juniper fruits were placed in a glass balloon and then subjected to hydrodistillation. During two hours, water was heated in a vessel with a flat bottom (steam generator) at a temperature of 100 (\pm 10) °C. The essential oil was separated in a Florentine bottle, where the separation of the two phases - oil and water phase - could be observed. The essential oil was separated by decantation, which was then stored in screw-cap vials with adequate labeling.

Gas-mass analysis of essential oil

Analyzes were performed on an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass-selective

detector (Agilent Technologies, Santa Clara, CA, USA) and a capillary column (HP5-MS, 30 m × 0.25 mm, 0.25 μm). As the mobile gas phase, helium (He) gas was used with a constant flow rate of 1 ml/min. The injector temperature was set to 230 °C, while the detector temperature was 250 °C. The column temperature was linearly changed in the range from 40 to 220 °C, at a rate of 3 °C/min. The injected volume of the sample (dissolved 1/1 in hexane (Fisher, UK), v/v) is 1.0 μl in a split ratio of 1:50. Mass spectra were recorded at 70 eV in the range m/z 40-450. The identification of the detected compounds was performed by comparing their mass spectra with spectra from the spectral database NIST08 (National Institute of Standards and Technology, Gaithersburg, MD, USA), which contains 192,108 spectra of different compounds. Quantification was performed by the method of normalization of the areas under the peaks, that is, based on the correlation of the areas of the peaks and the percentage representation. Qualitative and quantitative analysis of the chemical composition of the obtained essential oils was carried out in the laboratories of the Institute of Public Health in Kragujevac.

Antimicrobial activity

The antimicrobial activity of the essential oil was tested against nine microorganisms. The experiment involved eight strains of bacteria (five standard strains (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922) and three isolates (*Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enterica*)). Also, one yeast standard strain (*Candida albicans* ATCC 10231) was tested. All isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms were provided from the collection held by the Microbiology Laboratory Faculty of Science, University of Kragujevac.

The bacterial suspensions were prepared using the direct colony method. The turbidity of the initial suspension was adjusted with a densitometer (DEN-1, BioSan, Latvia). When adjusted to the turbidity of the 0.5 McFarland standard, the bacterial suspension contained approximately 10⁸ colony-forming units (CFU)/mL, while the yeast suspension contained 10⁶ CFU/mL. Ten-fold dilutions of the initial suspension were additionally prepared in sterile 0.85% saline.

Microdilution method

Antimicrobial activity was tested by determining the minimum inhibitory concentrations (MIC) and minimum microbicidal concentrations (MMC) using the microdilution plate method with resazurin (17). The 96-well plates were prepared by dispensing 100 μL of nutrient broth (Mueller–Hinton broth for bacteria and tryptone soy broth for yeast) into each well. A 100 μL aliquot of the stock solution of the tested essential oil, dissolved in Tween 40 at a 1:1 ratio, was added to the first row of the plate. Twofold serial dilutions were then performed using a multichannel pipette. The resulting concentration range for the **JC3** sample was 500 to 0.98

μL/mL, while for the **JC1**, **JC2**, and **JC4** samples, the range was 2000 to 3.91 μL/mL.

The microtiter plates were inoculated with suspensions to achieve final concentrations of 5 × 10⁵ CFU/mL for bacteria and 5 × 10³ CFU/mL for fungi. Microbial growth was monitored using resazurin (Alfa Aesar GmbH & Co., KG, Karlsruhe, Germany), a blue dye that turns pink when reduced by viable cells. Plates were incubated at 37°C for 24 hours (bacteria) and 28°C for 48 hours (yeasts). The MIC was the lowest extract concentration preventing the color change of resazurin. MMC was determined as the lowest concentration showing no microbial growth after plating samples onto nutrient agar. Each test included growth control and sterility control. All experiments were performed in duplicate.

Tetracycline and fluconazole were used as positive controls. The antibiotic tetracycline (Pfizer Inc., USA) was dissolved in a nutrient liquid medium, Mueller–Hinton broth (Torlak, Belgrade, Serbia), while the antifungal agent fluconazole (Pfizer Inc., USA) was dissolved in tryptone soya broth (Torlak, Belgrade, Serbia).

Antioxidant activity

Determination of DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical scavenging activity

The ability to scavenge free radicals was tested using DPPH radical according to the method described by Mishra et al (18). First, a solution of DPPH (Alfa Aesar GmbH & Co., Germany) in methanol (Fisher, UK) was prepared at a concentration of 0.05 mg/ml and stored in a darkened bottle in the refrigerator until the experiments were performed. Then, a series of standard solutions of the tested extracts and standards in methanol was made (ie 1000, 500, 250, 125, 62.5, and 31.25 μg/ml). Test tubes were filled with 200 μl of solutions containing either tested extracts or standards at specified concentrations, along with 2 ml of DPPH solution. After intensive mixing, the mixture was incubated in the dark for half an hour. After incubation, the absorbance of the solution was measured at 517 nm compared to the control. Ascorbic acid (Sigma-Aldrich, USA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Acros Organics, Belgium) were used as positive controls. The concentration of DPPH radicals was calculated according to the equation: Inhibition capacity of the DPPH radical (%) = 100 * (Ak–Au)/Ak

Where Ak denotes the absorbance of the control (which contains all reagents, except the tested extract or standard), and Au is the absorbance of the sample. Based on the obtained values, a nonlinear calibration curve was constructed, which was used to determine the concentration of the tested sample that inhibits 50% of DPPH radicals (IC₅₀).

Determination of the ability to neutralize ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radicals

The ability to neutralize free radicals was tested using ABTS radicals according to the previously described method by Tabassum et al. (19) with modification. During the preparation of the experiment, the mixture of 7 mM ABTS (Alfa Aesar GmbH & Co., Germany) and 2.45 mM potassium persulfate (Sigma-Aldrich, USA) was incubated at room temperature without the presence of light for 24 hours. This solution was diluted until an absorbance of 0.700 ± 0.02 at 734 nm was achieved. A volume of 300 μ l of extract or standard solution was mixed with 600 μ l of ABTS solution. This mixture was incubated at room temperature for 30 min. Absorbance was measured at 734 nm. Ascorbic acid and Trolox were used as positive controls. The ABTS radical concentration was calculated according to the equation: Inhibition capacity of the ABTS radical (%) = $100 * (A_k - A_u) / A_k$

RESULTS AND DISCUSSION

Essential oil yield

The information from the four samples of *J. communis*, including the collection sites, meteorological data, essential oil yields, and correlation coefficients between four environmental factors and the essential oil yields, is summarized in Table 1. The essential oil extracts from all four samples were colorless. Yields of essential oils (yields, g/g, dry fruit) obtained from the four samples ranged from 1.56 % to 1.98 %. The yield of **JC2** (1.98 %) was higher than the others, followed by **JC1** (1.71 %), **JC3** (1.58 %), and **JC4** (1.56%). Reports suggest that the yields of essential oils in plants can

be influenced by altitude, precipitation, temperature, and other ecological factors of their habitats. (20). Compared with the other three ecological factors, precipitation showed a strongly positive correlation (0.871) with essential oil yields, suggesting it could be a dominant environmental factor affecting the essential oil yields of *J. communis* from various regions. Labokas and Ložienė also observed the impact of precipitation on essential oil yield in plants (21).

Statistical analysis

ANOVA test with $\alpha=0.05$ was used to determine the differences between the assays. The correlation coefficients between yields and compounds of essential oils and the main ecological factors of collection locations were calculated. PCA was employed to identify the interrelations among the essential oils of *J. communis* obtained from the four places. HCA was performed based on the between-group linkage to classify the four oil samples examined. All statistical analyses were performed using SPSS 21.0.

Table 1. The collection sites, meteorological data, and yields of oils from the four *J. communis* samples and the correlation coefficients between four environmental factors and essential oil yields.

Sample	Place	Average Altitude (m)	Temperature ¹ (°C)	Precipitation ¹ (mm)	Humidity ¹ (%)	essential oil yield (wt%)
JC1	Kopaonik	1710	9.6	966.04	79.83	1.71
JC2	Mačkat	600	9.74	995.26	78.58	1.98
JC3	Takovo	330	11.7	818.82	75.83	1.58
JC4	Bavanište	80	13.38	768.34	69.83	1.56
Correlation coefficients		0.273	-0.756	0.871	0.637	

¹The annual average meteorological data (2015-2019) of four locations from the Republic Hydrometeorological Service of Serbia (annuals bulletin for Serbia, <https://www.hidmet.gov.rs>) and "Weather Atlas" (<https://www.weather-atlas.com>)

²Oil yields (wt.%) = Weight (crude oil)/Weight (dry fruit) \times 100;

CHEMICAL COMPOSITION OF ESSENTIAL OIL

Examination of the essential oil of juniper fruit from different locations in the Republic of Serbia included the preparation of plant material, isolation of the essential oil by hydrodistillation, phytochemical analysis of the mentioned oils by a combined chromatographic-spectroscopic method (gas chromatography-mass spectrometry) and comparison of the obtained results.

By analyzing the chemical composition of juniper essential oils in samples from Kopaonik, Mačkat, Takovo, and Bavanište, 23 compounds were separated and identified as shown in Table 2.

Examination of the chemical composition of these essential oils samples revealed the presence of several monoterpene and sesquiterpene compounds.

The most abundant compound in all samples is α -pinene (C2, 32.68-51.10 %), as shown in previous studies (22,23). β -pinene (C7), limonene (C8), α -cubebene (C10), α -elemene (C20), β and α -caryophyllene (C13, C14) were identified in all examined oils, but their quantitative representation differed. The content of β -pinene in the oil of fruits originating from higher altitudes (JC2 and JC1) was above 10 %, while its content was below 10 % in the oils obtained from the fruits of areas of lower altitudes (JC4 and JC3). The terpenes sabinene (C4, 17.82 %), aristolene (C22, 4.28%), and humulene (C16, 0.68 %) were identified in the drug oil originating from JC2, while their presence was not confirmed in the other oils. In the essential oil originating from JC3, the presence of the following compounds was confirmed: bicyclogermacrene (C19, 2.58 %), β -elemene (C12, 1.41 %), and copaene (C11, 1.18 %), which were not found in other oil samples. β -terpinene (C6) is a terpene that, with a percentage of 2.23%, was an integral part of only the essential oil obtained from juniper originating from JC4.

Table 2. Chemical composition of *J. communis* essential oils from different habitats

NO	Compound	Retention time (min)	Relative abundance (%)			
			JC1	JC2	JC3	JC4
C1	α - thujen	5.392	2.23	1.72	/	1.24
C2	α -pinene	5.559	32.68	41.95	51.10	46.82
C3	β -phellandrene	6.541	24.77	/	6.43	12.27
C4	Sabinene	6.547	/	17.82	/	/
C5	Tricyclene	6.605	2.19	2.57	2.23	/
C6	β -terpinene	6.593	/	/	/	2.32
C7	β -pinene	7.040	14.09	13.92	9.84	9.99
C8	D - limonene	8.005	4.16	3.78	4.86	3.81
C9	Terpinolene	9.660	1.40	1.02	/	1.10
C10	α -cubebene	16.798	0.76	0.80	2.08	0.76
C11	Copaene	17.480	/	/	1.18	/
C12	β - elemene	17.925	/	/	1.41	/
C13	β -caryophyllene	18.583	1.69	2.42	1.39	2.26
C14	α - caryophyllene	19.443	1.22	1.53	1.42	1.31
C15	γ -cadinene	20.130	9.56	6.45	/	/
C16	Humulene	20.488	/	0.68	/	/
C17	γ - elemene	20.494	0.80	/	/	0.65
C18	β - cubebene	20.136	/	/	11.09	4.36
C19	Bicyclogermacrene	22.452	/	/	2.58	/
C20	α - elemene	20.714	0.85	0.71	2.03	0.57
C21	Cadinene	21.164	0.70	0.88	1.18	/
C22	Aristolene	21.927	/	4.28	/	/
C23	Elixene	21.938	2.93	/	/	3.72

The representation of monoterpene secondary metabolites in all four essential oils is significantly higher than the content of sesquiterpenes. It has been shown that altitude has an influence on the composition of essential oils (24). α -pinene (C2), whose representation of over 50 % was the highest

in juniper fruit essential oil originating from JC3, may emphasize the consistency of certain chemical profiles within different geographical regions. There is a higher content of β -pinene (C7) in areas of higher altitude, as well as γ -cadinene (C15) which was identified only in oils from higher

altitudes. This can certainly be explained by the fact that different altitudes have different effects on the amount of precipitation, day and night temperatures, relative humidity, and exposure to wind. At high altitudes, there is greater exposure to sunlight and low temperatures, which in plants leads to changes in morphology and physiology, and therefore to changes in the production and composition of secondary plant metabolites. A noticeable difference is observed in the presence and concentration of other terpenes such as β -phellandrene (C3), sabinene (C4), β -pinene (C7), D-limonene (C8), α -cubebene (C10), and others, which further emphasizes the complexity of the influence of environmental factors on the chemical composition of essential oils. The high prevalence of α -pinene (C2) and β -phellandrene (C3), as the main components in the oil from JC1, corresponds to the qualitative analysis of the essential oil of juniper berries from the central part of Portugal where α -pinene and β -phellandrene were also identified as the two main components of the essential oil (25).

The identification of specific terpenes in different samples, such as sabinene (C4), aristolene (C22), and humulene (C16) identified only in the JC2, bicyclogermacrene (C19), β -elemene (C12) and copaene (C11) in the JC3, as well as β -terpinene (C6) in the JC4, indicates specific adaptations of plants to local conditions, which can be useful in identifying the geographical origin of essential oils and potentially useful in situations that require the application of certain chemical components.

Differences in the quantitative and qualitative composition of essential oils, which are the result of various factors such as altitude, geographical location, ripeness of the fruit, age of the plant, and production method, highlight the complexity of factors that affect the chemical profile of the essential oil (26,27). These factors not only affect the quality and characteristics of essential oil but also represent the basis of the individual biological properties of juniper essential oil (15,23). The present compounds have a high biopotential and previous studies have confirmed their various biological activities. This tells us that the essential oil of the juniper fruit is a source of natural active compounds with a wide application potential in the pharmaceutical industry as natural bioactive ingredients, in the cosmetic industry for the development of products with specific therapeutic properties, as well as in the food industry as natural preservatives or flavorings (14,15).

Principal component analysis and hierarchical cluster analysis

Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used to examine the variability in the chemical composition of four samples of juniper essential oils (JC1, JC2, JC3, and JC4). PCA was performed on a total of 23 identified compounds.

Principal component 1 (PC 1) explains 52.09 % of the variance and is strongly positively correlated with the following compounds: D-limonene (C8, 0.938), α -cubebene (C10,

0.998), copaene (C11, 0.999), β -elemene (C12, 0.999), β -cubebene (C18, 0.934), bicyclogermacrene (C19, 0.999) and α -elemene (C20, 0.980). All mentioned compounds belong to the group of sesquiterpenes except D-limonene (C8) which belongs to monoterpenes.

Principal component 2 (PC 2) explains 30.11 % of the variance and is strongly positively correlated with the following compounds: sabinene (C4, 0.893), humulene (C16, 0.893), and aristolene (C22, 0.893). Sabinene (C4) is the only compound that belongs to the monoterpene group, while the other compounds belong to the sesquiterpene group. The arrangement of identified compounds in the context of PC 1 and PC 2 is presented in Figure 1.

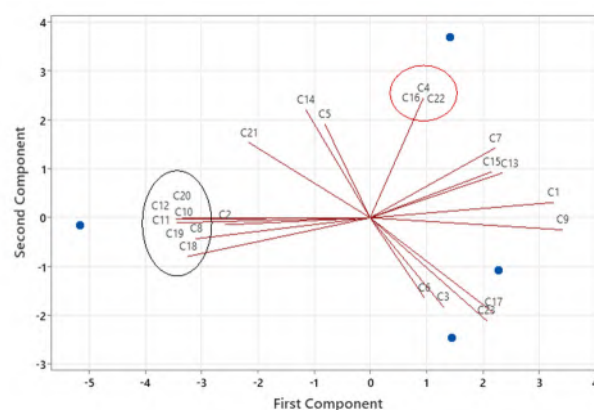


Figure 1. The loadings of each compound for principal components 1 and 2. The components with the highest correlation by component are encircled (PC 1 - black line; PC 2 - red line)

HCA was conducted using Centroid Linkage and Squared Euclidean Distance. It was shown that there are two clusters, where one cluster consists of essential oil from junipers collected on JC1, while the other cluster consists of essential oils obtained from junipers collected at the remaining three locations. Also, it was shown that essential oils from juniper originating from JC3 and JC4 are very similar. The HCA dendrogram is presented in Figure 2.

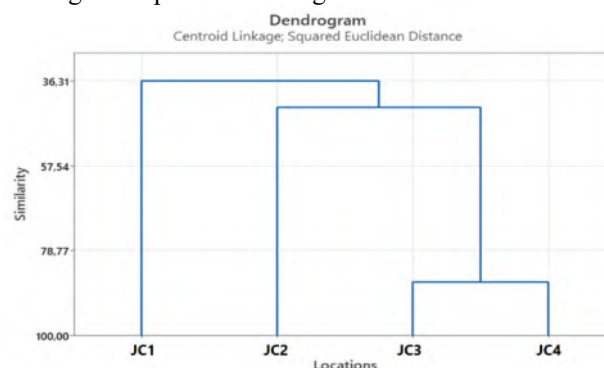


Figure 2. Dendrogram obtained by hierarchical cluster analysis of juniper essential oils from four different locations in the Republic of Serbia.

Antimicrobial activity

The results of *in vitro* testing of the antimicrobial activities of the tested essential oils, tetracycline, and fluconazole are shown in Table 3. The intensity of antimicrobial action varies depending on the microorganism species and the type of essential oil. In general, the tested juniper berry essential oil showed good activity, with the **JC4** sample being the most effective.

The **JC2** sample demonstrated the best antimicrobial effect (MIC ranging from <3.91 $\mu\text{l/ml}$ to 31.25 $\mu\text{l/ml}$), followed by the **JC4** sample (MIC ranging from <3.91 $\mu\text{l/ml}$ to 62.5 $\mu\text{l/ml}$). The **JC3** sample exhibited moderate activity (MIC ranging from <3.91 $\mu\text{l/ml}$ to 250 $\mu\text{l/ml}$), while the **JC1** sample showed a limited and selective effect (MIC ranging from 250 $\mu\text{l/ml}$ to >2000 $\mu\text{l/ml}$).

The strongest antibacterial effect of the **JC2** sample was observed against *E. coli* ATCC 25922 (MIC and MMC values at <3.91 $\mu\text{l/ml}$). The **JC4** sample showed the strongest effect on *P. mirabilis* ATCC 12453 and *E. coli* ATCC 25922 (MIC and MMC values at <3.91 $\mu\text{l/ml}$). The **JC3** sample exhibited stronger activity against *S. aureus* ATCC 25923 (MIC at <3.91 $\mu\text{l/ml}$; MMC at 7.81 $\mu\text{l/ml}$) and *P. aeruginosa* ATCC 27853 (MIC at <3.91 $\mu\text{l/ml}$; MMC at 15.65 $\mu\text{l/ml}$).

The **JC4** and **JC3** samples also demonstrated the best antifungal effects (MIC at <3.91 $\mu\text{l/ml}$; MMC at 15.65 $\mu\text{l/ml}$), followed with **JC2** sample (MIC at <3.91 $\mu\text{l/ml}$; MMC at 62.50 $\mu\text{l/ml}$).

