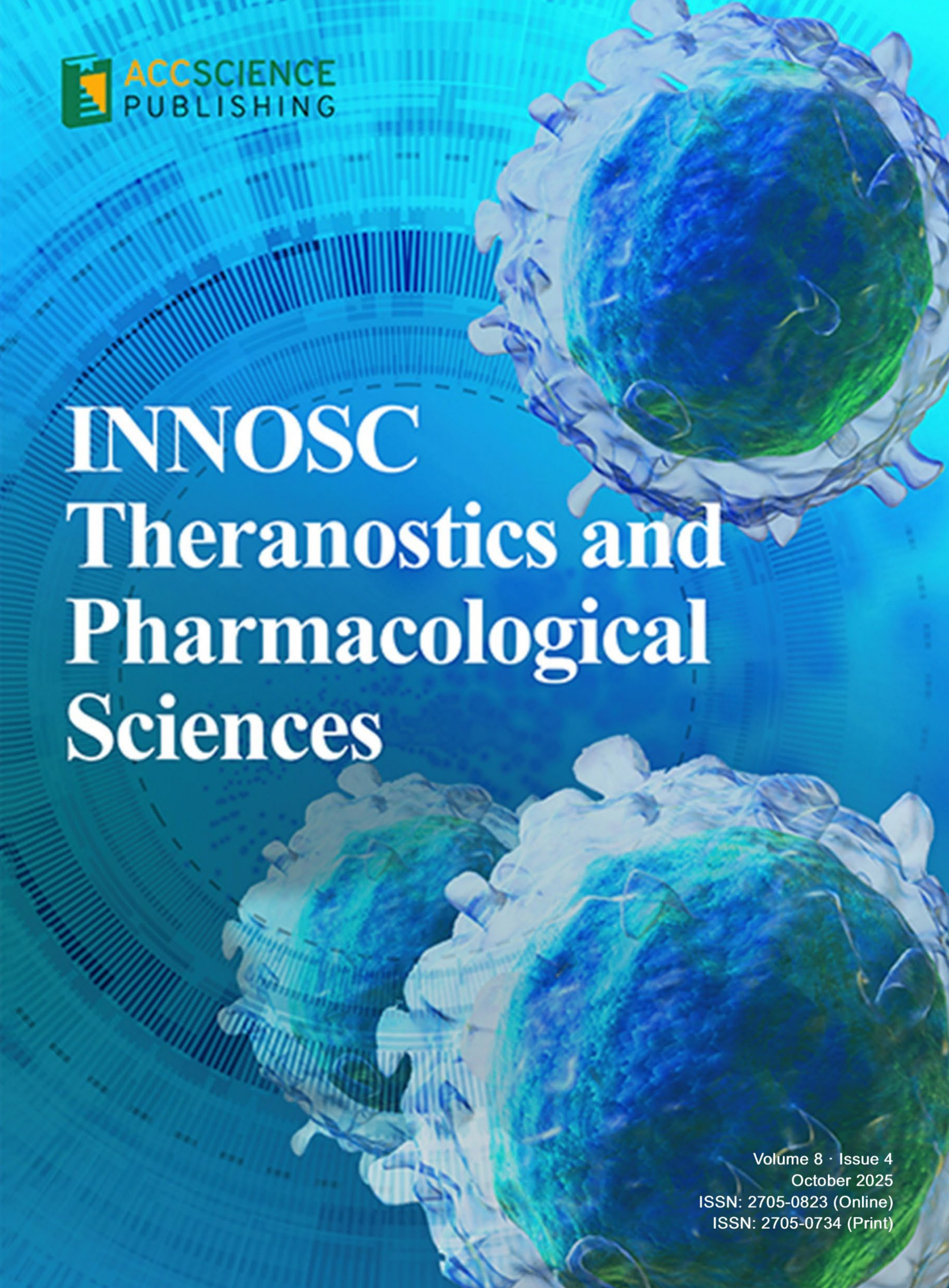
ACCSCIENCE
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INNOSC
Theranostics and
Pharmacological
Sciences
Volume 8 · Issue 4
October 2025
ISSN: 2705-0823 (Online)
ISSN: 2705-0734 (Print)