The positive antimicrobial effect of the essential oil from the **JC2** sample can be attributed to the high content of α -pinene (**C2**), as such indications have already been presumed (28,29). Sabinene (**C4**), identified only in the essential oil from **JC2**, has already shown effectiveness of inhibiting the growth of certain bacterial strains (30), so we can assume its additive antimicrobial effect in this oil. The same can be said for sesquiterpene such as β -caryophyllene (**C13**), present in all samples with the highest abundance in the oil sample from **JC2**, known for its anti-inflammatory properties, but studies also indicate its role in antimicrobial activity (31,32). Marčetić et al. (33) observed that the qualitative and quantitative composition of essential oils varies based on the substrates where the species were sampled. These variations explain the differing antimicrobial effects of essential oils from the same species collected in different localities. Furthermore, Hyldgaard et al. (34) highlighted that the antimicrobial activity of juniper essential oil is influenced not only by its major constituents but also by the interactions between major and minor constituents, which can lead to additive or synergistic effects.

Compared to Tetracycline, essential oils show significantly weaker antimicrobial activity. This is not surprising, given that synthetic antibiotics are specifically designed to target key pathways in pathogenic microorganisms with high efficiency. However, the importance of essential oils may lie in their application as complementary therapies, especially in the context of the growing problem of antibiotic resistance. Many studies have shown that combining essential oils with a conventional antibiotic can enhance the antimicrobial effect, suggesting a synergistic interaction between these agents (32,35).

Table 3. The antimicrobial activity of *J. communis* essential oils

Tested species	JC1		JC2		JC3		JC4		Tetracycline/ Fluconazole	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
<i>B. subtilis</i> ATCC 6633	500	500	15.63	62.50	125	1000	62.50	62.50	1.95	15.63
<i>S. aureus</i>	1000	>2000	<3.91	31.25	125	500	7.81	31.25	0.98	15.62
<i>S. aureus</i> ATCC 25923	500	1000	15.63	31.25	<3.91	7.81	7.81	62.50	0.22	3.75
<i>P. aeruginosa</i> ATCC 27853	500	500	7.81	62.50	<3.91	15.65	7.81	250	62.5	125
<i>P. mirabilis</i> ATCC 12453	500	1000	31.25	31.25	62.50	125	<3.91	<3.91	125	125
<i>E. coli</i>	>2000	>2000	7.81	31.25	31.25	62.50	<3.91	15.65	15.63	31.25
<i>E. coli</i> ATCC 25922	500	1000	<3.91	<3.91	7.81	15.63	<3.91	<3.91	15.63	31.25
<i>S. enterica</i>	>2000	>2000	7.81	62.50	250	500	<3.91	15.65	15.63	31.25
<i>C. albicans</i> ATCC 10231	250	1000	<3.91	62.50	<3.91	15.65	<3.91	15.65	31.25	1000

MIC-minimum inhibitory concentrations; MMC-Minimum microbicidal concentration; Values are expressed in $\mu\text{l/ml}$ for essential oils and in $\mu\text{g/ml}$ for tetracycline and fluconazole

Antioxidant activity

Antioxidant activities of the essential oils were analyzed using free radical scavenging (DPPH and ABTS assays and the results are presented in Table 4.

Table 4. The antioxidant activity of the *J. communis* essential oils

Samples/ Standards	DPPH	ABTS
	scavenging activity	scavenging activity
	IC ₅₀ (µg/ml)	
JC1	> 1000	896.11 ± 7.02
JC2	308.83 ± 3.85	237.74 ± 4.61
JC3	> 1000	> 1000
JC4	966.89 ± 10.83	793.66 ± 16.23
Ascorbic Acid	9.08±1.96	8.28±0.24
Trolox	14.26±3.81	12.40±0.40

*DPPH-1,1-diphenyl-2-picryl-hydrazyl free radical; ABTS-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical; IC₅₀- concentration of the tested sample that inhibits 50% of radicals;

Essential oil from **JC2** demonstrates a relatively low IC₅₀ value for both tests (308.83 µg/ml for DPPH and 237.74 µg/ml for ABTS), which indicates a significant ability to neutralize free radicals. **JC1** and **JC3** are characterized by IC₅₀ values greater than 1000 µg/ml for the DPPH test and high but measurable values for the ABTS test. Essential oil from **JC4** sample with IC₅₀ values of 966.89 µg/ml for DPPH and 793.66 µg/ml for ABTS, shows moderate antioxidant activity. α -Pinene (**C2**), present in high concentrations in all essential oils, is known for its antioxidant activity. Studies have shown that α -pinene effectively neutralizes free radicals and contributes to protection against oxidative stress (36, 37). It is present in the largest amount of essential oil **JC2**, which also showed the best antioxidant activity. β -Pinene (**C7**) is present in all tested samples. α -pinene and β -pinene belong to the group of monoterpene hydrocarbons, so their antioxidant effect can probably be explained by the presence of methylene groups in these molecules (37, 38). β -Phellandrene (**C3**) is present in significant amounts in three essential oil samples (24.77% **JC1**, 12.27% **JC4**, 6.43% **JC3**), and its antioxidant activity has already been confirmed (37). The absence of β -phellandrene in the **LC2** may indicate that the combination of different terpenes, and not the presence of a single compound, determines the overall antioxidant activity. D-Limonene (**C8**), in a previous study, showed its antioxidant activity even at low doses (39), and was also present in all oil samples tested. Its consistent presence may contribute to the baseline level of antioxidant activity in all oils tested, but variations in antioxidant activity suggest that the presence of other compounds plays a key role. β -Caryophyllene (**C13**), a sesquiterpene with observed antioxidant properties (40), although in small amounts, was present in all oil samples tested. The superior antioxidant activity of **JC2** can be attributed to the synergistic effects of its high α -pinene (**C2**) concentrations, the presence of D-limonene (**C8**), and the combination of β -caryophyllene (**C13**) and α -caryophyllene

(**C14**), which together form a complex mix of antioxidant active compounds. **JC1** and **JC4** with moderate to weak antioxidant activity, show that even the presence of high concentrations of potentially active components (such as α -pinene in **JC1**) is not sufficient for high antioxidant efficiency without appropriate synergy between the components. Essential oil **JC3** with the weakest antioxidant activity, indicates that the absence of key antioxidant terpenes and the potential presence of compounds that do not significantly contribute to antioxidant activity, or even inhibit the activity of other components, may result in lower overall efficacy. It is very difficult to attribute the antioxidant activity of essential oils to only one active principle, considering that essential oils are mixtures of different chemical components. It is important to emphasize that sometimes the biological activity of the oil is not only influenced by the most abundant active principles but that the less abundant components can also have a positive effect on the activity.

CONCLUSION

The current study compared the yields, compositions, and bioactivities of essential oils extracted from *J. communis* plants collected from distinct areas in Serbia. The research revealed that precipitation in the habitat was the most influential ecological factor affecting the essential oil yields of the samples. A total of 23 components, rich in monoterpenes and sesquiterpenoids, were identified in the essential oils from the four samples. The chemical variation among the essential oils from the four samples could be strongly influenced by the altitude of the habitats. The four samples could be divided into two clusters according to the variability of their components. The study assessed the antioxidant and antimicrobial activities of essential oils extracted from the four samples. The results suggested that the essential oils from *J. communis* possess promising potential for applications across several industries, such as food, cosmetics, and pharmacy, owing to

their inherent dual antioxidant and antimicrobial properties. The sample from Mačkat (JC2) exhibited superior performance in DPPH, ABTS, and antimicrobial assays, coupled with the highest yield. This implies that *J. communis* sourced from this location shows the greatest potential for further industrial utilization.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

FUNDING

None

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AWARENESS OF PERIODONTITIS AMONG UNDERGRADUATE STUDENTS AT A FACULTY OF MEDICINE IN BOSNIA AND HERZEGOVINA: A CROSS-SECTIONAL STUDY

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ABSTRACT

This study was aimed to investigate awareness of periodontitis among undergraduate students at a faculty of medicine in Bosnia and Herzegovina. This cross-sectional study was conducted among undergraduate students of the Faculty of Medicine Foca enrolled in three study programs (Medicine, Nursing, and Special Education and Rehabilitation). Knowledge and awareness of periodontitis were collected using a structured questionnaire. Approximately 58% of students had heard the term periodontitis. Higher percentage of Medicine and Nursing students identified Gum Recession, Bleeding Gums, Tooth Loss and Loose Tooth as common symptoms of periodontitis compared to Special Education and Rehabilitation students. Knowledge of risk factors for periodontitis differed between the groups, with Nursing students showing greater awareness but also incorrect recognizing of several risk factors. About one third of students answered that Diabetes Mellitus is related to periodontitis (32.4%). Significantly lower percentage of Nursing students assessed their knowledge of periodontitis as poor compared to Medicine and Special Education and Rehabilitation students. The findings revealed a lack of adequate knowledge of periodontitis across all the observed study programs. Strengthening periodontal education within undergraduate curricula is essential, particularly considering the established links between periodontal and systemic diseases.

Keywords: Periodontitis, risk factors, systemic diseases, education, students.

INTRODUCTION

Periodontitis is a chronic inflammatory disease caused by dysbiosis of the dental biofilm and is characterized by the progressive destruction of tooth-supporting structures (1). The disease results from a complex interaction between periodontal pathogens, host immune-inflammatory response, and various environmental and systemic risk factors (2).

It is among the most prevalent chronic infections globally and has a substantial impact on patients' quality of life (3). Recent review and meta-analysis of epidemiological studies conducted between 2011 and 2020 indicate that periodontitis affects 62% of adults, while severe forms occur in 23.6% (4). These findings suggest a notably higher prevalence than that reported in previous decades, with current estimates indicating that severe periodontitis affects approximately 1.1 billion people worldwide (5).

Despite its high prevalence and significant impact, periodontitis has historically been viewed as a localized inflammatory condition with limited systemic implications. However, contemporary evidence now strongly supports its understanding as a systemic disease, given its extensive impact on general health and its strong association with numerous chronic non-communicable diseases (6). Over the past decades, significant links between periodontal disease and cardiovascular disorders, diabetes mellitus, preterm birth and low birth weight, chronic obstructive pulmonary disease, and various autoimmune conditions have been demonstrated (7-9). Periodontal pathogens and their endotoxins are thought to enter the systemic circulation through damaged periodontal tissues, potentially affecting distant organs and contributing to systemic inflammation (10). Components of periodontal pathogens have been identified in various sites, including atherosclerotic plaques, placental tissue, the respiratory tract, pancreas, and colon (9,11).

Oral health has long been neglected in the broader context of global health. However, recognizing its significance, recent global health initiatives have increasingly emphasized the importance of oral health in overall disease prevention and health promotion. The 2021 WHO Oral Health Resolution highlights the urgent need for a preventive, risk-factor-oriented approach, timely and comprehensive care, strengthening of oral health systems, and improvement of public awareness of the oral health maintenance benefits (12).

Awareness of periodontitis has been reported to be insufficient among the general population (13). Studies have demonstrated that knowledge of periodontitis is highest among individuals aged 40–60 years. However, even in this group, understanding its etiology, signs, symptoms, and risk factors remains inadequate (14). Furthermore, recent evidence demonstrated that awareness and knowledge of periodontitis remain insufficient, not only among the general population but also among medical students and doctors (15).

Given the notably high and growing global burden of periodontitis, strengthening preventive strategies, particularly those centered on health education, has become imperative. In this context, understanding the level of knowledge among future healthcare professionals is essential for shaping effective preventive and educational approaches. Therefore, this study aimed to assess the awareness of periodontitis among undergraduate students at a faculty of Medicine in Bosnia and Herzegovina, with particular emphasis on risk factors, associations with systemic diseases, and students' attitudes toward preferred sources of information on this condition.

MATERIAL AND METHODS

This cross-sectional study was conducted at the Faculty of Medicine Foca, in Bosnia and Herzegovina, between March and July of 2025. The study included undergraduate students enrolled in three study programs of the faculty: Medicine (M) (from 1st to 6th year), Nursing (N) (from 1st to 4th year), and Special Education and Rehabilitation (SER) (from 1st to 4th year).

Before enrolment, the aim and purpose of the study were clearly explained to all respondents, after which written informed consent was obtained from all respondents. The Ethics Committee of the Faculty of Medicine approved the study protocol (approval no. 01-2-19/2025). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Insights into participants' knowledge of periodontitis, its risk factors, and its consequences were assessed using a structured questionnaire adapted from Kattel et al. (16). The questionnaire is developed based on a literature review of similar studies conducted globally and modified to suit our aims and study population. The questionnaire in this study comprised 10 questions. The first four questions covered demographic data: age (years), gender (male/female), study program (M, N, SER), and year of the study (1-6). The second part of the questionnaire included 6 questions: knowledge of the term periodontitis (yes/no), its signs and symptoms (Bad Breath, Oral Candidiasis, Gum Recession, Tooth Fracture, Tooth Decay, Bleeding Gums, Tooth Loss, Loose Tooth; multiple responses allowed), the most common periodontitis risk factors (Oral Candidiasis, Socioeconomic Status, Ehlers Danlos Syndrome, Papillon LeFevre Syndrome, Smoking, Sedentary Lifestyle, Pregnancy, Tooth Decay; multiple responses allowed), and the association between systemic diseases and periodontitis (Alzheimer's Disease, Rheumatoid Arthritis, Systemic Lupus Erythematosus, Sarcoidosis, Adverse Pregnancy Outcome, Osteoarthritis, Ectodermal Dysplasia, Chronic Kidney Disease, Diabetes Mellitus, Cardiovascular Disease Hospital-Acquired Pneumonia; multiple responses allowed), self-assessment of knowledge (poor/fair/neutral/good/excellent) and preferred sources of information to improve awareness of periodontal

health (Lectures, Information pack, Video, Web site; multiple responses allowed) (16).

The sample size was determined based on a previous study, which indicated that at least 215 subjects were required to achieve 95% power at a significance level of 0.05 (16).

Data were analyzed using SPSS 20.0 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp, USA). Data are reported as means and frequencies for each group. ANOVA with LSD post-hoc and chi-square test with post hoc Z-test with Bonferroni correction were used to determine the significance of differences between groups. Statistical significance was set at $p < 0.05$ in all analyses.

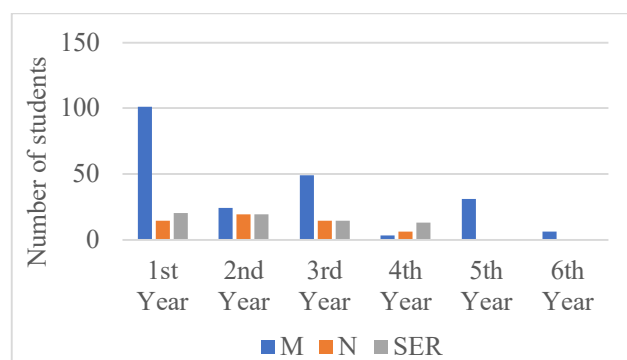
RESULTS

A total of 333 students from the Faculty of Medicine Foca, representing three study programs, participated in the study: 214 of M students, 53 of N students, and 66 of SER students (Figure 1). The mean age of all participants was 21.71 ± 1.77 years, ranging from 19 to 29 years. The majority of respondents were female (78.7%) ($p < 0.001$) (Table 1).

Of the total number of respondents, 42% reported that they were unfamiliar with the term periodontitis. The highest proportion of students who were familiar with the term periodontitis was recorded among N students (67.9%) (Table 2).

The highest number of students answered that Gum Recession (36.0%), Bleeding Gums (36.3%), Tooth Loss (20.7%) and Loose Tooth (25.2%) are common symptoms of periodontitis. Intergroup analyses showed that higher percentage of M and N students identified Gum Recession, Bleeding Gums, Tooth Loss and Loose Tooth compared to SER students, with significant differences for Gum Recession (M vs SER), Bleeding Gum (M and N vs SER), and Loose Tooth (M vs N vs SER). Oral Candidiasis was significantly more frequently recognized as a symptom of periodontitis by N students compared to SER students (Table 3).

Figure 1. Number of students by study year



Legend: M-Medicine students; N-Nursing students; SER- Special education and rehabilitation students

More than one-quarter of the students reported that Smoking (33.9%) and Tooth Decay (25.8%) are the most common periodontitis risk factors, followed by Oral Candidiasis (12.6%) and Pregnancy (11.1%). Intergroup analyses showed that significantly more N students answered that Papillon LeFevre Syndrome and Smoking are periodontitis risk factors in comparison to SER, and Sedentary Lifestyle and Pregnancy in comparison to M and SER students. Tooth decay is recognized significantly more often as the most common periodontitis risk factor among M and N students compared to SER students (Table 4).

About one third of students answered that Diabetes Mellitus is related to periodontitis (32.4%) followed by Cardiovascular Disease (12.6%). Intergroup comparisons showed significant differences between N and SER, M and N, M and SER students in linking periodontitis with Rheumatoid Arthritis, Chronic Kidney Disease, and Cardiovascular disease, respectively (Table 5).

A significantly lower percentage of N students (22.6%) assessed their knowledge of periodontitis as poor compared to M (41.6%) and SER (53.0%) students (Table 6).

While assessing the preferred sources for obtaining additional information about periodontitis, a similar number of respondents considered each of the offered options suitable, but a significantly higher number of N think that the information pack is better than SER students (Table 7).

Table 1. Distribution of undergraduate students by age and gender

Variable	M (n=214)	N (n=53)	SER (n=66)	TOTAL (n=333)	P
Age (mean \pm SD)	21.70 \pm 1.90	21.87 \pm 1.42	21.64 \pm 1.59	21.71 \pm 1.77	0.765
Gender n (%)					
Male	60 (28.0%)	7 (13.2%)	4 (6.1%)	71 (21.3%)	0.064
Female	154 (72.0%)	46 (86.8%)	62 (93.9%)	262 (78.7%)	

M-Medicine students; N-Nursing students; SER- Special education and rehabilitation students

Table 2. Knowledge of the term periodontitis among undergraduate students

Question	Response	M (n=214)	N (n=53)	SER (n=66)	TOTAL (n=333)	P
Have you heard of the term Periodontitis?	Yes	126 (58.9%)	36 (67.9%)	31 (47.0%)	193 (58.0%)	0.064
	No	88 (41.1%)	17 (32.1%)	35 (53.0%)	140 (42.0%)	

M-Medicine students; N-Nursing students; SER- Special education and rehabilitation students

Table 3. Knowledge of the most common periodontitis signs and symptoms among undergraduate students

Sign/Symptom	M (n=214)	N (n=53)	SER (n=66)	TOTAL (n=333)	P
Bad Breath	41 (19.2%)	12 (22.6%)	5 (7.6%)	58 (17.4%)	0.052
Oral Candidiasis	10 (4.7%) ^{a,b}	7 (13.2%) ^a	0 (0.0%) ^b	17 (5.1%)	0.004
Gum Recession	86 (40.2%) ^a	19 (35.8%) ^{a,b}	15 (22.7%) ^b	120 (36.0%)	0.036
Tooth Fracture	13 (6.1%)	4 (7.5%)	1 (1.5%)	18 (5.4%)	0.270
Tooth Decay	23 (10.7%)	5 (9.4%)	3 (4.5%)	31 (9.3%)	0.317
Bleeding Gums	84 (39.3%) ^a	23 (43.4%) ^a	14 (21.2%) ^b	121 (36.3%)	0.015
Tooth Loss	50 (23.4%)	11 (20.8%)	8 (12.1%)	69 (20.7%)	0.144
Loose Tooth	55 (25.7%) ^a	23 (43.4%) ^b	6 (9.1%) ^c	84 (25.2%)	<0.001

M-Medicine students; N-Nursing students; SER- Special education and rehabilitation students;
Different letters represent a statistically significant difference

Table 4. Knowledge of the most common periodontitis risk factors among undergraduate students

Risk factors	M (n=214)	N (n=53)	SER (n=66)	TOTAL (n=333)	P
Oral Candidiasis	29 (13.6%)	8 (15.1%)	5 (7.6%)	42 (12.6%)	0.370
Socioeconomic Status	19 (8.9%)	8 (15.1%)	4 (6.1%)	31 (9.3%)	0.226
Ehlers Danlos Syndrome	14 (6.5%)	3 (5.7%)	2 (3.0%)	19 (5.7%)	0.561
Papillon LeFevre Syndrome	9 (4.2%) ^{a,b}	5 (9.4%) ^a	0 (0.0%) ^b	14 (4.2%)	0.039
Smoking	74 (34.6%) ^{a,b}	24 (45.3%) ^a	15 (22.7%) ^b	113 (33.9%)	0.034
Sedentary Lifestyle	1 (0.5%) ^a	6 (11.3%) ^b	0 (0.0%) ^a	7 (2.1%)	<0.001
Pregnancy	21 (9.8%) ^a	12 (22.6%) ^b	4 (6.1%) ^a	37 (11.1%)	0.010
Tooth Decay	61 (28.5%) ^a	17 (32.1%) ^a	8 (12.1%) ^b	86 (25.8%)	0.015

M-Medicine students; N-Nursing students; SER- Special education and rehabilitation students
Different letters represent a statistically significant difference

Table 5. Knowledge of the association between systemic diseases and periodontitis among undergraduate students

Condition/Disease	M (n=214)	N (n=53)	SER (n=66)	TOTAL (n=333)	P
Alzheimer's Disease	9 (4.2%)	2 (3.8%)	0 (0.0%)	11 (3.3%)	0.242
Rheumatoid Arthritis	17 (7.9%) ^{a,b}	10 (18.9%) ^a	3 (4.5%) ^b	30 (9.0%)	0.017
Systemic Lupus Erythematosus	17 (7.9%) ^a	10 (18.9%) ^a	5 (7.6%) ^a	32 (9.6%)	0.044
Sarcoidosis	10 (4.7%)	5 (9.4%)	3 (4.5%)	18 (5.4%)	0.367
Adverse Pregnancy Outcome	8 (3.7%)	2 (3.8%)	2 (3.0%)	12 (3.6%)	0.962
Osteoarthritis	20 (9.3%)	6 (11.3%)	1 (1.5%)	27 (8.1%)	0.081
Ectodermal Dysplasia	11 (5.1%)	3 (5.7%)	0 (0.0%)	14 (4.2%)	0.162
Chronic Kidney Disease	10 (4.7%) ^a	8 (15.1%) ^b	3 (4.5%) ^{a,b}	21 (6.3%)	0.016
Diabetes Mellitus	73 (34.1%)	21 (39.6%)	14 (21.2%)	108 (32.4%)	0.070
Cardiovascular Disease	34 (15.9%) ^a	6 (11.3%) ^{a,b}	2 (3.0%) ^b	42 (12.6%)	0.022
Hospital-Acquired Pneumonia	6 (2.8%)	1 (1.9%)	1 (1.5%)	8 (2.4%)	0.807

M-Medicine students; N-Nursing students; SER- Special education and rehabilitation students
Different letters represent a statistically significant difference

Table 6. Self-evaluation of knowledge about periodontitis among undergraduate students

Knowledge Rating	M (n=214)	N (n=53)	SER (n=66)	TOTAL (n=333)	P
Poor	89 (41.6%) ^a	12 (22.6%) ^b	35 (53.0%) ^a	136 (40.8%)	
Fair	65 (30.4%) ^a	17 (32.1%) ^a	18 (27.3%) ^a	100 (30.0%)	
Neutral	51 (23.8%) ^a	15 (28.3%) ^a	11 (16.7%) ^a	77 (23.1%)	0.002
Good	8 (3.7%) ^a	6 (11.3%) ^a	2 (3.0%) ^a	16 (4.8%)	
Excellent	1 (0.5%) ^a	3 (5.7%) ^b	0 (0.0%) ^a	4 (1.2%)	

M-Medicine students; N-Nursing students; SER- Special education and rehabilitation students
Different letters represent a statistically significant difference

Table 7. Preferred sources of information about periodontitis among undergraduate students

Source of information	M (n=214)	N (n=53)	SER (n=66)	TOTAL (n=333)	P
Lectures	67 (31.3%)	20 (37.7%)	14 (21.2%)	101 (30.3%)	0.131
Information pack	52 (24.3%) ^{a,b}	19 (35.8%) ^a	9 (13.6%) ^b	80 (24.0%)	0.019
Video	67 (31.3%)	20 (37.7%)	29 (43.9%)	116 (34.8%)	0.151
Web site	74 (34.6%)	19 (35.8%)	16 (24.2%)	109 (32.7%)	0.256

M-Medicine students; N-Nursing students; SER- Special education and rehabilitation students

Different letters represent a statistically significant difference

DISCUSSION

The present study assessed the awareness and knowledge of periodontitis among students from three programs at the Faculty of Medicine Foca in Bosnia and Herzegovina. Our findings indicate that the overall knowledge of periodontitis, including its risk factors and association with systemic diseases, was suboptimal across all programs. Notably, 42% of students reported being unfamiliar with the term periodontitis, which contrasts with the findings of Kattel et al., where only 4.1% of medical students were unaware of the term. However, the study by Kattel et al. included not only final-year medical students (3rd to final year) but also interns, which may explain their higher overall knowledge of oral diseases (16). This observation is further supported by Márquez-Arrico et al., who reported that students with higher levels of education demonstrate greater awareness and knowledge of oral health (17).

Beyond general awareness, our findings regarding specific knowledge of periodontitis signs and symptoms offer further insights. M and N students, for instance, more frequently recognized Gum Recession, Bleeding Gums, and Loose Tooth as common symptoms than did SER students. Notably, a higher proportion of N students incorrectly identified oral candidiasis as a common symptom of periodontitis compared to M and SER students. These findings are consistent with a 2019 study involving 906 students from multiple faculties, which found a higher percentage of medical students correctly recognized bleeding gums as a symptom of periodontitis compared to students from other faculties (18). Early recognition of periodontitis signs and symptoms is crucial for preserving the supporting structures of the teeth and preventing tooth loss, as periodontitis is often a "silent" disease in its initial stages, with few or no symptoms (19). Patients frequently seek professional care only after significant impairment of the supporting structures, making treatment more complex and costly.

Our study results indicate that students demonstrated limited knowledge about risk factors for periodontitis onset. Interestingly, significantly more N students related Sedentary Lifestyle and Pregnancy with periodontitis than M and SER students. However, more M and N than SER students incorrectly recognized Oral Candidiasis as a risk factor for periodontitis. A finding that N students are much more likely to recognize common risk factors for periodontitis, but also to misrecognize them, probably can be related to the fact that many N students had completed secondary medical education, with respect to M and SER students. Our findings are in line with those of Yao et al., who suggested that insufficient understanding of the etiology of periodontitis may contribute to the high prevalence of bleeding gums among medical students (20).

This study shows insufficient knowledge among students regarding the association between periodontitis and systemic

diseases. Only a relatively small proportion of students were aware of the systemic implications of periodontitis. The highest recognition was observed for the link between diabetes mellitus and periodontitis; however, this proportion remained low (approximately 30%), with no statistically significant differences between the groups. In comparison, Kattel et al. reported that 76.6% of medical students correctly recognized this association (16). Considering the systemic impact of periodontitis, particularly its bidirectional relationship with diabetes mellitus, it is essential to improve our knowledge of the underlying mechanisms and clinical consequences of this interaction. Similarly, Parveen et al. found that more than half of the examined medical doctors (54.5%) had limited knowledge of the link between periodontitis and systemic diseases (15). Obulareddy et al. also reported a lack of clinician understanding regarding this association, highlighting a notable gap between scientific evidence and clinical practice (21). In a study among physicians in France, 75% of respondents were aware of the connection between periodontitis and diabetes, while approximately half recognized its association with cardiovascular, inflammatory bowel, and respiratory diseases (53–59%). However, 74.31% of these physicians rarely or never inquired about their patients' periodontal health (22). Consistent with our findings, Pisani et al. observed that medical students had limited knowledge regarding the oral-systemic health relationship; although approximately 60% were aware that diabetes is a risk factor for periodontitis, only 29.9% understood the bidirectional nature of this relationship (23).

The findings indicate that periodontal health is not given sufficient priority within the current educational framework, which may be due to the curriculum structure, limited clinical exposure, and the lack of formal interprofessional oral health education. Differences observed between study programs further highlight these educational gaps. Although medical and nursing students are expected to possess broader knowledge, their limited understanding of periodontitis may reflect insufficient coverage of oral health in their curricula. Additionally, the low level of awareness about the connection between periodontitis and systemic diseases emphasizes the need for preventive and educational strategies. Recognizing the relationship between periodontitis and systemic health can help develop integrated prevention and treatment approaches.

Analysis of the respondents' self-perceived knowledge of periodontitis revealed that approximately 71% rated their knowledge as poor or fair. A recent study demonstrated a significant improvement in knowledge and attitudes among clinicians following targeted training (15). As expected, studies including dental students reported that they exhibited superior knowledge and attitudes across all examined aspects compared with non-dental students (20,24).

Since knowledge of periodontitis, its complications, and associated risk factors is essential for prevention and

effective management, it is crucial to educate the wider population, particularly health professionals and medical students, as timely recognition of periodontitis, appropriate patient counseling regarding possible complications, and referral for further diagnosis and treatment are key steps in preserving oral and general health. Although M and N students demonstrated better knowledge in certain aspects than SER students, our findings emphasize the need for continuous improvement in periodontal health education across all three study programs at the Faculty of Medicine Foca. One potential approach is to incorporate dental health into the curricula of the observed programs, with particular emphasis on the relationship between periodontitis and systemic diseases. Such an approach not only promotes the improvement of patients' oral health but also supports the maintenance of oral health among future healthcare providers.

Since this study used a cross-sectional design, several limitations should be considered. It was conducted at a single center, limiting the extent to which the findings can be applied. Conducting research across multiple centers and including follow-up focus groups after educational interventions could produce more comprehensive data and support wider relevance. Additionally, the self-administered questionnaire was not validated, and the oral health status was not clinically assessed. In a self-administered questionnaire, design might have led to response bias, with participants possibly guessing or giving socially acceptable answers (25). However, the present results obtained from statistical analysis appear theoretically valid and offer a useful foundation for improving education curricula, aiming to boost clinicians' awareness and understanding of oral health and foster inter-professional collaboration.

CONCLUSION

The findings of this study revealed a lack of adequate knowledge of periodontitis across all the observed study programs. Strengthening periodontal education within undergraduate curricula is essential, particularly considering the established links between periodontal and systemic diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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IN *SILICO* DISCOVERY OF NOVEL CDK1 INHIBITORS FROM LINUM SPECIES FOR TARGETED CANCER THERAPY

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ABSTRACT

Targeting cell cycle regulators such as cyclin-dependent kinase 1 (CDK1) represents a promising strategy for anticancer drug discovery. This study employed an integrated computational approach to identify natural CDK1 inhibitors from the ethyl acetate extracts (EtOAc) of *Linum numidicum* Murb. (LN) and *Linum trigynum* (LT), which have previously been shown to induce G2/M arrest in PC3 prostate cancer cells. We hypothesized that this phenotype results from the direct inhibition of CDK1. To test this hypothesis, a structure-based virtual screening of 78 phytoconstituents identified from LN and LT was conducted against the CDK1/Cks2 and CDK1/cyclin B1/Cks2 complexes. Molecular docking simulations were performed using the Molecular Operating Environment (MOE), with ligand structures optimized and their pharmacokinetic properties evaluated using SwissADME. The CDK1/cyclin B1/Cks2 complex exhibited stronger binding affinities (binding energy < -7.0 kcal/mol) than the CDK1/Cks2 apoenzyme. Notably, several flavonoid glycosides, including 6,4'-dimethoxy-scutellarein-7-neohesperidoside, 8,3',4'-trihydroxyflavone-7-O-6"-O-p-coumaroyl)- β -D-glucopyranoside, and vicenin-2 and its isomers, showed higher binding affinities to CDK1 than the known inhibitors NU6102 and dinaciclib. These findings provide the first *in silico* evidence identifying specific LN and LT metabolites as high-affinity CDK1 ligands, thereby validating the initial hypothesis and providing a strong rationale for the purification and experimental validation of these lead compounds as potential chemotherapeutic agents.

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INTRODUCTION

Cancer remains the leading cause of death worldwide, accounting for nearly 10 million deaths in 2022. Among the most common cancers, lung and prostate cancers predominate in men, while breast cancer is the most prevalent among women (GLOBOCAN 2020, IARC). This disease is characterized by uncontrolled cell proliferation, often associated with deregulation of the mechanisms governing the cell cycle⁽¹⁾.

The cell cycle is a fundamental biological process essential for growth and tissue homeostasis. It is finely regulated by a series of molecular events that ensure orderly cell division^{(2),(3)}. This regulation largely depends on the coordinated action of CDKs and their regulatory partners, cyclins^{(4),(5)}. These proteins drive cell cycle progression by activating phase-specific checkpoints. Among CDKs, cyclin-dependent Kinase 1 (CDK1) plays a pivotal role in the G2/M transition by forming a complex with cyclins A and B. This complex initiates mitosis through the phosphorylation of key substrates involved in chromosome condensation, nuclear envelope breakdown, and mitotic spindle formation⁽⁶⁾.

CDK activity is counterbalanced by specific inhibitors (CDKIs), whose alterations are frequently observed in various cancers⁽⁴⁾. Overexpression of CDK1, or inactivation of its inhibitors, disrupts cell cycle regulation, leading to uncontrolled proliferation and tumorigenesis. Given its central and highly conserved role in the cell cycle, CDK1 deregulation has major repercussions and has been implicated in the development of several cancers, including breast, colorectal, ovarian, liver, gastric, esophageal, and oral cancers⁽⁷⁾⁻⁽⁹⁾.

Consequently, the development of selective CDK1 inhibitors represents a promising strategy to restore cell cycle homeostasis and suppress tumor growth. Recent studies have focused on plant-derived bioactive compounds for their anticancer potential. The *Linum* genus, belonging to the Linaceae family, is known for its richness in bioactive secondary metabolites and is attracting increasing attention in molecular oncology due to its diverse biological activities⁽¹⁰⁾⁻⁽¹³⁾.

In parallel, *in silico* approaches have emerged as powerful tools for the discovery of new therapeutic agents, particularly through drug repositioning. These computational methods

include signature matching, molecular docking, genetic analysis, signaling pathway mapping, and data integration from various sources⁽¹⁴⁾. Specifically, molecular docking predicts the optimal binding conformation between a ligand and its protein target, subsequently evaluated using scoring functions that estimate binding affinity and stabilizing interactions^{(15),(16)}.

Such structure-based approaches significantly reduce the time and cost required for new drug development⁽¹⁷⁾. The present study aims to investigate the inhibitory potential of compounds identified in the ethyl acetate (EtOAc) extracts of two *Linum* species, *L. numidicum* Murb. (LN) and *L. trigynum* L. (LT), against CDK1, using an in-silico approach. The objective is to evaluate their interactions with this key cell cycle regulator and to determine their potential as therapeutic agents in cancer treatment.

MATERIALS AND METHODS

Preparation of target proteins

The three-dimensional structures of CDK1/Cks2 and CDK1/cyclin B1/Cks2 were obtained from the Protein Data Bank (PDB), identified by PDB IDs 6GU7 and 5LQF, respectively. These complexes represent key molecular targets for the development of anticancer therapeutics. Table 1 summarizes the crystallographic properties of 6GU7 and 5LQF. Protein energy minimization was carried out using the Austin Model 1 (AM1) Hamiltonian implemented in the Molecular Operating Environment (MOE) software, with force fields derived from the Merck Molecular Force Field (MMFF94x). Water molecules were removed from the protein surface to maintain exposure of the contact region during docking. Docking simulations were focused on the canonical ATP-binding pocket of CDK1. This site was explicitly defined according to the coordinates of the co-crystallized high-affinity inhibitors found in the PDB structures: AZD5438 for 6GU7 and NU6102 for 5LQF (Figures 1 and 2). The centroid X, Y and Z for co-crystallized ligands 6GU7 [328.345, 213.852, 191.967], 5LQF receptor A [33.777, -67.413, 185.099] and 5LQF receptor D [-26.487, -101.353, 233.771], residues within a 6 Å radius of the ligands were considered as part of the active site and used for subsequent docking studies.

Table 1. Crystallographic properties of enzymes.

Enzyme	PDB ID	Classification	Organism	Expression system	Resolution	Method	TSW (kDa)	Chain
CDK1/Cks2	6GU7	Cell Cycle	<i>Homo sapiens</i>	<i>Spodoptera frugiperda</i> , <i>Escherichia coli</i>	2.75 Å	XRD	179.75	1) A, C, E, G 2) B, D, F, H
CDK1/cyclinB1/CKS2	5LQF	Transferase	<i>Homo sapiens</i>	<i>Escherichia coli</i>	2.06 Å	XRD	153.23	1) A, D 2) B, E 3) C, F

PDB, Protein Data Bank; TSW, Total structure weight; Å, Angstrom; XRD, X-ray diffraction.

Preparation of ligands

The main chemical constituents of LN and LT were identified in our previous study⁽¹⁰⁾. These compounds have demonstrated anticancer activity by inducing cell cycle arrest and apoptosis. To evaluate the drug-like properties of the active compounds, a drug-likeness prediction was performed using the SwissADME online tool to determine their

physicochemical parameters, including molecular weight, lipophilicity, hydrogen bond donors, and hydrogen bond acceptors⁽¹⁸⁾. The chemical structures were drawn using ChemDraw (Mol format) and subsequently converted into 3D structures in the MOE software for further analysis. Table 2 lists the ligands used in this study.

Table 2. Chemical compounds and physicochemical parameters of compounds identified by LC-HRMS/MS analysis in ethyl acetate extracts of *L. numidicum* (LN) and *L. trigynum* (LT).

Compounds Name	MW (g/mol)	Conse Log $P_{o/w}$	TPSA (\AA^2)	GI Abs	P-gp Sub	Lipinski	CYP3A4 inhibitor
6,4'-dimethoxy- scutellarein-7-neohesperidoside	622.57	-0.01	227.2	low	Yes	No	No
8,3',4'-trihydroxyflavone-7-O-6(6''-O-p-coumaroyl)- β -D-glucopyranoside	594.52	1.15	216.58	low	No	No	No
Foliasalacioside B1	504.57	-0.57	175.37	low	No	No	No
Isovitexin 2''-O-arabinoside	564.49	-1.26	239.9	low	yes	No	No
Luteolin-7,3'-di-O- β -D-glucoside	610.52	-1.49	269.43	low	yes	No	No
Malvidin 3-O- β -galactoside	493.44	-0.74	191.67	low	No	No	yes
Olivil 4'-O- β -D-glucoside	538.54	0.1	187.76	low	Yes	No	No
Rutin	610.52	-1.29	269.43	low	Yes	No	No
Vicenin-2 isomer 2	594.52	-1.98	271.2	low	No	No	No
Vicenin-2 isomer 3	594.52	-1.98	271.2	low	Yes	No	No
Violanthin	578.52	-1.43	250.97	low	Yes	No	No
Vitexin-2''-rhamnoside	578.52	-0.99	239.97	low	Yes	No	No

MW, Molecular weight; Conse log $p_{o/w}$, Consensus Log Partition Coefficient (octanol/water); TPSA, Topological Polar Surface Area; GI Abs, Gastrointestinal Absorption; P-gp Sub, P-glycoprotein Substrate; CYP3A4, Cytochrome. P450 3A4 inhibitor.