INNOSC Theranostics and Pharmacological Sciences

Print ISSN: 2705-0734

Online ISSN: 2705-0823

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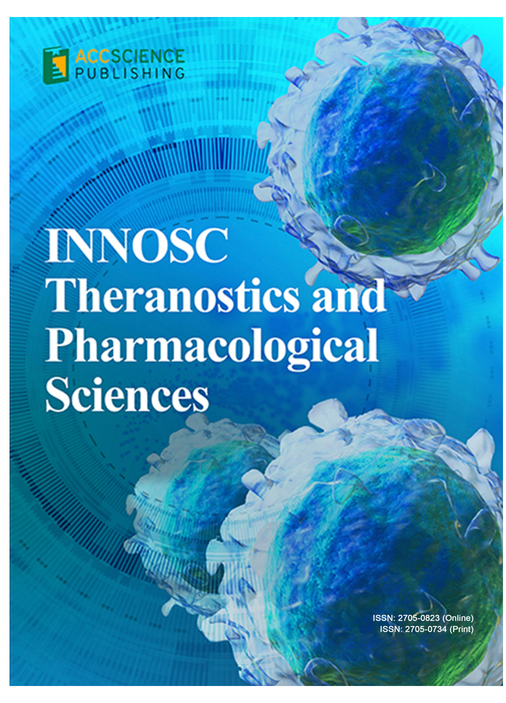

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Volume 8 • Issue 4 • October 2025
ISSN 2705-0734 (print) ISSN 2705-0823 (online)

INNOSC Theranostics and Pharmacological Sciences

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INNOSC THERANOSTICS AND PHARMACOLOGICAL SCIENCES

ISSN: 2705-0734 (print)

ISSN: 2705-0823 (online)

Editorial and Production Credits

Publisher: AccScience Publishing
Managing Editor: Jennifer Fang
Production Editor: Sharmila Velapasamy
Article Layout and Typeset: Sinjore Technologies (India)
Cover Design: ProPub (China)

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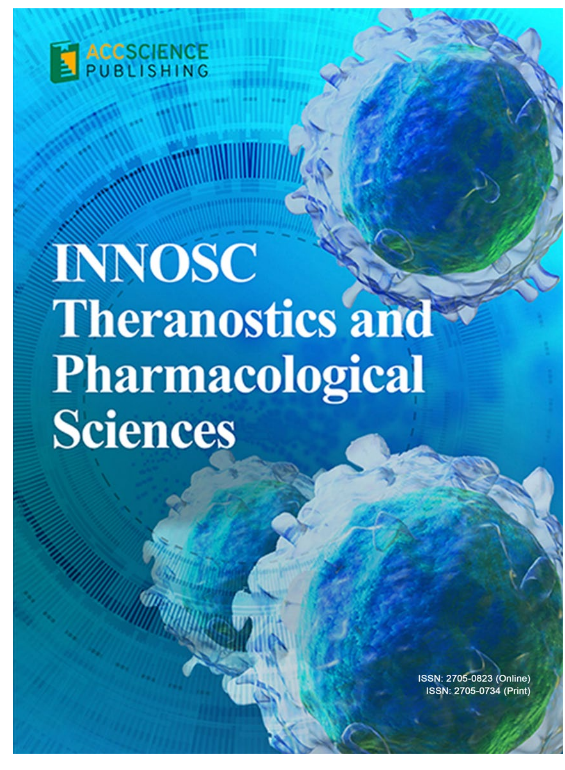

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INNOSC Theranostics and Pharmacological Sciences

REVIEW ARTICLE

FTO as an epigenetic regulator in metabolic and inflammatory diseases

Sunita Giri and Vijay Kumar*

Department of Molecular and Cellular Medicine, Institute of Liver and Biliary Sciences, New Delhi, India