The chemical structures and physicochemical properties of drugs currently in clinical trials for cancer therapy specifically targeting CDK1 were presented in Table 3. This table provides key insights into the characteristics and features of these drugs, facilitating the understanding and evaluation of their potential efficacy against cancer^{(19)–(23)}.

Table 3. Chemical structures and physicochemical parameter of main proposed drugs for cancer treatment.

Drugs name	Pub chem ID	MW (g/mol)	Conse Log $P_{o/w}$	TPSA (\AA^2)	GI Abs	P-gp Sub	Lipinski	CYP3A4 inhibitor
Flavopiridol	44297210	401.8	2.78	94.14	high	Yes	Yes	Yes
Dinaciclib	46926350	396.49	1.84	91.15	high	Yes	Yes	No
AZD5438	16747683	371.46	2.51	98.15	high	Yes	Yes	No
NU6102 (4SP)	4566	402.47	2.22	144.26	Low	Yes	Yes	No

Drugs name	Pub chem ID	MW (g/mol)	Conse Log $P_{o/w}$	TPSA (\AA^2)	GI Abs	P-gp Sub	Lipinski	CYP3A4 inhibitor
Indirubin	135398511	365.38	2.38	110.43	high	Yes	Yes	No
Roniciclib	45380979	430.44	3.74	116.57	Low	Yes	Yes	Yes
Podo- phyllotoxin	10607	414.41	2.28	92.68	high	No	Yes	Yes

MW, Molecular weight; Conse log $p_{o/w}$, Consensus Log Partition Coefficient (octanol/water);

TPSA, Topological Polar Surface Area; GI Abs, Gastrointestinal Absorption;

P-gp Sub, P-glycoprotein Substrate; CYP3A4, Cytochrome. P450 3A4 inhibitor.

Molecular docking

To evaluate their potential interactions with the target proteins, natural ligands identified from the EtOAc fractions of LN and LT, along with the reference drugs, were subjected to energy minimization under standard conditions (temperature 300 K, pH 7). Molecular docking was then carried out using MOE 2015.10 against the CDK1/Cks2 (PDB ID: 6GU7) and CDK1/cyclinB1/CKS2 (PDB ID: 5LQF) complexes. The Triangle Matcher method was employed for ligand placement, generating 75 poses per ligand. The resulting poses were refined and scored using the GBVI/WSA dG scoring function, and the best three poses for each compound forming higher interactions were selected for further analysis.

RESULTS AND DISCUSSION

The results obtained by Mouna et al.⁽¹⁰⁾ demonstrated that the EtOAc extracts of LN and LT induced cell cycle arrest in PC3 cells at the G2/M phase. This blockage already suggested an interference with key cell cycle regulators, particularly CDKs, notably CDK1, which plays an essential role in the G2/M transition⁽²⁴⁾. To further investigate these observations, we conducted a complementary molecular docking study to evaluate the affinity of the compounds identified in the extracts toward CDK1, a major therapeutic target in several cancers, including prostate cancer. For each ligand, 75 docking poses were initially generated. These poses were scored and ranked, and the top three poses per ligand were selected for further analysis. The final ranking of the compounds was determined based on a consensus between the best docking score (ΔG) and the formation of key hydrogen bonds with crucial residues in the ATP-binding pocket. The

compound exhibiting the most favorable balance between these two parameters was identified as the top candidate.⁽²⁵⁾

The in silico pharmacokinetic profiling revealed a distinct ADME (Absorption, Distribution, Metabolism, and Excretion) divergence between the investigated natural compounds (Table 2) and the established synthetic CDK inhibitors (Table 3). The natural compounds consistently exhibited high molecular weights (MW > 500 Da), negative consensus Log P values, and large topological polar surface areas (TPSA > 175 \AA^2). Collectively, these characteristics explain their uniformly predicted low gastrointestinal absorption and their frequent classification as P-glycoprotein substrates, suggesting significant limitations in oral bioavailability.⁽²⁶⁾ In contrast, the synthetic drugs generally complied with Lipinski's Rule of Five, showing lower MW and positive Log P values, which correlate with higher predicted gastrointestinal absorption (GI Abs) in most cases. However, this enhanced permeability is counterbalanced by a greater risk of drug–drug interactions, as several synthetic inhibitors (e.g., Flavopiridol, Roniciclib) are predicted to inhibit CYP3A4, a key metabolic enzyme.⁽¹⁸⁾ Consequently, while the natural compounds may display poor absorption, the synthetic agents could pose toxicity risks associated with cytochrome P450 inhibition.⁽²⁷⁾

Molecular docking studies

The molecular docking study carried out on the 78 compounds identified in EtOAc extracts of both *Linum* species assessed their affinity for the CDK1/Cks2 (PDB ID: 6GU7) and CDK1/cyclin B1/CKS2 (PDB ID: 5LQF) complexes, 12 most promising were listed in the (Table 5). These results were compared with reference inhibitors (Table 4) to identify the most promising molecules as potential CDK1 inhibitors.

Table 4. Docking results of drugs under clinical test and reference ligands inhibitors.

Drugs and reference ligands	6GU7		5LQF receptor A		5LQF receptor D	
	E score	RMSD	E score	RMSD	E score	RMSD
Flavopiridol	-7.4933	1.6222	-7.6560	2.0977	-7.5930	1.1823
Dinaciclib	-7.5641	2.9463	-8.6410	1.6370	-7.7471	2.7987
AZD5438	-6.7570	1.4343	-8.2058	1.1977	-7.6836	1.4969
NU6102 (4SP)	-7.0154	1.4603	-9.2294	1.8517	-8.7623	2.1990
Indirubin	-5.9587	1.17179	-6.5945	0.8525	-6.4472	0.8468

Drugs and reference ligands	6GU7		5LQF receptor A		5LQF receptor D	
	E score	RMSD	E score	RMSD	E score	RMSD
Roniciclib	-7.1375	1.75631	-8.9343	0.8115	-8.6737	2.6749
podophyllotoxin	-6.6977	2.1235	-7.6797	2.0758	-7.3931	2.1096

E score, binding energy score (kcal/mol); RMSD, Root Mean Square Deviation (Å).

Table 5. Results of docking of compounds identified in ethyl acetate extracts of *L. numidicum* and *L. trigynum* with 6GU7 and 5LQF targets

Ligands	6GU7		5LQF/A		5LQF/D	
	E score	RMSD	E score	RMSD	E score	RMSD
6,4'-dimethoxy-scutellarein-7-neohesperidoside	-8.7922	2.4460	-9.8332	1.4417	-9.6457	1.6701
8,3',4'-trihydroxyflavone-7-O-6(6''-O-p-coumaroyl)-β-D-glucopyranoside	-8.8836	1.5144	-9.8308	2.1867	-9.6374	2.0696
Foliasalacioside B1	-8.1466	2.2304	-7.9735	2.0343	-9.0780	1.5581
Isovitexin 2''-O-arabinoside	-8.2867	2.2208	-8.7986	1.0639	-9.0273	1.3896
Luteolin-7,3'-di-O-β-D-glucoside	-8.4639	1.5872	-9.7184	1.4914	-9.3306	2.0331
Malvidin 3-O-β-galactoside	-7.5680	1.7951	-8.7742	2.6041	-8.9311	1.8049
Olivil 4'-O-β-D-glucoside	-8.0439	1.5787	-8.8431	2.1685	-8.5054	1.7226
Rutin	-8.5279	1.7807	-9.5872	2.1477	-9.1845	1.8575
Vicenin-2 isomer 2	-8.9413	1.3524	-9.7820	1.4479	-9.6997	1.1453
Vicenin-2 isomer 3	-8.5247	1.2757	-8.7863	1.8631	-9.6332	1.3865
Violanthin	-8.0624	1.2929	-9.1498	2.5548	-9.5624	1.5645
Vitexin-2''-rhamnoside	-8.5079	2.4076	-8.6167	3.7997	-8.9943	1.4957

E score, binding energy score (kcal/mol); RMSD, Root Mean Square Deviation (Å).

Analyses revealed that the CDK1/cyclin B1/CKS2 complex (5LQF) exhibited overall lower binding energy values than the CDK1/CKS2 complex (6GU7), indicating more stable interactions with the tested ligands. Among the inhibitors evaluated, NU6102 (4SP) showed the lowest binding energies for the 5LQF sites A and D, with values of -9.2294 kcal/mol and -8.7623 kcal/mol, respectively. Conversely, for the 6GU7 complex, dinaciclib displayed the highest binding affinity, with a score of -7.5641 kcal/mol (Table 4).

Among the compounds studied, most ligands showed greater affinity for the 5LQF complex than the reference inhibitor NU6102 (4SP), with binding energies below -9.0 kcal/mol. For active site A, five compounds exhibited particularly low binding energy values: **6,4'-dimethoxy-scutellarein-7-neohesperidoside** (-9.8332 kcal/mol), **8,3',4'-trihydroxyflavone-7-O-6(6''-O-p-coumaroyl)-β-D-glucopyranoside** (-9.8308 kcal/mol), **vicenin-2 isomer2** (-9.7820 kcal/mol), **luteolin-7,3'-di-O-β-D-glucoside** (-9.7184 kcal/mol), and **rutin** (-9.5872 kcal/mol).

With regard to active site D, six compounds also demonstrated high affinity: **Vicenin-2 isomer2** (-9.6997 kcal/mol), **6,4'-dimethoxy-scutellarein-7-neohesperidoside** (9.6457 kcal/mol), **8,3',4'-trihydroxyflavone-7-O-6(6''-O-p-coumaroyl)-β-D-glucopyranoside** (-9.6374 kcal/mol), **vicenin-2 isomer3** (-9.6332 kcal/mol), **violanthin** (-9.5624 kcal/mol), and **luteolin-7,3'-di-O-β-D-glucoside** (-9.3306 kcal/mol).

While several compounds exhibited higher binding affinity than dinaciclib (-7.5641 kcal/mol), six stood out with significantly lower energy scores, testifying to particularly stable interactions with CDK1 (6GU7): **vicenin-2 isomer 2** (-8.9413 kcal/mol), **8,3',4'-trihydroxyflavone-7-O-6(6''-O-p-coumaroyl)-β-D-glucopyranoside** (-8.8836 kcal/mol), **6,4'-dimethoxy-scutellarein-7-neohesperidoside** (-8.7922 kcal/mol), **rutin** (-8.5279 kcal/mol), **vicenin-2 isomer 3** (-8.5247 kcal/mol), and **vitexin-2''-rhamnoside** (-8.5079 kcal/mol). The compound **8,3',4'-trihydroxyflavone-7-O-6(6''-O-p-coumaroyl)-β-D-glucopyranoside**, identified exclusively in the LN EtOAc extract, exhibited lower binding

energies than all inhibitors tested against both 5LQF (sites A and D).

The results also revealed that several major compounds from the EtOAc extracts of both *Linum* species⁽¹⁰⁾, notably vicenin-2 and its isomers, showed particularly low energy scores for 5LQF (sites A and D) and 6GU7. The low root means square deviation (RMSD) values (consistently below 2.0 Å for the top-ranked poses), together with these high-affinity interactions, indicate a high degree of pose reliability and geometric consistency, thereby reinforcing the robustness and significance of these findings. These findings align with computational benchmarks for kinase inhibitors. Our docking scores compare favorably with those reported in validated studies using similar methodologies, where successful CDK1 inhibitors typically exhibit scores between -8.5 and -10.5 kcal/mol.^{(28),(29)} Successful posture reproduction was defined as RMSD < 2.0 Å for 75% of re-docked ligands in the Comparative Assessment of Scoring Functions (CASF) 2016 benchmark⁽²⁹⁾. Our vicenin-2 isomers' geometric consistency (RMSD 1.1-1.4 Å) was superior than flavonoid glycosides' mean RMSD of 2.3 Å in the KLIFS kinase-ligand interaction database investigation of 45 CDK-inhibitor complexes⁽³⁰⁾. Results align with docking validation studies that reference the PDBbind database⁽³¹⁾ as a standard benchmark, where RMSD < 2.0 Å is commonly used as a criterion for successful pose reproduction. Additionally, our findings demonstrate similar pose accuracy than Patel (2025)⁽³²⁾, who reported RMSD values of 1.8-2.3 Å for 1574 natural compounds from the NPACT database targeting CDK1.

Ligands-Protein Interactions

Interactions of ligands with 6GU7

The interaction between vicenin-2 isomer 2 (binding energy: -8.9413 kcal/mol) and 6GU7 revealed six potential hydrogen bonds: five hydrogen-donor interactions involving the amino acid residues Asp128, Asp146, Asp86, Ser84, and Asp128, and one hydrogen-acceptor interaction with Lys89. The corresponding interaction energies were -0.8, -2.6, -0.8, -1.8, -1.2, and -2.4 kcal/mol, with hydrogen bond distances of 3.18, 2.76, 3.24, 2.8, 3.34, and 2.81 Å, respectively (Figure 3).

This extensive hydrogen-bonding network aligns with computational studies of high-affinity CDK1 inhibitors, where interaction with Asp86 and Asp146 in the hinge region is consistently reported as critical for sub-micromolar activity⁽³³⁾. The binding energy scores falls within the range reported for known flavonoid-based CDK1 inhibitors (-8.5 to -10.2 kcal/mol) in similar docking studies using MOE.⁽³⁴⁾

The highest number of hydrogen bond interactions between ligands and the 6GU7 complex was observed with 8,3',4'-trihydroxyflavone-7-*O*-6(6''-*O*-*p*-coumaroyl)-β-D-glucopyranoside (Figure 4), which achieved the second-lowest binding energy among all ligands tested (-8.8836 kcal/mol). This compound formed six hydrogen-donor interactions with the residues Asp128 (two bonds), Ile10, Asp86,

and Asp146, as well as two hydrogen-acceptor interactions with Lys89, and one additional interaction with Thr14. The interaction energies were -2.0, -4.1, -2.1, -1.0, -3.9, -5.5, -3.8, -1.0, and -1.4 kcal/mol, with corresponding bond distances of 2.81, 2.69, 2.88, 3.16, 2.65, 2.74, 2.84, 3.30, and 4.26 Å.

Moreover, most compounds identified from the EtOAc extracts of *Linum* species (LN and LT) established at least four potential hydrogen bonds with the 6GU7 complex. The detailed interaction data for best 12 compounds are provided in the supplementary Information (Table S1).

Interactions of ligands with 5LQF

The interaction between 6,4'-dimethoxy-scutellarein-7-neohesperidoside and the 5LQF receptor A (binding energy: -9.8332 kcal/mol) revealed seven hydrogen bond interactions. These included two H-donor bonds with amino acids Ile10 and Glu12, and three H-acceptor bonds with Gln132, LEU83, and Glu12. Additionally, two π-H interactions were observed with Val18 and Ala145. The corresponding interaction distances were 2.83, 2.79, 2.92, 3.43, 2.97, 4.28, and 4.22 Å, with respective interaction energies of -3.0, -1.7, -1.7, -1.4, -2.4, -0.6, and -0.8 kcal/mol (Figure 5). This binding affinity (-9.83 kcal/mol) is particularly notable, as it exceeds the threshold of -9.0 kcal/mol that Sankket et al⁽³⁴⁾ correlated with sub-micromolar CDK1 inhibition in their benchmark study of 15 flavonoids as anticancer.

For other receptor D, the interaction with Vicenin-2 isomer 2 binding energy: -9.6997 kcal/mol (Figure 6), exhibited two hydrogen bonds and two π-H interactions. These included one H-donor and one H-acceptor bonds with Leu83, as well as two π-H interactions involving Val18 and Ala145. The corresponding distances were 3.09, 3.40, 4.33, and 4.19 Å, with associated interaction energies of -0.7, -1.4, -0.7, and -0.8 kcal/mol, respectively (Figure 4b). Furthermore, the other compounds interactions with the 5LQF/A complex were described in supplementary Tables S2 and S3).

The results of this study confirm that several compounds present in the *Linum* EtOAc extracts exhibit a strong affinity for CDK1 complexes, particularly for the CDK1/cyclin B1/CKS2 (5LQF) complex, suggesting their potential role as CDK1 inhibitors. Comparative analysis between the two protein complexes studied revealed that 5LQF is the most stable with the ligands tested. This may be attributed to the synergistic stabilizing effect of cyclin B1 and CKS2, which maintain the CDK1 conformation, thereby enhancing its interaction with inhibitors⁽³⁵⁾.

One of the most promising compounds, 8,3',4'-trihydroxyflavone-7-*O*-6(6''-*O*-*p*-coumaroyl)-β-D-glucopyranoside, was identified exclusively in the EtOAc extract of LN. This compound displayed the lowest binding energies at both 5LQF sites A and D, which may explain the stronger cell cycle blockade observed for the LN EtOAc extract compared to the LT EtOAc extract, as previously reported⁽¹⁰⁾. Moreover, the major compounds identified in the EtOAc

extracts of LN and LT, notably vicenin-2 and its isomers, showed high affinity for both CDK1 complexes (5LQF and 6GU7). The affinity ranges and interaction patterns of flavonoid derivatives in CDK1 docking studies were similar, with substantial hydrogen bonding and hydrophobic interactions impacting inhibitory potential. Retamal & Caballero 2016⁽³⁶⁾ found that flavonoid scaffolds docking simulations reveal different binding orientations and activity patterns due to hydrogen bond donors and hydrophobic interactions. Moreover, many biochemical tests shown that nitrogen-containing flavonoid analogues inhibit CDK1/Cyclin B, highlighting the importance of flavonoid scaffolds for CDK1 suppression.⁽³⁷⁾

Binding mode analysis confirms competitive inhibition

To confirm that the investigated compounds act as competitive ATP inhibitors, we analyzed their binding modes in comparison with the co-crystallized reference inhibitors NU6102 (in 5LQF) and AZD5438 (in 6GU7). Docking simulations revealed that all top-ranked compounds bind directly within the ATP-binding pocket and exhibit a high degree of pose similarity with these known competitive inhibitors. This spatial overlap with established ATP-competitive inhibitors provides strong computational evidence for a competitive mechanism, consistent with validation protocols used in kinase inhibitor discovery pipelines⁽³⁸⁾

The binding pose superpositions in Figure 7 demonstrate that Vicenin-2 isomer 2 not only occupies the ATP-binding pocket but also forms a more robust hydrogen-bonding network than the reference compounds. Vicenin-2 forms six hydrogen bonds (described above) including the critical hinge interaction, compared to four for AZD5438 (Figure 7a) and only one for Dinaciclib (Figure 7b). This enhanced interaction profile within the same site provides compelling evidence that Vicenin-2 acts as a competitive inhibitor.

Also 6,40-dimethoxy scutellarein-7-neohesperidoside demonstrate a superior interaction profile compared to NU6102. In the ATP-binding pocket of 5LQF receptor A (Figures 8a), our compound forms seven hydrogen bonds (described above), one more than the reference ligand. In receptor site D (Figures 8b), both compounds perform equally well, each forming seven hydrogen bonds, confirming the high affinity of our compound for the target sites. The findings align with earlier research indicating that dinaciclib is a powerful ATP-competitive inhibitor of CDK1 Parry et al., 2010⁽³⁹⁾ and that NU6102 suppresses CDK1/2 in cancer cells under treatment, the increased affinity of NU6102 for 5LQF may result from the stabilizing effects of cyclin B1 and CKS2, establishing a dependable criterion for assessing natural compounds as prospective CDK1 inhibitors.