Abstract

Obesity has emerged as a global health crisis in the 21st century, driven by rising obesity rates and associated metabolic disorders. The genome-wide association studies led to the identification of a gene associated with obesity and thus named the fat mass and obesity-associated (*FTO*) gene. In humans, the *FTO* gene is expressed in various tissues, including the brain, liver, and adipose tissue. It is known to play a major role in regulating energy balance, appetite, and body fat mass. *FTO* encodes an N6-methyladenosine RNA demethylase that regulates key biological processes, including adipogenesis, energy homeostasis, and glucose metabolism, through a dynamic RNA modification process involving splicing, export, decay, and/or translation. Polymorphisms in the *FTO* gene, particularly within intron 1, are strongly associated with increased risk of body mass index and obesity. In addition, *FTO* has been implicated in the pathogenesis of type 2 diabetes mellitus, cardiovascular disease, chronic kidney disease, and various cancers, where it could function both as an oncogene and a tumor suppressor gene. Pre-clinical studies have established the functional relevance of FTO, where its overexpression promotes obesity, whereas its deletion allows maintenance of a normal body phenotype. This review covers the recent advances in the development of specific FTO inhibitors and highlights their potential as therapeutic agents for obesity and metabolic disorders, although their development is still in its early stages. Despite the significant progress made in understanding the role of FTO in metabolic diseases, further research is needed to elucidate the precise mechanisms linking *FTO* variants to specific pathological outcomes and to optimize targeted therapies.

Keywords: FTO; N6-methyladenosine; Metabolic dysfunction-associated steatotic liver disease; Obesity; RNA demethylase; Inhibitors; Single nucleotide polymorphisms; RNA demethylation

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Citation: Giri S, Kumar V. *FTO* as an epigenetic regulator in metabolic and inflammatory diseases. *INNOSC Theranostics and Pharmacological Sciences*. 2025;8(4):1-15.
doi: 10.36922/ITPS025140019

Received: April 1, 2025

1st revised: June 17, 2025

2nd revised: July 2, 2025

Accepted: July 4, 2025

Published online: August 20, 2025

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1. Introduction

Overweight and obesity pose significant health risks that are characterized by abnormal or excessive accumulation of adipose tissue, both in size (hypertrophy) and number (hyperplasia).[1] These conditions are commonly classified based on body mass index (BMI), where BMI >25 is defined as overweight, and BMI >30 is categorized as obese.[2] According to the World Health Organization (WHO), the prevalence of overweight and obesity has continued to rise in both adults and children, and over 1 billion people worldwide will become obese by 2030.[3]

Obesity is a chronic multifactorial disease that adversely affects overall health and quality of life. It is influenced by genetic predisposition, physiological mechanisms, environmental factors, and lifestyle choices, including diet and physical activity levels.[4] The primary underlying cause is sustained energy imbalance, wherein excessive caloric intake leads to lipid accumulation and adipocyte hypertrophy, resulting in increased secretion of pro-inflammatory cytokines.[1] Chronic inflammation contributes to insulin resistance and impaired glucose and lipid metabolism, leading to metabolic syndrome (MetS), a key factor in obesity-related complications.[5,6] Obesity is associated with increased risk of numerous medical conditions, including cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), hypertension, dyslipidemia, nonalcoholic fatty liver disease, and several malignancies.[5] Besides, it has profound psychological and psychosocial consequences that affect self-esteem and mental health.[6]

The genome-wide association studies have shown that the fat mass and obesity-associated (*FTO*) gene has a strong association with the obesity risk.[7,8] Genetic variations in the *FTO* gene are strongly associated with increased BMI and obesity risk, as well as a higher risk of developing different cancers in diverse populations.[8] The role of single-nucleotide polymorphisms (SNPs) in the pathophysiology of obesity and cancer is increasingly becoming clear.[2,9] At the molecular level, it involves dynamic regulation of N6-methyladenosine (m6A) modifications in nucleic acids, particularly RNAs.[9] FTO is a member of the Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenase superfamily,[7] primarily functioning as an oxidative demethylase by catalyzing the conversion of m6A to N6-hydroxymethyladenosine and then to adenosine through N6-formyladenosine intermediate (Figure 1).[10]

Recently, FTO has gained attention as a potential RNA demethylase or an "eraser" that has a profound influence on mRNA stability, protein translation, and disease development (Figure 2). The FTO protein selectively interacts and co-localizes with RNA-binding proteins and splicing factors in an RNA-independent manner across the transcriptome, facilitating m6A demethylation in proximity to splicing factor, proline-, and glutamine-rich (SFPQ) binding sites.[11] SFPQ binds and interacts with the CUGUG consensus sequence on RNA and recruits FTO. The C-terminal domain (CTD) of FTO facilitates this interaction, enhances RNA substrate selectivity, and mediates localized demethylation. The reversible nature of m6A marks in gene transcripts makes it a promising therapeutic target for FTO-related interventions.