This affinity, which exceeds that of the reference inhibitors, suggests a promising inhibitory potential likely responsible for the observed antiproliferative effects. The docking results also revealed that several other compounds present in the EtOAc extracts of LN and LT, including 6,4'-dimethoxy-

scutellarein-7-neohesperidoside, luteolin-7-O- β -D-glucoside, rutin, violanthin, foliasalacioside B1, isovitexin 2''-O-arabinoside, vitexin-2''-rhamnoside, and malvidin 3-O- β -galactoside, exhibited strong binding affinities toward CDK1, with binding energies lower than those of the reference inhibitors tested. This aligns with computational studies showing that 37 flavonoids often outperform inhibitors in CDK1 docking due to their extended interaction networks⁽³⁶⁾. This high affinity reinforces our previous findings: inhibition of CDK1 prevents activation of the cyclin B/CDK1 complex, a key regulator of the G2/M transition, thereby explaining the G2/M cell cycle arrest observed in PC3 cells following treatment with the EtOAc extracts from both plants. Similar correlations between CDK1 docking scores and G2/M arrest have been reported for other natural products.⁽⁴⁰⁾

These findings suggest that the identified natural compounds could serve as promising candidates for the development of CDK1-targeted inhibitors in innovative cancer therapies.

Analysis of the molecular interactions revealed a complex network of bonds, mainly hydrogen bonds, but also hydrophobic and polar interactions. These multiple interactions confer notable stability to the formed complexes, an essential requirement for effective CDK1 inhibition.

Hydrogen bonds ensure precise anchoring of the ligands within the active site, promoting an optimal orientation for interaction, whereas hydrophobic interactions significantly contribute to the stabilization of the ligand-protein complex. This multimodal binding mechanism plays a key role in the molecular recognition and selective inhibition of enzymatic targets. The combination of hydrophobic filling, electrostatic steering, and optimized hydrogen bonding represents a comprehensive binding strategy observed in successful kinase inhibitor designs.⁽⁴¹⁾

In line with the findings of Sahu et al.⁽⁴²⁾, hydrophobic forces play a fundamental role in the final phase of the binding process, filling hydrophobic cavities within the active site, reducing void spaces, and reinforcing complex cohesion through van der Waals interactions. Electrostatic forces also contribute to the initial long-range molecular recognition, facilitating the orientation of ligands toward the catalytic pocket.

Another factor influencing complex stability lies in the specific geometry of the binding systems. As demonstrated by Rupniak et al.⁽⁴³⁾, a temporarily stabilized ring system, dependent on the conformation of donor and acceptor groups, can reduce the energetic penalty associated with desolvation. Such a conformation may enable ligands like vicenin-2 to navigate the protein structure more effectively, thereby enhancing their capacity to establish long-lasting and specific interactions with CDK1.

Finally, the work of Thorson et al.⁽⁴⁴⁾ emphasizes that even a single additional hydrogen bond can enhance the biological activity of a ligand by up to a hundredfold,

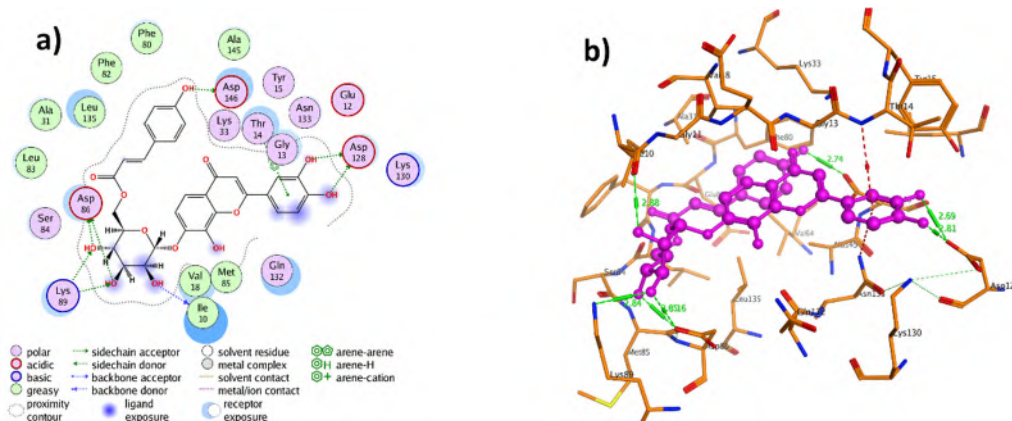


Figure 4. a) 2D and b) 3D interactions of 8,3',4'-trihydroxyflavone-7-O-(6''-O-p-coumaroyl)- β -D-glucopyranoside in the binding site of 6GU7. 3D: Ligand in purple, residues pocket in orange, hydrogen bonds in green, pi interactions in red.

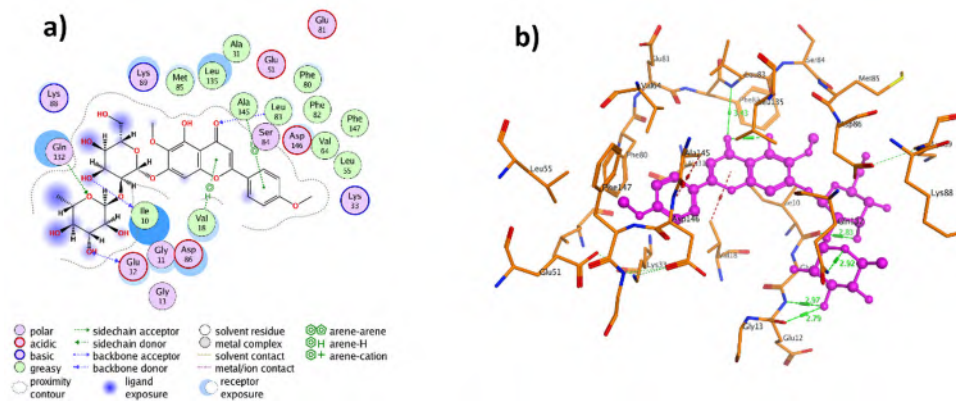


Figure 5. a) 2D and b) 3D interactions of 6,4'-dimethoxy in the binding sites of 5LQF receptor A. 3D: Ligand in purple, residues pocket in orange, hydrogen bonds in green, pi interactions in red.

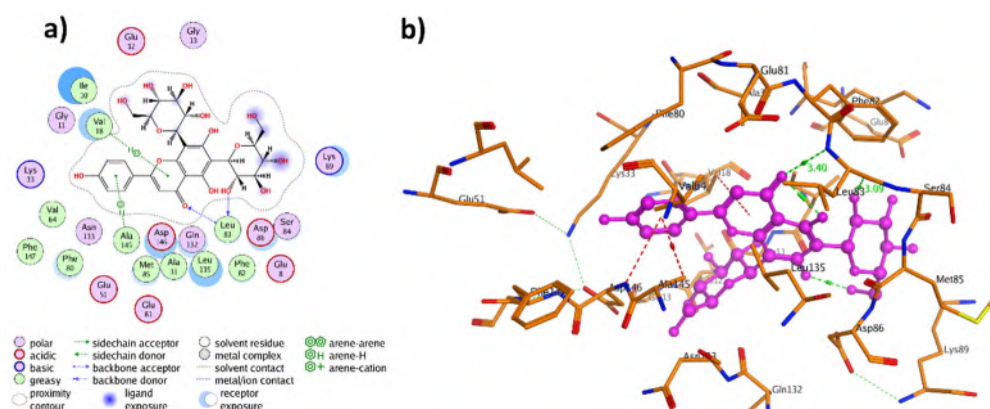


Figure 6. a) 2D and b) 3D interactions of vicenin-2 isomer 2 in the binding sites of 5LQF receptor D. 3D: Ligand in purple, residues pocket in orange, hydrogen bonds in green, pi interactions in red.

rhamnoside, olivil 4'-*O*- β -D-glucoside and malvidin 3-*O*- β -galactoside demonstrated binding affinities comparable to, or even greater than, those of the reference inhibitors NU6102 and dinaciclib.

These *in silico* findings provide a plausible molecular explanation for the previously observed G2/M cell cycle arrest induced by *Linum* extracts in PC3 cells. They identify potential bioactive constituents that may act as CDK1 inhibitors and justify further investigation. However, as molecular docking offers predictive rather than experimental evidence, future work should include molecular dynamics simulations and *in vitro* kinase inhibition assays to validate the biological relevance of these interactions.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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THE EFFECTS OF SELECTIVE SEROTONIN REUPTAKE INHIBITORS ON MOTILITY OF PERIPHERAL SMOOTH MUSCLES

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ABSTRACT

According to the World Health Organization, part of the quality of life is the perception of one's body concerning the culture and values of each individual. Limb amputation affects the quality of life through several defined aspects. Observing the available statistical data, after the amputation of the lower extremities, only 65% survived the first year, 52% survived the first two years, 37% survived three years, and five or more years, only up to 15% of patients with amputated limbs. Epidemiological data on the frequency of lower limb amputations are pretty similar to those in developed countries. Modern prostheses enable daily use, intending to replace the missing limb. Many factors influence the outcome of rehabilitation treatment and the result of functional recovery. Among the factors that influence the favorable outcome of rehabilitation and independence in activities of daily living is the level of amputation, age, and comorbidities. For patients after amputation of the lower extremities, the goal in rehabilitation is to achieve as much independence as possible within the activities of daily life. Our study aimed to examine daily functionality and activity through the Barthel and functional independence indices-FIM tests during post-operative rehabilitation and after one month.

Keywords: Amputation, Barthel index, FIM test.

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INTRODUCTION

According to the World Health Organization, part of quality of life includes the perception of one's own body in relation to the culture and values of each individual. Limb amputation affects quality of life through multiple multidimensional aspects: psychological, physical, social, familial, housing, and sense of security. When considering statistics post-amputation, only 65% survive the first year, 52% the first two years, 37% three years, and five or more years only up to 15% of patients with amputated limbs. The high mortality rate among amputees is primarily attributed to depression, which influences patients to reject further therapeutic treatment methods. Epidemiological data on the frequency of lower limb amputations are quite similar to those in developed countries, with differences in the number of amputations, type, and cause. (1) Amputation originates from the Latin word "amputare" and can be literally defined as the complete or partial removal of a certain part of the body. (2) In rehabilitative medicine, the main goal for patients after lower limb amputation is to achieve better functionality in daily activities and walking with the aid of a prosthesis. (3) Modern prostheses enable daily use with the aim of replacing the missing limb. An important aspect is comprehensive and quality rehabilitation, enabling patients to return to everyday work activities, social life, or work with minimal functional limitations. The outcome of rehabilitation treatment and functional recovery results are influenced by numerous factors such as the cause and level of amputation, prosthesis design, presence of phantom pain, and the patient's psychosocial status. (4) Factors influencing favorable rehabilitation outcomes and independence in daily activities include the level of amputation, age, and comorbidities. (5) The goal of rehabilitation for patients after lower limb amputation is to achieve as much independence as possible in daily activities. (6) After lower limb amputation, adequate care for the residual limb, reduction of swelling and pain if present, and initiation of early rehabilitation and pre-prosthetic preparation of patients are necessary. (7)

The aim of our study was to investigate functionality and daily activities using the Barthel Index and the Functional Independence Measure (FIM) test during pre-prosthetic rehabilitation and after one month.

MATERIALS AND METHODS

Study Design and Study Population

The study was designed as a clinical prospective study, including participants of both sexes, aged 18 to 75 years, who were treated and underwent rehabilitation, both on an outpatient and inpatient basis, at the Department of Physical Medicine and Rehabilitation of the Clinical Center Kragujevac. The study was conducted from April 2023 for the next year, and the data used for the study were collected from April to November 2023.

The Ethics Committee of the University Clinical Center approved the study number 01-23/167 by decision of 08.05.2023. years. Patients were referred by a vascular surgeon or a chosen physician to a physiatrist after lower limb amputation. Each patient first underwent a consultative examination, and after establishing indications, was referred for hospitalization for further pre-prosthetic and prosthetic preparation for functional recovery. Patients signed informed consent for the study before inclusion. Inclusion criteria for study participants were:

- patients with unilateral lower limb amputation above the ankle level who were admitted for stationary pre-prosthetic and prosthetic rehabilitation at the Department of Physical Medicine and Rehabilitation;
- patients with unilateral transtibial or transfemoral and partial foot amputation according to Chopart;
- patients who were first fitted with a prosthesis (including patients who received the prosthesis during rehabilitation);
- patients who understand and speak the Serbian language.

Exclusion criteria for study participants were:

- cognitively impaired patients, with a score lower than 27 on the MMSE;
- individuals who refuse to participate in the study if there is another objective reason preventing or complicating participation in the study (other diseases and conditions affecting the general health condition and functionality of participants – infectious diseases, tuberculosis, malignancies, presence of deformities after trauma...);
- patients with bilateral transtibial or transfemoral amputations;
- patients with incomplete medical documentation.

Sampling of the study population was performed from the entire population of patients referred for rehabilitation after lower limb amputation. Each patient, upon admission for hospitalization and rehabilitation, was examined by the investigator successively to determine inclusion and exclusion criteria. Patients who met the criteria were included in the study up to the number calculated for the total sample size.

On the first day of hospitalization, after signing the consent to participate in the study, a physiatrist took a complete medical history from the patient and assessed the activities of daily living using the Barthel Index, FIM test, as well as the cognitive status using the MMSE.

During hospitalization, a kinesiotherapy program for functional recovery and pre-prosthetic preparation was conducted, including: residual limb bandaging, strengthening of the muscles of the residual limb, strengthening of the muscles of the contralateral lower limb, upper limbs, and patient verticalization. The physiotherapist, upon discharge or during hospitalization, assessed the mobility of the patient after amputation using the Amputee Mobility Predictor (AMP) test,

and if the patient had an adequate level of mobility according to the test, the prescription of an aid or prosthesis was indicated.

Upon discharge, the patient received the prescribed aid or prosthesis and in home conditions, with the help of a physiotherapist, continued the kinesiotherapy program for functional recovery, or if the aid was received during hospitalization, the program began stationary and continued in home conditions. One month after receiving the aid or prosthesis, patients came for a control examination, as outpatients, for functional testing of the aid, and on that occasion, a control testing was done by the investigator using the Barthel index and FIM test.

The Barthel Index was introduced into everyday practice in 1965 and is one of the most commonly used tools for assessing functional status, primarily for assessing activities of daily living. It scores personal hygiene, bathing, feeding, using the toilet, climbing stairs, dressing, bowel control, bladder control, transferring from chair to bed, mobility, and wheelchair mobility. The maximum score is 100, indicating complete independence in performing activities of daily living. The lowest score is 0, representing complete dependence. Specifically, 0-20 indicates complete dependence, 21-60 indicates severe dependence, and 61-90 indicates moderate dependence. (8)

FIM is a functional index that measures the motor and cognitive functioning of the patient. Items are divided into 6 groups: self-care, sphincter control, mobility, locomotion, communication, and social integration. Considering the required amount of assistance for each of the 18 items, a score from 1 (complete assistance) to 7 (complete independence) can be given. By summing up the scores, the total FIM can range from a minimum of 18 to a maximum of 126 points. The questionnaire consists of two parts. The first part comprises motor functions, and the second part comprises the aforementioned cognitive functions. Within motor functions, there are 4 major sections: self-care, sphincter control, mobility, and locomotion. Within them, there are a total of 13 smaller parts, each individually scored from 1 to 7. Thus, the number of points for motor functions must be between 13 and 91. Cognitive functions consist of two major sections: communication and social cognition. Within them, there are a total of 5 smaller parts, also scored from 1-7, so the number of points for cognitive functions must be between 5 and 35. Patients receive 6 and 7 points for a specific action, depending on whether they can perform it independently. 7 points are awarded if performed completely independently, and 6 if performed with the assistance of aids. Points 1 to 5 are given if assistance from another person is needed. 5 points are given if the subject does everything independently but requires supervision, 4 points are given if another person provides a little help, while 3 points are given when another person helps significantly, but the subject still does more than 50% of the work on their own. 1 and 2 points are given when the subject is entirely dependent, with 2 points awarded if the subject does more than 25% of the work themselves, and 1 point

awarded if they do less than 25% on their own. Looking at the entire FIM questionnaire, we see that it consists of 18 parts, which are evaluated from 1 to 7 according to the rules mentioned above. The higher the number, the more independent the patient is and the better their functional status. (9)

The AMP index assesses the mobility of individuals after lower limb amputation and their existing or potential functional capabilities. Each item included in the AMP is selected for its contribution to the overall assessment of function in individuals after amputation. The AMP questionnaire is based on objective measurements and examines six different domains of mobility: sitting balance, transfers, standing balance, walking, stair climbing, and the use of assistive devices, through a total of 21 items. (10)

Statistical Analysis

The collected data were organized and entered into the statistical software SPSS version 20. The data were first descriptively analyzed; for continuous variables, measures of central tendency and measures of variability were determined, while for categorical variables, the frequency of individual categories was assessed. The significance of differences in observed values of continuous variables was tested using paired t-tests in the case of normal distribution (which was previously examined by the Kolmogorov-Smirnov test), or the Wilcoxon signed-rank test for results that do not follow a normal distribution.

RESULTS

In our study, there were 32 participants, of whom 8 (25%) were female and 24 (75%) were male. The largest percentage of participants were aged 61 to 70 years, accounting for 37.5% (12 participants), followed by the age group of 41 to 50 years, representing 31.3% (10 participants), the age group of 71 to 75 years, comprising 18.8% (6 participants), and the smallest percentage was the age group of 51 to 60 years, with 12.5% (4 participants). Participants with completed secondary education dominated at 56.3% (18 participants), followed by those with a university degree at 25% (8 participants), and those with completed primary education at 18.8% (6 participants). The majority of participants were in a marital union, comprising 68.8% (22 participants), while 18.8% were divorced (6 participants). There were 6.3% each in the widowed and single groups (2 participants each). Within a multi-member family, 68.8% of participants lived (22), while 25% lived alone (8 participants), and 6.3% (2 participants) lived in a nursing home. Participants' occupations were predominantly retirees at 56.3% (18 participants), followed by professionals in their field at 18.8% (6 participants). Farmers and the unemployed were represented equally at 12.5% each (4 participants each) (Table 1). The majority of participants were cigarette smokers at 62.5% (20 participants), while 37.5% were non-smokers (12 participants). The average length of hospitalization for participants was 17.25 ± 4.040 days. Half of the participants had a right transfemoral

amputation (16 participants), 37.5% of participants had a left transfemoral amputation (12 participants), while 12.5% of participants had a right transtibial amputation. In the case of the majority of participants, 75% (24), amputations occurred as a result of vascular diseases, while trauma was the cause of amputations in 25% (8) of participants.

Table 1. Presentation of patients based on the type and cause of amputation

Type of amputation	N	%
Right transfemoral	16	50.0
Left transfemoral	12	37.5
Right transtibial	4	12.5
Left transtibial	0	0
Cause of amputation	N	%
Vascular diseases	24	75.0
Trauma	8	25.0

The functionality of the subjects was assessed using the Barthel Index and the Functional Independence Measure (FIM) test on the first day of hospitalization and one month after completion of hospital treatment and arrival of aids. The results indicate statistically significant improvement in functionality measured by the Barthel Index (<0.001), as well as by the FIM test (<0.001), motor sub-score, and overall FIM test. The cognitive sub-score remained identical at the first and follow-up assessments with a value above 26, according to the MMSE scale. The mobility index of patients after amputation (AMP) was 13.00 at the initial assessment, and it showed a statically significant increase during the follow-up assessment, reaching 16.50 (Table 2).