Significant efforts have focused on developing small-molecule inhibitors to block the demethylase activity of FTO and regulate gene expression involved in pathophysiological changes. However, the clinical relevance of FTO in obesity and metabolic diseases is still unclear. This review explores the key roles of FTO in health and disease and in managing metabolic disorders.

2. *FTO* gene and protein

2.1. Discovery of the *FTO* gene

The *FTO* gene was originally discovered as one of six genes that naturally localized within a 1.6 Mb region on chromosome 8 in a fused toe (Ft) mouse model.[7] Earlier, it was known as *Fatso*, on account of its large size, but was later dubbed as *FTO* (Figure 3).[10,12]

2.2. Origin and evolution of the *FTO* gene

The evolutionary lineage of the *FTO* gene spans approximately 450 million years. Sequence homology analyses across species, from mammals to unicellular organisms (such as diatoms and green algae), reported good sequence conservation at both the nucleotide and amino acid levels.[7,8] *FTO* is present as a single copy gene with no variants or isoforms in all vertebrates, except for humans (based on the National Center for Biotechnology Information [NCBI] database). Homology analysis of the FTO protein revealed a high degree of sequence conservation among different vertebrate species, each of which carries two conserved protein sequence motifs, namely "MAVSWHHDENLV" and "PRFSSTHRVA," in the middle region (NCBI database; Figure 4). The human and mouse *FTO* genes share a close sequence alignment, with each containing nine exons. While the *FTO* gene is localized on chromosome 16q12.2 in humans, it is present on chromosome 8 in mice.[7] Sequence homology between mouse and human FTO proteins is approximately 87%, whereas mouse and rat FTO proteins share 96% similarity (NCBI database). In addition, the human FTO protein and its vertebrate homologs are globular in structure and contain a nuclear localization signal, indicating a primary nuclear function.[12]

The *FTO* gene is expressed in various animal tissues and is widely dispersed across a variety of biological taxa.[10] Notably, it is highly expressed in the skeletal muscle and adipose tissue.[10,12] Its most prominent expression is observed in the hypothalamic arcuate nucleus, which is crucial for regulating energy balance and may play a key role in appetite regulation and metabolic processes.[7,10] The *FTO* gene encodes a protein with RNA demethylase activity essential for controlling a wide range of biological and metabolic processes. The gene is highly polymorphic, with many variations listed in the NCBI dbSNP database. FTO polymorphisms, especially those found in intron 1, are associated with (MetS; Table 1). These genetic loci are

Figure 1. Biochemistry of the N6-methyladenosine (m6A) demethylation process. Adenosine undergoes methylation at the sixth position, a process facilitated by N6-adenosine methyltransferase-like 3/14/16 (METTL3/14/16) enzymes. Fat mass and obesity-associated protein catalyzes oxidative demethylation reactions of m6A. The N6-methyl group undergoes oxidation in the presence of Fe(II) and α-ketoglutarate, leading to the formation of N6-hydroxymethyladenosine (hm6A) and N6-formyladenosine (f6A). In contrast, methylation at the first position of adenosine occurs through an endogenous alkylating agent and can be reversed by alpha-ketoglutarate-dependent dioxygenase alkB homolog 3 demethylase. Figure was created with BioRender.com (https://BioRender.com/z9rg2dy).
Abbreviation: m1A: N1-methyladenosine.

Table 1. ***FTO*** **single-nucleotide polymorphisms (SNPs) and their associated diseases**

SNP	Location in gene	Associated traits/diseases	References
rs9939609	Intron 1	Increased BMI, obesity, T2DM, metabolic syndrome, CVD, CAD, atherogenic lipid profile, elevated C-reactive protein, hypertension, increased risk of certain cancers (lung, renal, breast, prostate, pancreatic, endometrial, and melanoma)	9,15-17
rs1421085	Intron 1	Increased BMI, obesity, T2DM, metabolic syndrome	17
rs17817449	Intron 1	Increased BMI, obesity, T2DM, metabolic syndrome, CVD	9,17
rs8043757	Intron 1	Increased BMI, obesity	2

Abbreviations: BMI: Body mass index; CAD: Coronary artery disease; CVD: Cardiovascular disease; T2DM: Type 2 diabetes mellitus.