Table 2. The functionality of the patients

	Admission to the hospital	After one month of admission	p
FIM	85.38±14.630	90.44±15.629	<0.001*
FIM motor score	66.00±13.090	71.06±13.891	<0.001*
FIM cognitive score	19.38±1.540	19.38±1.738	
Barthel Index	75.0 (20)	80.0 (20)	<0.001**
AMP	13.00 (10)	16.50 (12)	<0.001**

*Paired-Samples T Test; **Wilcoxon Signed Ranks Test

DISCUSSION

The results of our study showed a statistically significant improvement in the mean value of the Barthel index and the FIM test after one month compared to the values during pre-prosthetic rehabilitation. This finding is in line with recent studies that highlight the effectiveness of pre-prosthetic rehabilitation in enhancing patient outcomes. For instance, in a study conducted by Gailey et al. it was demonstrated that early and comprehensive rehabilitation interventions significantly improve functional independence in patients after lower limb amputation, particularly in terms of mobility and self-care activities (10). Similarly, a meta-analysis by Silva et al. further emphasized that structured rehabilitation programs lead to improved scores on functional assessments like the Barthel index and FIM, reinforcing the importance of these interventions in post-amputation care (11).

Pre-prosthetic rehabilitation aims to optimize the functional status of the residual limb and prepare the patient for the use of a prosthesis. This comprehensive approach includes wound care, pain management, range of motion exercises, strengthening exercises, gait training, and activities of daily living (ADL) training (12). Addressing these aspects is critical in preparing patients for successful prosthesis use. Studies have shown that interventions such as muscle strengthening, proprioception training, and balance exercises are pivotal in reducing the risk of falls and ensuring long-term functional independence (14). These elements are essential for improving muscle strength, balance, coordination, and proprioception, all of which are key factors for successful prosthetic use and overall mobility outcomes.

The Barthel index and the FIM test are widely used tools for assessing functional status and independence in performing daily activities, and they are crucial in evaluating the effectiveness of rehabilitation interventions (14). The Barthel index assesses basic activities such as bathing, dressing, grooming, feeding, and toileting, providing a focused evaluation of self-care abilities. On the other hand, the FIM test offers a broader scope, assessing mobility, locomotion, communication, and social integration. This dual assessment approach gives a comprehensive picture of a patient's recovery and adaptation following amputation. Our findings align with those of previous studies that have reported improvements in both scores following pre-prosthetic rehabilitation, further underscoring its importance. For example, a randomized control trial by Meier et al. showed that patients who participated in pre-prosthetic rehabilitation exhibited significant improvements in walking ability, ADL independence, and overall quality of life compared to those who did not receive such interventions (12). These results support the notion that pre-prosthetic rehabilitation plays a crucial role in fostering functional recovery and independence, especially in the early stages of post-amputation care (11,14).

The statistically significant improvement in the Barthel index and FIM test observed in our study is consistent with the growing body of literature advocating for early, structured rehabilitation interventions for patients after lower limb amputation. By improving muscle strength, balance, and proprioception, pre-prosthetic rehabilitation serves as a cornerstone for enhancing patient independence and quality of life, making it an essential component of post-amputation care (11,14).

CONCLUSION

Our study shows significant improvements in functionality and independence, as measured by the Barthel Index and FIM test, after one month of pre-prosthetic rehabilitation. Mobility increased, while cognitive function remained stable, highlighting the importance of rehabilitation in enhancing outcomes for patients following lower limb amputation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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POSSIBILITIES AND LIMITATIONS OF X-RAY DIAGNOSTICS IN CASES OF WHOOPING COUGH

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ABSTRACT

Pertussis was a dreaded disease, especially for the very young, having a high risk of death for infants under the age of less than a year. It is characterized by paroxysms of cough, inspiratory whoop, and posttussive vomiting, with more severe forms leading to apnea in infants. The World Health Organization (WHO) describes pertussis as "an extremely contagious respiratory tract disease caused by Bordetella pertussis," a microorganism specific to humans. Pertussis affects people of all ages, especially children, and is one of the leading causes of death in infants under one year of age. The incubation period is usually 7 to 10 days, and clinical characteristics are associated with age, duration of infection, immune status, and antibiotic therapy. Chest X-ray imaging plays a significant role in the diagnosis of pertussis, especially in terms of rapid availability and ease of examination. The availability of chest X-ray imaging allows clinicians to quickly make diagnosis and timely initiation of appropriate treatment. One of the main problems with chest X-ray imaging of patients with pertussis is coughing attacks during the procedure, inefficiency in detecting disease in the early stage, exposure of the patient to ionizing radiation, and interpretation errors of the images.

Keywords: Radiographic diagnostics, pertussis, respiratory infection, diagnosis, interpretation error, additional tests.

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INTRODUCTION

Pertussis, which literally means “a whooping cough”, also known as “the 100-day cough”, was for the first time described in the 1578 Paris epidemic. The disease agent, *Bordetella Pertussis*, was discovered in 1906, and the vaccine was developed in 1940s (1). Pertussis was a dreaded disease, especially for the very young, having a high risk of death for infants under the age of less than a year. It is characterized by paroxysms of cough, inspiratory whoop, and posttussive vomiting, with more severe forms leading to apnea in infants. Before the vaccine development, Pertussis was the major cause of the morbidity and mortality of newborns (2). *Bordetella Pertussis* is a Gram-negative coccobacillus which gets in touch with ciliated respiratory epithelial cells. Local inflammatory changes occur in the respiratory tract mucous membrane. Toxins (Pertussis toxin, dermonecrotic toxin, adenylate cyclase toxin and tracheal cytotoxin), which have a local and systemic effect, are released even though the agent itself does not penetrate the respiratory tract completely and is almost never found in the hemoculture (3). Furthermore, the disease was highly contagious, leading to strict quarantining of the affected child. Lack of availability of treatment for pertussis resulted in clinical and psychosocial burden for both patient and the community. Numerous drug experiments were tried to accelerate recovery and enhance survival; however, none of the experiments achieved a significant therapeutic utility. Even though the whooping cough disease has been reduced to sporadic cases by vaccination, it is evident that the cases of the disease are reoccurring nowadays. One of the key reasons for the reintroduction of complex whooping cough cases is the insufficient level of vaccination, especially in the high-risk population, displaying high vaccination skepticism. The lack of vaccination or untimely vaccination contribute to the growth of the pertussis cases in the population (4). The pertussis cases often present a challenge due to various differential diagnoses. The whooping cough symptoms are frequently similar to other respiratory infection symptoms, which makes the precise clinical-symptom diagnosis more difficult. Therefore, it is essential to have a reliable diagnostic tool which can provide the relevant information about the patient’s condition. X-ray diagnostics has been used as one of the key modalities in the chest/lung disease evaluation for decades. The availability, the low price and the possibility of the chest anatomic structure visualization make x-ray diagnostics one of the most common diagnostic methods in the respiratory disease evaluation. Chest x-ray images provide the data on the inflammation presence or other pathological changes which could be the result of pertussis. Nevertheless, despite its wide application, x-ray diagnostics also demonstrates certain limitations. It can only provide a two-dimensional image of organs, which makes the precise localization and characterization of the lesions. Also, x-ray imaging does not procure a detailed overview of soft tissues surrounding the lungs, which can result in the negligence of certain pathological changes. This paper focuses on the advantages and disadvantages of x-ray diagnostics employed in the diagnosis of whooping cough patients, including the possible errors during the interpretation process and

the need for additional diagnostic modalities. The analysis includes the factors which can affect the preciseness and reliability of x-ray findings as well as the need for the integration of other diagnostic methods aimed at providing an overall picture of the patient’s condition.

METHODS

In this overview paper, the electronic databases Google Scholar Advanced Search, Consortium of Serbian Libraries for Coordinated Purchase and and the PubMed platform are used. The search included the following key words: x-ray diagnostics, whooping cough, pertussis, interpretation errors. The 2014 to 2024 publications in Serbian and English have been used. The results of the studies related to x-ray diagnostics in cases of whooping cough are presented in a narrative form.

WHOOPIING COUGH

The World Health Organization (WHO) describes pertussis as "an extremely contagious respiratory tract disease caused by *Bordetella pertussis*," a microorganism specific to humans. Pertussis affects people of all ages, especially children, and is one of the leading causes of mortality in infants under the age of one year. The incubation period usually ranges from 7 to 10 days (from one to three weeks), and clinical characteristics are associated with age, duration of infection, immune status, and the applied antibiotic therapy (4). Whooping cough in children is characterized by paroxysmal cough accompanied by the typical inspiratory sound and post cough vomiting. Namely, as stated earlier, the clinical course can be susceptible to many factors, including the vaccination history and age. Adolescents and adults often show atypical symptoms and can only be faced with the persistent extended cough. The WHO estimates that a total of 151, 074 whooping cough cases worldwide were recorded in 2018, and in the previous years (2008) up to 89,000 mortalities were reported. Now, vaccination is the best available strategy to fighting the disease (1-3) (Figure 1).

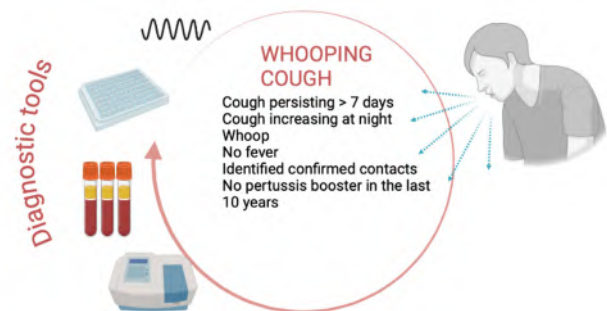


Figure 1. Clinical signs and diagnostic methods of whooping cough

Moreover, the severity of the disease is inversely proportional to the patient age (4, 5). Namely, pertussis has a predictable course in unvaccinated children and can result in grave symptoms and complications. The prognosis is particularly poor during the first and second year of age, when the

hospitalization incidence and mortality are the highest (mortality rate: 0.2% in the developed and 4% in the developing countries) (6). The disease can have a mild and atypical course in vaccinated children, adolescents, and adults, which causes a rare disease detection. Namely, the abovementioned patients can pose a powerful infection source for little children, especially infants under the age of one, when the immune system is still maturing.

The pertussis infection has shown immunomodulatory effects, in the recent research focused on the role of adenylate cyclase toxins (CyaA) and the secretion system effector proteins type III (TTSS), which can affect the pathogenesis of the basic chronic conditions/diseases, especially the chronic inflammatory diseases (8, 9). The evidence suggests the *Bordetella pertussis* infection can impact the asthmatic response (10), respiratory diseases (8) and other atopic conditions (11).

Pertussis in patients with chronic diseases such asthma or chronic obstructive pulmonary disease can be related to the complications which can incur additional treatment costs (12). Besides, pertussis can be an immediate causative agent of other diseases. A recent study indicates that 60% of elderly people in Europe suffer from at least two chronic diseases. The elderly are frequently susceptible to infectious diseases and the resulting complications for a few reasons, including the absence of previous immunization or incomplete vaccination or weakened immunity.

Pertussis testing is not always available as part of the primary healthcare, nor is it as part of certain medical emergency services. A nasopharyngeal swab and Polymerase Chain Reaction (PCR) can procure the laboratory confirmation, however, given the existing conditions, these tests appear to be quite demanding. Also, slow-growing *Bordetella* organisms demand specialized means, and the cultures usually are not positive from 3 to 7 days. In adults, at the time around the suspected diagnosis, cultures are usually negative (96%), and the overall culture sensitivity is only 20% to 40% (13, 14). PCR is more sensitive and more specific than the culture, and the testing is not widely used.

From the differential diagnostics standpoint, whooping cough should be taken into account in patients with extended coughing period, especially if occurring in paroxysms or accompanied by the inhalation "sound" or vomiting following the cough. During the early paroxysmal stage, leukocytosis (often between 25,000 and 60,000 per mL) with lymphocytosis can raise suspicion of pertussis (15-17). The chest radiography findings are not specific and can point out to peribronchial thickening, overemphasized reticular perihilar image, lung parenchyma consolidation, a different level lung atelectasis and lymphadenopathy.

THE ADVANTAGES OF X-RAY DIAGNOSTICS IN PATIENTS WITH WHOOPING COUGH

The Ministry of Health and the available whooping cough diagnostics protocols recommend that chest x-ray imaging should be done in patients under the age of four suspected of whooping cough to make the diagnosis process easier and detect potential complications. Nevertheless, despite all these recommendations, some 20% of the patients included in the investigation carried out by Lima et al. (2022) had not been chest x-rayed, which could have been a result of medical workers' unawareness of this recommendation (18). Still, the abovementioned study identified changes in 57.5% of chest x-rayed patients, with peribronchial thickening and overemphasized reticular perihilar image being the most common findings. Namely, a small number of studies described the chest x-ray changes in patients suspected of whooping cough. Nonetheless, the most common chest x-ray image findings in patients with acute respiratory infections are: peribronchial thickening, overemphasized reticular perihilar image and lung parenchyma consolidation (19, 20).

Chest x-ray imaging is fairly significant for the whooping cough diagnostics, especially having in mind the rapid availability and the simplicity of the medical examination execution. The availability of x-ray imaging enables clinicians to be provided with the lung image immediately upon the examination indications. Additionally, it results in the rapid diagnosis and timely initiation of the adequate treatment (21).

In the case of whooping cough whose symptoms may overlap with other diseases, an urgent condition evaluation is essential, and x-ray imaging can procure the key information concerning anatomic chest/lung changes. The simplicity of x-ray examination also contributes to its significance in the whooping cough diagnostics. Chest x-ray imaging is a non-invasive procedure using ionizing radiation aimed at providing the chest's/lung's internal image. This method is routinely used at many medical centers and hospitals, that is, this diagnostic tool is easily accessible. Due to its availability and execution simplicity, chest x-ray imaging is a vital step for the evaluation of the disease's clinical course.

Peribronchial structure thickening is often found in the whooping cough patients. The x-ray image shows the thickened peribronchial space as a more conspicuous peripheral bronch due to the inflammation and swelling of the surrounding tissue (18, 22). These findings can be a consequence of the direct infection presence in the patient's respiratory system, but also it can occur on account of an inflammation due to the mechanic bronchial wall irritation caused by sudden pertussis cough attacks. Besides the peribronchial structure thickening, x-ray images are also likely to indicate other pertussis-specific changes.

Atelectasis (the collapse of lung alveoli) and alveolar shadowings (the accumulation of liquid or substances in the lung parenchyma) can also be detected during the chest x-ray imaging in the pertussis-stricken patients. Atelectasis is a

common disease complication occurring as a result of the increased pressure in the alveoli during the forceful and long-lasting coughing attacks, which results in their collapse and the limited gas exchange inside the alveoli. An x-ray image manifests atelectasis as decreased parenchyma transparency which, in fact, represents the lung segment without air (19, 23). Atelectasis may be localized or diffuse depending on the size of the stricken lung area (24). More severe cases can lead to the atelectasis of the whole lung. Such chest x-ray image findings point out to a severe lung function disorder in the pertussis-stricken patients (19). Consequently, atelectasis can lead to the lower organism airflow, which can cause symptoms such as shortness of breath, fatigue and weakness.

Alveolar opacification are also common findings related to chest x-ray imaging in pertussis patients (14, 25). They represent liquid or substance accumulation in the lung parenchyma. Berdetella Pertussis itself, via its toxins and other metabolic products, may cause the lung inflammation and irritation, which causes increased mucous production. This thick mucous may be accumulated in the lung alveoli as well, which x-ray imaging presents as the occurrence of alveolar opacifications, which can be coupled with other disease manifestations such as peribronchial structure thickening and atelectasis (26). The combination of these changes shown in the x-ray images may indicate a severe lung function disorder in patients. Due to the limited airflow and inefficient gas exchange, alveolar infiltrations are clinically manifested by symptoms such as shortness of breath, coughing and dysfunctional breathing.

THE LIMITATIONS OF X-RAY DIAGNOSTICS IN PATIENTS WITH WHOOPING COUGH

One of the key problems of whooping cough patient x-ray imaging is coughing attacks during the imaging procedure. This may lead to artefacts which make the findings interpretation more difficult. Thereby, the poor image quality may require additional imaging, which can be an exhausting and unpleasant experience to patients who are in no control of the coughing attacks.

Another disadvantage of x-ray imaging of whooping cough patients is inefficiency of an early-stage detection of the disease, even though it may prove useful for the detection of later complications such as pneumonia or pneumothorax. Namely, in the early disease stage, cough may seem to be mild and bear resemblance to the cold-related cough. These initial stages can be vital for a timely diagnosis and the initiation of the adequate treatment aimed at the further prevention of disease spreading. However, x-ray imaging is usually not sufficiently sensitive to detect the mild changes occurring within this period (27-29).

Regarding x-ray imaging, another limitation is the patient's exposure to ionizing radiation, which may potentially damage the body cells. Overexposure to this radiation may increase the risk of cancer and other disease development. In the event of whooping cough, with x-ray imaging not

required for the early-stage diagnosis, the patient's exposure to this type of radiation may be inefficient and unnecessary (30).

Regarding the chest x-ray interpretation in the whooping cough diagnostics, certain errors affecting the diagnosis precision are possible to take place. Some of the possible problems are:

- Insufficient sensitivity
- Non-specific aspects of the findings
- Diagnostic confusion,
- The findings variation during the disease.

Namely, chest/lung radiography may be less sensitive to mild changes detection occurring in the early-stage pertussis. This may lead to false negative findings with the image showing the lack of infection and may result in making an inaccurate diagnosis. Also, in patients with whooping cough, it may indicate non-specific changes such as peribronchovascular image thickening or slighter alveolar shadowing. These changes are not whooping cough specific and may be found in other respiratory infections or conditions (31, 32). Sometimes, the pertussis symptoms may overlap with other respiratory infections or diseases making the similar demonstration such as bronchitis or asthma, thus may cause the so-called diagnostic confusion. In such cases, an x-ray image itself will not be sufficiently discerning in such overlapping cases, so additional evaluation and clinical assessment are needed. Additionally, whooping cough has different stages over the course of time, and x-ray findings may also vary depending on the disease stadium of the x-rayed patient. For instance, the early infection stages may have normal x-ray findings, whereas the latter stages may indicate consolidations as well. With a view to minimizing the chest radiography interpretation errors suspected of pertussis, it is important to have an overall approach including the patient's clinical history (including the presence of whooping cough specific episodes), laboratory tests and the monitoring of symptoms over the course of time aimed at making the right diagnosis and initiating the adequate treatment (33-35).

With regard to overriding and diminishing the limitations of whooping cough x-ray diagnostics, it is significant that a few alternative methods as potential solutions should be considered provided there is a choice possibility: computerized tomography (CT), bronchoscopy and functional breathing tests.

CONCLUSION

In conclusion, several key concluding points regarding the possibilities and limitations of x-ray diagnostics in cases of whooping cough can be drawn. Chest radiography is frequently used as the first method for the evaluation of patients with whooping cough symptoms on account of its speed, simplicity and relative availability. With regard to this, the advantages of x-ray diagnostics are numerous. Firstly, chest x-ray imaging procures the visualization of the lung structure and detection of potential abnormalities such as peribronchial

structure thickening, alveolar shadowings, pneumonia and atelectasis. Besides, it can be used for the tracking of the applied therapy effects. Nevertheless, it is important to underline certain x-ray diagnostics limitations in whooping cough patients as well. Firstly, an x-ray image is not always sufficient for making a proper diagnosis or detecting the severity of the patient's condition. Some cases may require additional diagnostic procedures such as computerized tomography (CT) or magnetic resonance imaging (MRI). Also, one must be aware of the fact that x-ray radiation poses a certain risk level to patients due to the ionizing radiation exposure. Therefore, it is important to make a benefit and risk assessment before applying this modality. Based on the advantage and disadvantage analysis of chest x-ray imaging in the whooping cough cases, a further investigation of the topics related to the technology improvement and x-ray diagnostic methods is suggested. Additional research may also be oriented towards the understanding of specific features of certain pathological conditions causing whooping cough aimed at improving the findings interpretation during the routine chest radiography. Given the fact that the majority of pertussis patients are made up of little children, it is important that x-ray diagnostics be applied in the case of grave necessity with benefits exceeding the potential risks. In cases of easier methods used for condition diagnostics, such as early-stage pertussis, alternative methods should be preferred in order to avoid the unnecessary ionizing radiation exposure, with x-ray methods used for the potential complication detection.