categorized as intronic polymorphisms (e.g., rs9939609 and rs17817449), exonic polymorphisms (e.g., rs79206939), and promoters (e.g., rs62048369).[13] Moreover, strong correlations have also been found between obesity and MetS biomarkers, such as waist circumference, blood pressure, and levels of triglycerides, high-density lipoprotein, and fasting blood glucose, in association with variations within intron 1, such as rs993960976.[14]

2.3. Structure and function of *FTO* protein

The *FTO* gene variants exhibit a significant variability in their structure, size, and function. The human *FTO* gene encompasses 12 known isoforms, whereas mice have only one isoform, reflecting the complexity of FTO regulation in humans. The function and expression of mouse FTO protein (502 aa) share a similar pattern to that of human FTO-3 (505 aa). The domain structure of the FTO protein was elucidated after the resolution of the crystal structure of the human FTO isoform 3.[10] This isoform carried a catalytic core N-terminal domain (NTD) with demethylase activity (amino acids 32–325). The CTD (amino acids 326–505) adopts a three-helix bundle configuration, one end of which interacts extensively with the NTD to provide structural stability and to regulate the enzymatic

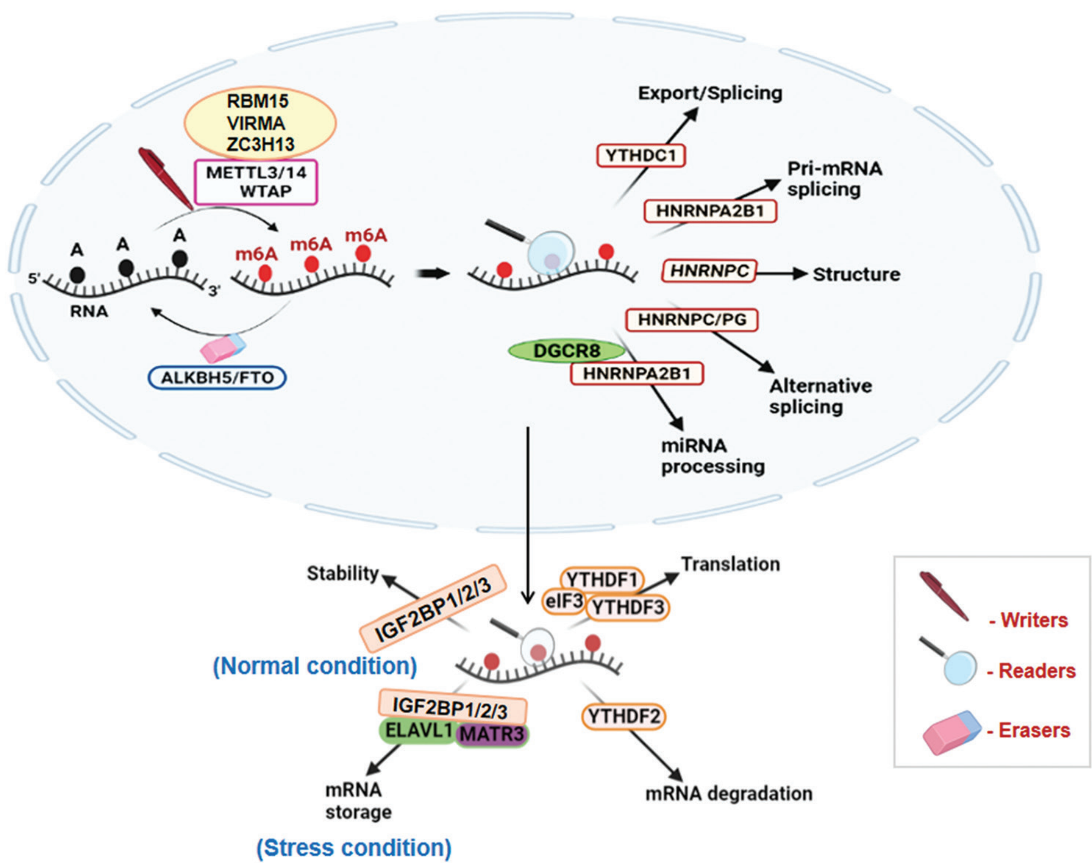

Figure 2. Dynamic regulation of N6-methyladenosine (m6A) modification in mRNA. Methyltransferases, including N6-adenosine methyltransferase-like 3/14 and WT1-associated protein, behave as writers and incorporate m6A signatures into gene transcripts. Demethylases such as fat mass and obesity-associated protein and alpha-ketoglutarate-dependent dioxygenase alkB homolog 5 act as erasers by removing m6A signatures from transcripts, whereas readers such as YTH family and others determine the transcript fate. Figure was created with BioRender.com (https://BioRender.com/eei3fb1).
Abbreviations: DGCR8: DiGeorge syndrome critical region 8; IGF2BP1: Insulin-like Growth Factor 2 mRNA Binding Protein 1; eIF3: Eukaryotic translation initiation factor 3; ELAVL1: ELAV-like protein 1; HNRNP: Heterogeneous nuclear ribonucleoproteins;; MATR3: Matrin-3; mRNA: Messenger RNA; miRNA: micro RNA; RBM15: RNA-binding protein 15; VIRMA: vir like m6A methyltransferase associated; YTHDC1: YTH domain-containing protein 1; YTHDF: YTH domain-containing family protein; ZC3H13: Zinc finger CCCH domain-containing protein 13.

activity of FTO (Figure 5).[7] Highly conserved residues, such as His231, Asp233, and His307, within the double-stranded β-helix of the NTD play a crucial role in Fe^{2+} chelation. Although the structure–function relationship of FTO-3 has been investigated in detail, the functions and sites of expression of other isoforms remain unknown. Nevertheless, these findings offer structural insights into FTO's substrate specificity and establish a framework for the rational design of FTO inhibitors.[10]

The NCBI database suggests that among the 12 FTO isoforms in humans, FTO-1 is the longest (526 aa) isoform, whereas FTO-11 (334 aa) is the shortest isoform containing an "unstab_antitox" module (Figure 6). However, the organ-specific functions of FTO isoforms in obesity-associated diseases need to be investigated as their biological functions remain poorly understood.

3. Roles of *FTO* in metabolic regulation and disease susceptibility

3.1. *FTO* and insulin resistance

Insulin is the only hormone that lowers blood glucose levels.[18] Impaired β-cell function and insufficient insulin production in the body lead to insulin resistance (a hallmark of T2DM), glucose-sensitive organ damage, and vital function impairment. It has been reported that obesity and higher BMI increase the risk of developing T2DM.[19-21] Excess body fat increases the release of free fatty acids, inflammatory cytokines, and other metabolic byproducts that disrupt insulin signaling, leading to insulin resistance[20] (Figure 7). In animal models, hepatic *FTO* mRNA levels were increased in fasting mice with reduced body weight and low blood glucose levels,[7] whereas