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IMPLANT-SUPPORTED PROSTHETIC REHABILITATION OF THE EDENTULOUS MAXILLA USING THE OT BRIDGE EQUATOR SYSTEM: A CASE REPORT

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ABSTRACT

Edentulism of the maxilla presents significant clinical and functional challenges due to limited bone quality, anatomical constraints, and inadequate retention commonly associated with conventional dentures. Low-profile abutment systems, such as OT Equator and OT Bridge, enable the fabrication of fixed full-arch prostheses with improved mechanical stability and simplified maintenance. A 60-year-old male patient with terminal-stage periodontitis in the maxilla underwent full-arch implant-supported rehabilitation. After the extraction of compromised teeth, six implants were placed in strategic positions and allowed to heal. Upon radiographic confirmation of osseointegration, OT Equator abutments were selected based on soft-tissue height and installed according to the manufacturer's recommendations. A two-phase open-tray impression was taken, followed by fabrication of a Co-Cr framework and a zirconia-based definitive prosthesis. The prosthesis was retained using the OT Bridge system, combining elastic primary retention via Seeger rings with secondary screw retention. Standard and angulated extragrade abutments were used to compensate for implant divergence and ensure passive fit. The patient reported excellent aesthetic and functional satisfaction using the OT Bridge Equator System. Follow-up examinations showed stable soft-tissue healing, absence of inflammation, and no mechanical complications. The applied system provided predictable retention, favorable stress distribution, and facilitated oral hygiene. This case demonstrates that the OT Equator and OT Bridge systems offer a reliable solution for full-arch fixed rehabilitation of the edentulous maxilla. Their low-profile design, divergence compensation, and dual-retention mechanism support predictable outcomes and represent a valuable alternative to traditional multi-unit abutments in anatomically demanding cases.

Keywords: Maxillary implant rehabilitation, OT Bridge System, full-arch implant rehabilitation, dental implant.

INTRODUCTION

The state of toothlessness, especially in the upper jaw, represents a significant clinical and functional problem that can have profound consequences for patients' quality of life. Conventional complete dentures in this region often fail to provide satisfactory retention and stability, and palatal extension can lead to discomfort, a gag reflex, as well as a decrease in taste and masticatory function (1, 2).

Using dental implants, the prosthetic rehabilitation of edentulous patients has been significantly improved. Implant-supported dental restorations provide greater stability, better functionality, and greater psychological security for patients. These benefits are especially important in complex clinical conditions; therefore, implant-supported restorations are the therapy of choice for patients with advanced ridge resorption and unfavorable anatomy (3, 4). Unlike the lower jaw, in the upper jaw, due to the lower bone density and different distribution of forces, it is usually recommended to install four or more implants for optimal load distribution and long-term success of therapy (5, 6). Additionally, retention elements such as locators/equators enable a stable, predictable, and easily maintainable prosthetic restoration. Among retention systems, low-profile OT equators stand out for their extremely low height (about 2.1 mm total height with housing), especially useful in situations with reduced interocclusal space. In addition to good mechanical resistance and the ability to compensate for implant divergence up to 80°, they are characterized by simple replaceability, multiple retention values, and durability (7, 8).

The manufacturer's specification states that the components are made of high-quality titanium with a titanium-nitride coating (hardness ~ 1600 Vickers), are compatible with most implant systems, and offer matrices with different levels of retention (600-2700 g), enabling personalized adjustment and simple chairside servicing. Contemporary studies also indicate that resilient or elastic retention mechanisms improve load distribution within implant-supported prostheses, resulting in lower peri-implant microstrain and reduced mechanical stress transferred to the implant-prosthesis assembly (9). This system also shows a low affinity for plaque accumulation, which contributes to better gingival healing, especially in cases where immediate loading is applied. This establishes a stable biological barrier between the abutment and soft tissue, which over time prevents the penetration of bacteria and significantly reduces the risk of developing peri-implant complications, including peri-implantitis (10, 11, 12).

However, in recent decades, innovative retention concepts have emerged that allow the fabrication of fixed restorations on OT Equator abutments, which were previously used mainly for mobile restorations. This approach combines the functionality of fixed work with the advantages of low-profile abutments, allowing compensation of implant divergences, stress reduction, and a simpler technical approach during fabrication and maintenance (13, 14). A special

advantage of the OT Equator system in fixed restorations is the possibility of combining passive retention (via seeger) and secondary retention (via screwing), which achieves a high degree of stability, simple oral hygiene maintenance, such as aesthetics without compromise (15, 16).

Regarding all the data above, the present study aimed to provide a clinical presentation of the implant-prosthetic rehabilitation of the edentulous upper jaw using the OT bridge equator system.

CASE REPORT

A 60-year-old male patient was referred to the Department of Dentistry, Faculty of Medical Sciences, University of Kragujevac, due to prosthetic rehabilitation in the upper jaw. In medical history, it was observed that the patient had arterial hypertension, which the attending specialist adequately treated. In family history, the patient indicated the absence of the same or similar diseases.

Intraoral examination and radiographic analysis showed the presence of teeth 11, 21, 13, 15, and 23, of low biological value, in the terminal stage of periodontitis, and the presence of a metal ceramic prosthetic bridge in regio 35-45. To better assess the density and volume of the bone, the patient underwent a Cone Beam Computer Tomography (CBCT) scan (Sirona Dental Systems GmbH, Bensheim, Germany) (Figure 1). Based on the medical history, clinical and radiographic examination, implant prosthetic therapy of the upper jaw - full arch fixed implant-supported prostheses was proposed.

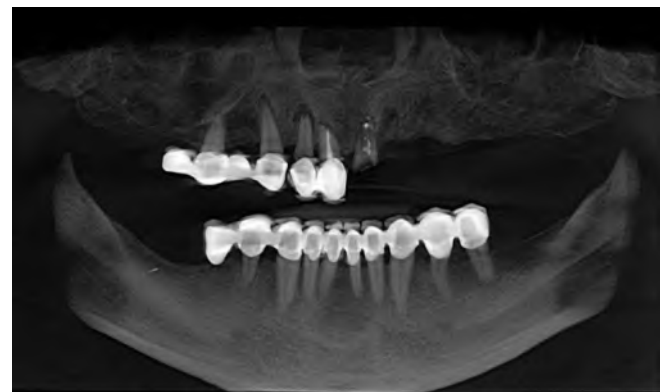


Figure 1. Pre-surgical CBCT images.

After the CBCT image analysis determined the possibility of implementing the planned implant-prosthetic therapy, and the patient's consent to it, oral-surgical intervention was initiated. As part of the above intervention, periodontally affected teeth in the upper jaw were extracted, and dental implants (B&B Dental, Bologna, Italy) were placed in the following positions: 11 (4.0x10), 21 (4.0x10), 13 (4.0x10), 23 (4.0x10), 14 (3.5x10), and 25 (3.5x10) (Figure 2). Primary closure was achieved using sutures. After radiographic confirmation of osseointegration of the implants after a period of 4 months, they were opened, and sulcus formers were placed.



Figure 2. Post-surgical orthopantomography.

As the prosthetic treatment plan included the use of the OT Bridge® system and the use of an OT Equator abutment (Rhein 83, Bologna, Italy), a universal “C.H.” gauge (Rhein 83, Bologna, Italy) was used to determine the tissue height above the implant. Six low-profile OT Equator abutments were selected and placed in the appropriate positions, and tightened according to the manufacturer's instructions to 25 Ncm (Figure 3).



Figure 3. Intraoral image of an OT Equator abutment placed in the appropriate position in the upper jaw.

In the next phase, an analog two-phase simultaneous impression in an open tray was taken at the level of the abutments, after previously splinting them with the help of a self-polymerizing acrylic resin (Pattern Resin LS, GC, Tokyo, Japan). An additional silicone combination of DMG Honigum Pro Putty Soft and DMG Honigum Pro regular (DMG Chemisch-Pharmazeutische Fabrik GmbH, Hamburg, Germany) mass was used for impression taking (Figure 4).

Upon determination of intermaxillary relationship, a framework made of Co-Cr alloy was tested, with indirect seating achieved using extragrade abutments. Due to the greater angulation of the implants in the anterior segment, extragrade abutments inclined at 15 degrees were used in positions 11, 21, 13, 23, while standard extragrade abutments were used in regions 14 and 25. The passive fit of the framework was assessed through clinical and radiographic evaluations (Figure 5 a, b).

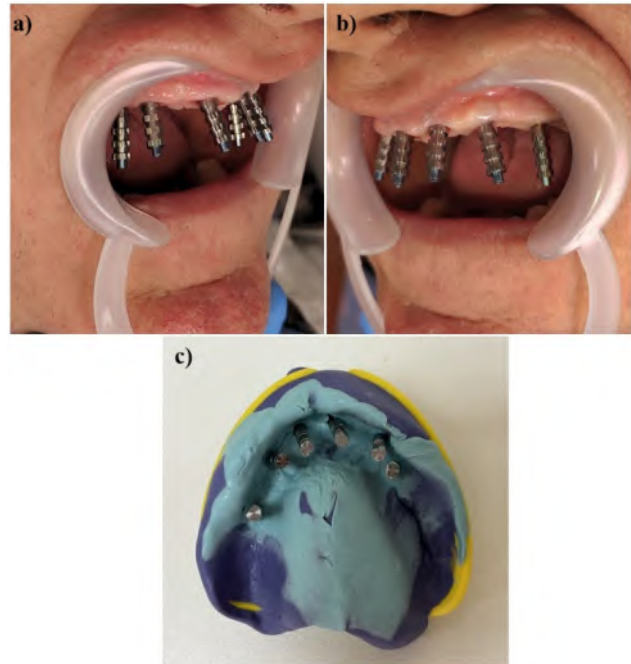


Figure 4. OT Equator abutment level impression: (a) intraoral right lateral view with transfer impression coping, (b) intraoral left lateral view with transfer impression coping, (c) extraoral analog two-phase simultaneous impression of the upper jaw.

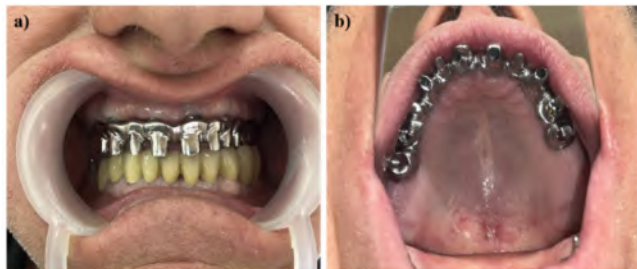


Figure 5. Intraoral image of a framework made of Co-Cr material with extragrade abutments in the upper jaw: (a) frontal view, (b) occlusal view.

A prototype of the future teeth was made in polymer (BreCam, Multicom, Bredent srl, Bolzano, Italy) and tried on; the previously registered intermaxillary relationships were confirmed, and the occlusion was corrected (Figure 6).



Figure 6. Intraoral image of a prototype of the future teeth in the upper jaw.

During this phase, tooth and gingiva shade selection was also performed. Definitive superstructure of fixed implant-supported prosthesis was made of zirconium-oxide ceramic, while gingiva characterization was performed by composite (Figure 7a,b).

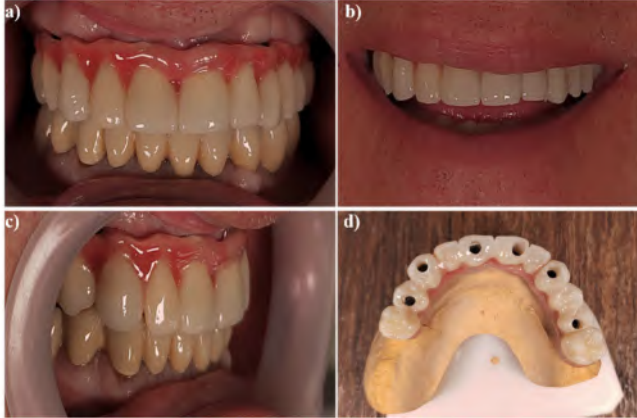


Figure 7. Intraoral image of the definitive superstructure of a fixed implant-supported prosthesis: (a, b) frontal view, (c) lateral view and (d) extraoral image of the definitive superstructure of a fixed implant-supported prosthesis: occlusal view.

The specificity during the delivery of the definitive restoration - OT Bridge fixed prosthesis involved the placement of white Seeger rings (standard) that provide primary retention of the restoration (Figure 8).



Figure 8. Extraoral image of the definitive superstructure of a fixed implant-supported prosthesis with Seeger rings, a palatal view.

In contrast, secondary retention is based on a screw. Retention screws were tightened according to manufacturer instructions (15 Ncm). Access holes are protected with Teflon tape and closed with composite. Additionally, the specific feature of the present OT-Bridge superstructure supported by six implants was the presence of four connection screws, two of which (at positions 11 and 15) were omitted, with the connection being entrusted solely to the Seeger system (Figure 9).



Figure 9. Orthopantomography control at the definitive superstructure of a fixed implant-supported prosthesis.

At follow-up, the patient received a protective night guard and oral hygiene instructions. The patient reported full functional and esthetic satisfaction post-rehabilitation.

DISCUSSION

This case demonstrates that carefully planned implant-supported prosthetic rehabilitation of the edentulous maxilla can give predictable and stable outcomes, even under conditions of limited interocclusal space and unfavorable anatomy. In this patient, six strategically positioned implants using the OT Bridge Equator system provided optimal occlusal load distribution and mechanical stability, along with high-quality esthetic characterization.

During the past decade, advances in scientific research have markedly improved traditional approaches to implant loading (17). The anatomical limitations of the maxilla, combined with the high esthetic requirements and its distinctive pattern of bone resorption, contribute to the challenges of treatment planning. These elements make optimal implant placement challenging and limit the feasibility of constructing a conventional screw-retained prosthesis (13). Accordingly, the application of the OT Bridge system, using OT Equator abutments and double retention (primary Seeger ring and secondary screw fixation), ensures secure retention while allowing for easy removal of the prosthesis for maintenance (18). A one-year multicenter clinical study (19) demonstrated that the OT Equator system (Rhein'83) for fixed maxillary prostheses on four to six implants provides high therapeutic success, with excellent implant and prosthetic survival rates, minimal complications, high patient satisfaction, and stable biological parameters, with average bone remodeling of only 0.2 mm after one year, which is consistent with the results of our study. In addition, Mohamed et al. (16) reported that the OT Bridge system exhibits biomechanical efficiency even when approximately one-third of the anchoring screws are omitted in an all-on-six configuration. Their findings indicate that the exclusion of certain screws does not compromise prosthetic stability. Nevertheless, both the number and geometric arrangement of the omitted screws were shown to affect stress distribution within the OT Bridge Equator framework. In all-on-six restorations, unilateral removal of two screws was associated with a better stress distribution pattern, which is consistent with the report of the current study.

Furthermore, the present study indicated that early follow-ups showed high esthetic and functional satisfaction, with no signs of soft tissue inflammation or screw loosening. Literature supports that OT Equator systems, owing to their low-profile design and elastic Seeger rings, reduce stress concentration on peri-implant tissues and help preserve marginal bone levels in fixed prostheses (20). Tallarico et al. (21) reported similar clinical and biomechanical advantages of OT Equator abutments over other systems in patients with implant-supported overdentures, highlighting reduced spatial requirements and improved retention properties, contributing to predictable prosthetic rehabilitation. Likewise, Montanari et al. (22) confirmed that a fixed prosthetic system built on OT Equator, which reduces spatial requirements, represented a significant relief in clinical work and laboratory processing, while improved retention properties contributed to greater reliability and predictability of prosthetic rehabilitation.

An additional advantage in the current case was the combination of standard and angulated extragrade abutments, enabling adequate screw fixation even for highly angled implants, thereby correcting implant divergence. Such flexibility is critical in the maxilla, particularly for esthetic considerations where anatomical limitations and screw emergence must be addressed (23). The literature data confirmed that the capacity of extragrade OT Equator abutments to compensate for implant divergence up to 80° using Seeger rings (24). Moreover, *in vitro* biomechanical studies demonstrated passive prosthesis seating and stability, even in the absence of screws, through mechanical retention provided by Seeger rings (25). While multi-unit abutments are widely used in implantology for their ability to compensate for implant divergence and facilitate passive fit, studies indicate they may induce localized stress concentrations, increasing the risk of micromovement and potential complications over time (26, 27). Conversely, the OT Bridge system using OT Equator abutments provides more favorable stress distribution and reduces the likelihood of micromotion. Studies show that the OT Bridge system can effectively reduce prosthetic stresses, even when one of four screws is absent, without compromising prosthetic stability (16, 28).

Although the OT Bridge Equator system demonstrated favorable clinical performance in the present case, potential mechanical and biological complications should be acknowledged. As reported in systematic reviews of implant-supported fixed prostheses, mechanical events such as screw loosening, preload loss, or framework-related complications may occur under functional loading (30, 31). Finite element and *in vitro* studies specific to the OT Bridge system have further indicated that stress distribution may be influenced by the number and position of prosthetic screws, particularly when screw omission protocols are applied (16, 27). From a biological perspective, peri-implant mucositis and peri-implantitis remain risks in all implant-supported rehabilitations, although titanium nitride-coated components have demonstrated a favorable soft-tissue response in experimental studies (10, 11). Therefore, careful prosthetic execution and regular maintenance are essential to ensure long-term stability.

Based on clinical follow-up and patient-reported outcomes, this therapeutic protocol demonstrated high predictability and long-term efficacy with minimal complications. Nonetheless, extensive prospective clinical studies with larger cohorts and longer observation periods are necessary to validate long-term stability and biological safety (29). However, it is important to note that the choice between OT Bridge and multi-unit abutments should be guided by clinical indications, anatomical constraints, and patient-specific esthetic demands, as each system has distinct advantages and limitations.

CONCLUSION

This case report demonstrates that the application of the OT bridge Equator system enabled successful full-arch fixed prosthetic rehabilitation supported by multiple implants. The system provided stable prosthesis retention, simplified screw access and component management, enhanced overall clinical procedures, and improved aesthetic characterization. In addition, the biomechanical performance remains excellent even with the absence of one-third of the anchoring screws. Importantly, this case highlights the clinical value of the OT system by providing evidence of its practicality and reliability in complex full-arch rehabilitations, thereby offering clinicians an effective treatment option in cases requiring fixed, implant-supported restorations.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

INFORMED CONSENT STATEMENT

Informed consent was obtained from the participant or their representatives.

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HBOT - AN EFFECTIVE OPTION FOR THE TREATMENT OF CHRONIC WOUNDS IN DIABETES MELLITUS: A CASE REPORT

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ABSTRACT

Diabetes mellitus is a chronic metabolic disease characterized by chronic hyperglycemia and disorder of carbohydrate, fat and protein metabolism, caused by an absolute or relative lack of insulin secretion and/or action. It is manifested by a characteristic clinical picture, and in the further course of the disease, complications occur in small blood vessels (microangiopathy) and large blood vessels (macroangiopathy). Diabetic foot syndrome is "foot ulceration, associated with peripheral neuropathy and peripheral vascular disease of varying degrees and infection". It is the most common and serious complication associated with this chronic metabolic disease. Hyperbaric oxygen therapy is inhaling 100% oxygen under carefully controlled elevated pressure conditions. It has found its significant place as one of the treatment modalities for several pathological conditions characterized by tissue hypoxia, such as diabetic wounds. In this report we presented a 59-year-old male patient and a 73-year-old female patient with different kinds of diabetic wounds on which hyperbaric oxygenation had an evident positive effect.