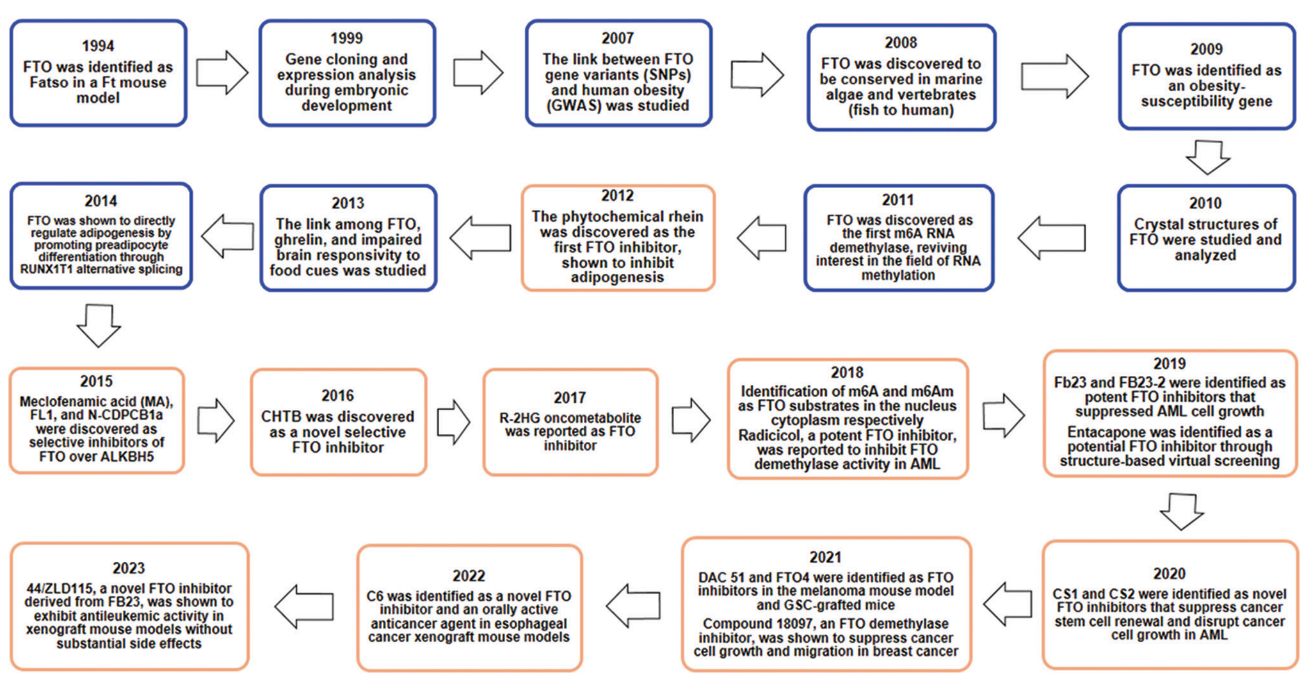

Figure 3. Timeline of fat mass and obesity-associated (*FTO*) gene and protein research. The events related to FTO discovery and biology are shown in blue boxes. Novel and FTO-selective inhibitors are shown in brown boxes. Image created by the authors using Microsoft PowerPoint.

Abbreviations: ALKBH5: Alpha-ketoglutarate-dependent dioxygenase alkB homolog; AML: Acute myeloid leukemia; CHTB: 4-Chloro-6-(6'-chloro-7'-hydroxy-2',4',4'-trimethyl-chroman-2'-yl)benzene-1,3-diol; FL1: Floricaula/leafy homolog 1; Ft: Fused toe; GWAS: Genome-wide association studies; GSC: glioblastoma stem cells; m6A: N6-methyladenosine; m6Am: N6, 2-O-dimethyladenosine; N-CDPCB1a: N-(5-chloro-2,4-dihydroxyphenyl)-1-phenylcyclobutanecarboxamide; R-2HG: (R)-2-hydroxyglutarate; RUNX1T1: RUNX1 partner transcriptional co-repressor 1; R-2HG: R-2-hydroxyglutarate; SNP: Single-nucleotide polymorphism.

hyperglycemic mice showed reduced levels of hepatic *FTO* mRNA than the normoglycemic controls.[19] In contrast, humans with hyperglycemia and metabolic dysfunction-associated steatotic liver disease (MASLD) showed higher *FTO* mRNA and protein levels than healthy individuals.[7] In addition, the dual function of FTO in controlling adipose tissue, as well as energy balance, is linked to the progression of obesity to diabetes.[20,21]

In patients with T2DM, glucose increases FTO expression and decreases global m6A content. The m6A methylation in gene transcripts plays a crucial role in β-cell biology, neonatal β-cell mass development, T2DM pathogenesis,[7] and glucose homeostasis regulation by pancreatic β-cells.[22,23] It also influences the maturation of β-cell insulin secretion and cell survival.[7] It has been shown that the downregulation of FTO impairs autophagy, suggesting FTO as a biomarker and therapeutic target in diabetic wound healing.[24] Furthermore, FTO influences the expression of several metabolism-associated genes, such as *FOXO1*, *FASN*, *G6PC*, and *DGAT2*, which in turn affects the pathophysiology of T2DM.[7,19,25] Research has demonstrated a negative correlation between m6A content and *FTO* mRNA expression and a positive correlation between fasting glucose levels and FTO mRNA expression in white blood cells.[7]

A genome-wide screen for T2DM susceptibility genes has identified several FTO variants that predispose individuals to obesity through their effect on BMI.[26,27] For example, FTO variants rs8050136 and rs9939609A>C are strongly associated with important markers of inflammation, obesity, and insulin resistance in an adiposity-dependent manner.[26]

3.2. *FTO* in appetite and energy balance

The prevalence of overweight and obesity has reached pandemic levels worldwide, affecting 60–70% of the adult population in industrialized nations, with numbers continuing to rise.[19] Among the genetic factors linked to obesity, the *FTO* gene has been implicated in the development of obesity and related metabolic disorders.[28] It was the first gene to be conclusively linked to obesity through GWAS, which identified SNPs within the gene that correlates with BMI, body fat rate, waist and hip circumference, as well as energy intake.[9]