Keywords: Diabetic wounds, Hyperbaric oxygenation, Case report.

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease characterized by chronic hyperglycemia and disorder of carbohydrate, fat and protein metabolism, caused by an absolute or relative lack of insulin secretion and/or action. It is manifested by a characteristic clinical picture, and in the further course of the disease, complications occur in small blood vessels (microangiopathy) and large blood vessels (macroangiopathy) (1). In the last three decades, the number of new cases and prevalence of DM has increased dramatically in all countries, regardless of the level of development. About 422 million people worldwide have DM, but predominantly in underdeveloped and developing countries. Every year, about 1.5 million deaths are directly related to this disease (2). In 2021, there were 529 million (95% uncertainty interval [UI] 500–564) people living with diabetes worldwide, and the global age-standardized total diabetes prevalence was 6,1% (5,8–6,5). Total diabetes prevalence—especially among older adults—primarily reflects type 2 diabetes, which in 2021 accounted for 96,0% (95,1–96,8) of diabetes cases and 95,4% (94,9–95,9) of diabetes DALYs worldwide. By 2050, more than 1,31 billion (1,22–1,39) people are projected to have diabetes, and 89 (43,6%) of 204 countries and territories will have an age-standardized rate greater than 10% (3).

According to the definition of the World Health Organization (WHO), diabetic foot syndrome (DF sy) is "foot ulceration (distal to the ankle and including the ankle), associated with peripheral neuropathy and peripheral vascular disease of varying degrees and infection" (4). It is the most common and serious complication associated with DM, occurring in 15% of patients and the cause of 50% of lower limb amputations (5). In the early stage of DF, there is mainly a disorder of the sensorium of the skin of the lower limbs and cold lower limbs, which, if ignored, can progress to diabetic foot ulcer (DFU) and gangrene. The final outcome can be amputation of the affected lower limb or even death in severe cases, which represents a great burden to the family and society. Peripheral neuropathy, peripheral vascular disease and infection are three factors that are seen together in 60% of patients with DF and in combination with factors from the external environment, such as inadequate foot care and hygiene and wearing inadequate footwear, lead to the formation of wounds, i.e. ulcerative changes on lower limbs that heal slowly. Other risk factors for the occurrence of DFU are smoking, obesity, alcohol abuse (6,7). Due to the complexity of the wound healing process, their treatment should be approached in several ways. The main modalities of DFU treatment primarily include optimal glycemic control to slow progression, wound treatment including local debridement of the wound, regular dressings, offloading, i.e. reduction of pressure on the foot, then control of infection with the use of targeted antibiotics, revascularization in case of need and nutritional support (8,9). The main characteristic of chronic wounds is the low partial pressure of oxygen in their center, and it has been proven that oxygen plays a central role in wound healing. It is not only necessary for cellular respiration, but also as a source of free oxygen radicals, which are

vital in several physiological processes, such as cellular communication, bactericidal activity and promotion of angiogenesis. In addition, low subcutaneous oxygen pressure is associated with a higher risk of infection. For this very reason, hyperbaric oxygen therapy (HBOT) has found its significant place as one of the treatment modalities for several pathological conditions characterized by tissue hypoxia, such as diabetic wounds, carbon monoxide poisoning, gas gangrene, necrotizing fasciitis, compartment syndrome, intracranial abscesses, burns and the consequences of radiation treatment or osteomyelitis. In this sense, it has been shown that healing of diabetic foot wounds progress favorably after HBOT treatment. Furthermore, HBOT prior to surgery has been reported to reduce complications and hospital stay. HBOT is a type of therapy during which the patient inhales 100% oxygen under a pressure of at least 1.4 ATA (absolute atmosphere), usually between 2ATA and 3ATA, in special chambers designed for that. HBOT increases the availability of oxygen to body tissues, including plasma, and increases the capacity of blood to transport oxygen relative to the concentration under normobaric conditions, which is often associated with its pharmacological effects. The aim of this paper is to demonstrate the contribution of HBOT as an adjuvant therapy to the healing of diabetic foot wounds through examples from practice.

CASE REPORT 1

A 73-year-old female patient was first admitted in November 2023. to the Department of Baromedicine at the Zemun Clinical Hospital Center due to a non-healing wound on her right foot. The ulcer was located on the inside of the right foot in the projection of the head of the first metatarsal bone, 6 cm in diameter, with signs of infection and necrotic content (Figure 1). The patient gives information that the change occurred about a month before hospitalization and that it was a minor wound, probably the result of blisters. Until admission, she was treated in a regional health institution with bandages and antibiotic therapy. The skin of both feet is trophically altered, pedal pulsations are not palpable. The patient has been diabetic for 18 years, on combined insulin and oral therapy. She is being treated for hypertension, hyperlipoproteinemia and psoriasis as well. A swab of the wound was taken upon admission. After an examination by a cardiologist and otorhinolaryngologist and an x-ray of the heart and lungs, the patient was treated with HBO according to the regular protocol included. HBOT was conducted in a one-piece chamber that accommodates only a single patient, in which he lies only in his cotton underwear, under a pressure of 2 ATA for 60 minutes once a day. During treatment, the pressure is raised by the first 15 minutes at a rate of 0.3 ATA per minute, the next half hour, the patient breathes 100 % O₂ under constant pressure from 2 ATA, and last 15 minutes gradual decompression is also made at 0.3 ATA per minute. A total of 20 HBO therapies were conducted. Immediately upon admission, empiric antibiotic therapy was included, which was corrected in accordance with the results of the wound swab, from which *Pseudomonas aeruginosa* was isolated, and the antibiogram. Wound care with hydrogen and povidone-iodine solution were performed daily, with occasional debridement of

necrotic tissue and drainage of the contents. During hospitalization, leukocytes ranged from 9.4-7.1 ($10^9/L$), CRP 74.7-28.6 (mg/L). At the end of the treatment, the patient was discharged in good general condition with local findings improving, without signs of infection and with the appearance of clear granulation tissue in the area of the ulcer, with a recommendation to continue treatment and bandages in the regional health institution. Two months after her discharge, the patient comes for a follow-up examination, stating that she regularly received bandaging and antibiotic treatment from the attending physician through the Community health center's home healthcare service. The local findings show a wound that is almost completely filled with granulation tissue, with macerated edges, apparently without infection (Figure 2). By pressing in the region of the root of the thumb plantar path of the wound, a larger amount of purulent content is obtained. The patient was hospitalized at the end of February 2024. in the Baromedicine Service again and treated with hyperbaric oxygen therapy according to the same protocol as the previous time. A total of 15 HBOTs were conducted. A swab of the wound was taken, from which *Pseudomonas aeruginosa* was isolated again, and it was treated with antibiotic therapy based on the results of the swab and the antibiogram, the wound was regularly bandaged with curettage of the place where the purulent content was obtained and its drainage. X-ray of the foot did not show the presence of gas in the soft tissue structures of the foot or signs of bone infection (Image 1,2). During the second hospitalization, leukocytes ranged from 8.1-6.4 ($10^9/L$), CRP 8.1-10.1 (mg/L). At the end of the therapy, the patient was discharged in a good condition, with a significant improvement in the local findings and minimal slightly cloudy secretion (Figure 3). Further control and follow-up by the attending surgeon was advised. Six months after the last cycle of HBOT, the wound has completely healed (Figure 4).



Figure 1. Local finding at the beginning of the first cycle HBOT



Figure 2. Local finding at the beginning of the second cycle HBOT



Figure 3. Local finding at the end of the second cycle HBOT



Figure 4. Local finding six months after last cycle of HBOT



Image 1. Anteroposterior X-ray image of the right foot



Image 2. Lateral X-ray image of the right foot

CASE REPORT 2

A 59-year-old patient was first admitted to the Department of Baromedicine because of a necrotic wound after a partial amputation of the right foot. In September 2023, after the removal of hyperkeratotic changes on the right foot, a wound appeared. The wound was complicated by the development of an infection and then gangrene. Partial amputation of the right foot was performed according to Lisfranc in UCC “Zvezdara” on November 15th 2023. Until admission, he was treated with antibiotic therapy, and according to the recommendation of a vascular surgeon, he comes for HBO therapy. At the reception, the condition after the partial amputation of the right foot is found, the wound is visible partially covered with necrotic deposits (Figure 5). Pedal pulsations are not palpable. The patient is being treated for diabetes and hypertension, in 2014 he had an acute myocardial infarction, and in 2015 he suffered a cerebrovascular insult. A swab of the wound was taken upon admission. After an examination by a cardiologist and otorhinolaryngologist and an x-ray of the heart and lungs, the patient was treated with HBO according to the regular protocol included. HBOT was conducted in a

one-piece chamber that accommodates only a single patient, in which he lies only in his cotton underwear, under a pressure of 2 ATA for 60 minutes once a day. During treatment, the pressure is raised by the first 15 minutes at a rate of 0.3 ATA per minute, the next half hour, the patient breathes 100 % O₂ under constant pressure from 2 ATA, and last 15 minutes gradual decompression is also made at 0.3 ATA per minute. A total of 20 HBO therapies were conducted. Immediately upon admission, empiric antibiotic therapy was included, which was corrected in accordance with the results of the wound swab, from which *Klebsiella* sp., *Enterococcus* sp. and *Acinetobacter* sp. were isolated, and the antibiogram. Toileting and dressing of the wound with hydrogen and povidone-iodine solution were performed daily, with occasional debridement of necrotic tissue and drainage of the contents. During hospitalization, leukocytes ranged from 17.7-12.6 (10⁹/L), CRP 9.6-5.4 (mg/L). At the end of the treatment, the patient was discharged in good general condition with local findings improving, without signs of infection and with the appearance of clean granulation tissue in the wound area. He was advised to continue treatment and dressing at regional hospital. In June 2024, after a regular check-up, the

patient was hospitalized for the second time. He stated that he regularly received bandaging and antibiotic treatment from the attending physician through the Community health center's home healthcare service. In the local findings in the area of the amputation stump, a wound about 5 cm in diameter was observed, the bottom of the wound was covered with fresh granulations, without visible signs of infection (Figure 6). Pedal pulsations were not palpable. During hospitalization, a total of 20 HBO treatments were performed according to the same protocol as the previous time. It is regularly bandaged with Aquacel Ag+ dressings and Aquacel foam dressings. Leukocytes ranged from 13.6-12.7 ($10^9/L$), CRP 2.4-1.3 (mg/L). At the end of the therapy, the patient was discharged in a good general condition, with a local finding in significant improvement, the wound area decreased by about 2/3 in diameter, the bottom of the wound was clean with fresh granulations (Figure 7). Further control and follow-up by the attending surgeon was advised.



Figure 5. Local finding at the beginning of the first cycle HBOT.



Figure 6. Local finding at the beginning of the second cycle HBOT



Figure 7. Local finding at the end of the second cycle HBOT

DISCUSSION

Although the pathogenesis of diabetic foot is still unclear, the current theory is that in patients with long-term hyperglycemia, there is a tendency for thrombus formation due to atherosclerosis of blood vessels of the lower extremities and their occlusion, which results in local ischemia and hypoxia. Peripheral neuropathy, which leads to a loss of

protective sensitivity, as well as a tendency to infection of the lower extremities contributes to the whole process (6, 7). Wound healing in diabetics is a complex process that includes three overlapping phases: inflammation, proliferation and remodeling. Severe acute hypoxia can stimulate cell proliferation and tissue repair, but on the other hand, chronic

hypoxia can lead to inhibition of angiogenesis, re-epithelialization and extracellular matrix synthesis, thus compromising healing (8). Under physiological conditions, hemoglobin is saturated with 97% oxygen and therefore increasing hemoglobin saturation does not lead to a significant improvement in tissue oxygen supply. According to Henry's law, at a constant temperature, the amount of dissolved gas in a liquid is directly proportional to the partial pressure of that gas in contact with the liquid, so an increase in the partial pressure of oxygen will increase the amount of oxygen dissolved in the plasma that can be transported to the tissue. In this regard, hyperbaric oxygen therapy can significantly increase the concentration of dissolved oxygen in plasma, and increasing tissue oxygenation appears as a key therapeutic strategy (5). During HBOT, it is possible to bring additional amounts of oxygen dissolved in the plasma to the target tissue, even if the blood vessel is theoretically narrowed so much that not even an erythrocyte can pass, but the liquid component of the blood can pass, which is now multiple times saturated with dissolved oxygen. It is clear that the increased flow of oxygen to the tissue can only be achieved in hyperbaric conditions, when the main carrier of oxygen is no longer hemoglobin in the erythrocyte, but plasma. Ischemic tissue now does not depend on erythrocytes, which in such tissue often cannot reach the target tissue due to their size (10, 11). Several studies have shown that HBOT restores and improves the functions of oxygen-dependent cells such as leukocytes, fibroblasts, osteoblasts, nerve cells (12, 13, 14, 15). This type of treatment has a direct bacteriostatic and bactericidal effect on anaerobic microorganisms, because their level of antioxidant defense is weakened. HBOT also acts as a broad-spectrum, non-specific antibiotic, because by increasing the production of free oxygen radicals in leukocytes, it enhances their phagocytic ability. Given this improvement in the ability of leukocytes to kill bacteria, it helps the local ischemic tissue to keep under control potential infection, which in these conditions would lead to increased tissue destruction and reduce the possibility of recovery of reversibly altered tissue. In general, HBOT improves blood circulation by reducing plasma viscosity, platelet aggregation, and accelerating neocapillarization, which means it improves vascular blood flow in the lower extremities as well, promotes local blood and oxygen supply, enhances tissue metabolism, reduces inflammatory exudation, and reduces or eliminates tissue edema (7). In addition to the above, HBOT has also been found to modulate various growth factors such as vascular endothelial growth factor VEGF, epidermal growth factor EGF, platelet growth factor PDGF, interleukin-1 α , fibroblast growth factor FGF-2, etc., which stimulates angiogenesis and arteriogenesis. Moreover, HBOT has shown its effectiveness in activating fibroblasts and endothelial cells through signaling pathways such as hypoxia-inducible factor 1 α HIF-1 α and nuclear factor NF- κ B, thereby accelerating the healing process (8). In one study, it was found that HBOT directly influences the gene expression of several potent antioxidants and pro-inflammatory cytokines, thus favoring angiogenesis and blood circulation in the extremities. It induces the gene expression of SOD1, SOD2, and GPX2, and significantly increases the expression of pro-inflammatory cytokines IL-1 β , IL-4, and IL-

12, although the expression of TNF α decreases significantly. Therefore, it demonstrates that HBOT could significantly alter the inflammatory response by modulating the gene expression of antioxidants and inflammatory cytokines (16). At the end of the discussion, I must emphasize that the outcome in these two presented cases is in accordance with a recent prospective, randomized, controlled study in which it was proven that at the end of the study, HBOT combined with standard wound care led to a significantly greater decrease in pain score and wound size as well as a significantly greater proportion of patients who developed healthy granulation tissue in the wound bed compared to standard wound care alone. Moreover, HBOT combined with standard wound care led to a significantly lower incidence of amputation. (17) On the other hand, according to the first systematic review that focuses specifically on patients with DFUs in combination with peripheral arterial occlusive disease (PAOD), HBOT appears to have some beneficial effect as adjunctive therapy to treat DFUs with PAOD as it decreases the major amputation rate, but requires a good general condition and stamina among eligible patients. Future research should focus on patient selection and the effectiveness of HBOT as standard adjunctive treatment in ischemic DFUs. (18, 19) And finally when it comes to costs of additional hyperbaric oxygen therapy compared to standard care the majority of HBOT studies have reported that HBOT is cost-effective, particularly based on a long-term perspective. Currently, there is a limited number of pharmacoeconomic evaluations for the cost-effectiveness of HBOT in DFU, because of that extensive cost-effectiveness evaluation for the topic is fundamental and further studies should combine clinical application of interventions with concomitant economic assessment (20).

CONCLUSION

The problem of DFU is considered as a serious challenge for modern medicine, it is the most challenging complication for both medical professionals and patients in the treatment of DM. From the high rates of failed treatments and consequent amputations (patients with DM are at 15 times higher risk for DE amputation compared to the rest of the population) it is clear that the desired results in the treatment of diabetic foot wounds have not yet been achieved. Therefore, it is necessary to take all preventive measures, to diagnose changes in an early stage of the disease, to provide adequate treatment, to educate patients and help them become aware of the need for a quick reaction when all the above-mentioned warning symptoms appear. A healthcare is obliged to provide equal access to all treatment modalities, including hyperbaric oxygen therapy, to all patients who are at risk or have already developed this complication.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Manuscripts should be submitted in *Microsoft Office Word*. The authors should use normal, plain *Times New Roman* font (12pt) for text. Pages should be numbered automatically. Italics may be used for emphasis. Abbreviations should be defined at the first mentioning in the text and used consistently thereafter (do not use a separate subtitle for abbreviations only). Please use no more than three levels of displayed headings. International System (SI) of Units should be used (imperial, US customary and other units should be converted to SI units).

INFORMATION FOR AUTHORS

Original research articles should contain following sections: Introduction, Materials and Methods, Results, Discussion, Conclusions, Acknowledgments, Conflict of Interest, and References. *Reviews* may require different formats, while *Case reports* manuscripts should follow the [CARE](#) guidelines.

Introduction. This section should contain context or background for the study, rationale, clear aim of research or tested hypothesis.

Materials and Methods. This section should provide sufficient detail for replication of the study. If more than one method is used in the research, use subsections with appropriate subheadings. The *Materials and Methods* section should also contain following statements:

- a) **Informed Consent Statement.** In cases where the identification of personal information is necessary for scientific reasons, authors should obtain informed consent from all individuals included in the study
- b) **Human Right Statement.** Manuscripts containing information related to human should clearly state that the research has complied with all relevant international and national regulations and institutional policies and has been approved by the authors' institutional Ethics committee.
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For details and examples of statements please see part 'Research and publication ethics'.

Results. The results should be presented in logical sequence in the manuscript. Do not repeat all the data in the tables or figures in the text.

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Acknowledgments. Acknowledgments of people, grants, funds, etc. should be placed in a separate section after the *Conclusions* section. The names of funding organizations should be written in full. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflict of Interest. Authors must declare all relevant interests that could be perceived as conflicting. If there is no conflicts exist, the authors should state this. Submitting authors are responsible for coauthors declaring their interests.

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The examples of correct referencing:

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For journal papers by DOI:

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For books:

Kleiner FS, Mamiya CJ, Tansey RG. 2001. *Gardner's art through the ages* (11th ed.). Fort Worth, USA: Harcourt College Publishers.

For chapter in an edited book:

Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. *Introduction to the electronic age*, New York: E-Publishing Inc; 2009, p. 281–304.

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Tables should always be cited in text in consecutive numerical order. For each table, please supply a table caption (title) explaining the components of the table. Identify any previously published material by giving the original source in the form of a reference at the end of the table caption. Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Figures

Please submit each figure as an individual file separate from the manuscript text. All figures are to be numbered using Arabic numerals. Figures should always be cited in text in consecutive numerical order. Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.

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RESEARCH AND PUBLICATION ETHICS

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigation was carried out following the rules of the Declaration of Helsinki of 1975 (<https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/>), revised in 2013. As a minimum, a statement including number of approval and name of the ethics committee must be stated in Section 'Statement of Human Rights' of the article. In addition, the protection of privacy is a legal right that must not be breached without individual informed consent. In cases where the identification of personal information is necessary for scientific reasons, authors should obtain full documentation of informed consent, including written permission from the patient prior to inclusion in the study.

Example of Statement of Human Rights: "The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Name of the Institution (No. number of approval)."

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For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all

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