FTO expression in the arcuate nucleus of the hypothalamus is regulated by feeding and fasting cycles[9] and has been shown to influence food intake in rats when selectively altered.[19] Inhibition of hypothalamic FTO activates signal transducer and activator of transcription

Figure 4. Sequence homology of the fat mass and obesity-associated (FTO) protein from representative vertebrate species. Alignment of amino acid sequences of the nine FTO proteins using the Clustal Omega (version 1.2.4; http://www.clustal.org/) multiple sequence alignment software. Gaps (--) were introduced to optimize the alignment of identical amino acid residues. All species encode two conserved protein sequence motifs, "MAVSWHHDENLV" and "PRFSSTHRVA" (highlighted in yellow), and six conserved cysteine residues (highlighted in blue). The gene accession numbers for the nine different FTO sequences are NP_001017273.1-frog (498 aa), NP_001172076.1-chicken (507 aa), NP_001034802.1-rat (502 aa), NP_036066.2-mouse (502 aa), NP_001350823.1-human FTO 1 (526 aa), NP_001186297.1-rabbit (504 aa), NP_001106162.1-pig (505 aa), NP_001098401.1.1-sheep (505 aa), and NP_001091611.1-cattle (505 aa). Note: Data retrieved and modified from National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov).

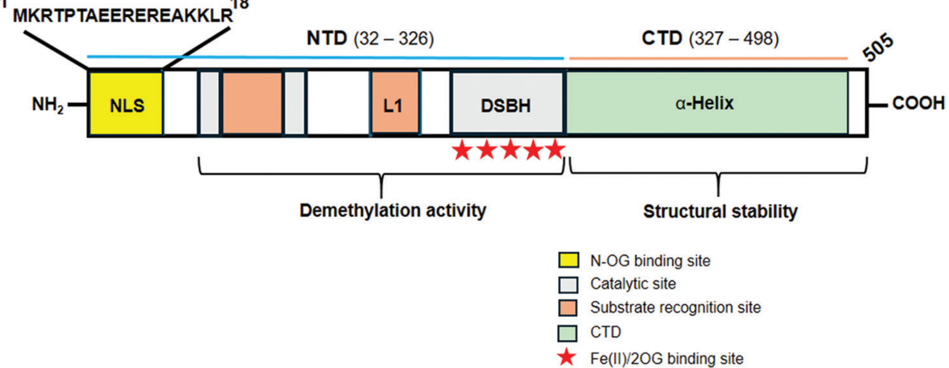

Figure 5. Functional domains of the fat mass and obesity-associated (FTO) protein. The human FTO protein, comprising 505 amino acids, contains two primary domains: the N-terminal domain (NTD) spanning 32–326 aa residues and the C-terminal domain (CTD) covering 327–498 aa residues. NTD also contains nuclear localization signals (NLS) rich in K (lysine) and R (arginine) spanning from 1 to 18, two distinct substrate recognition lids encompassing 77–102 aa residues and 103–116 aa residues, and a unique loop (L1) exclusive to FTO located between residues 210 and 223, mediating substrate specificity. The catalytic core within the NTD features a highly conserved double-stranded β-helix (DSBH) fold, resembling the structural characteristics of Fe(II)/2-oxoglutarate (OG)-dependent oxygenase. CTD interacts with the OG-binding domain in NTD for demethylation activity and maintains the structural stability of the protein. Image created by the authors using Microsoft PowerPoint.

3 (STAT3) through extracellular signal-regulated kinase 1/2 signaling, resulting in reduced food intake and body weight.[29] In addition, FTO has been implicated in the regulation of ghrelin, a hunger hormone. *FTO*-knockout mice exhibit elevated circulating ghrelin levels after prolonged fasting, whereas *FTO* overexpression in cell cultures shows reduced m6A methylation of *Ghrl* mRNA, thereby increasing ghrelin production.[13] A similar pattern is observed in humans carrying high-risk *FTO* alleles, where peripheral blood cells show increased *FTO* and *Ghrl* mRNA expression.[19] Together, these findings underscore the role of FTO in the regulation of appetite and energy balance.

3.3. *FTO* in adipogenesis

In addition to its role in regulating food intake and satiety, FTO is also involved in adipogenesis. During adipocyte differentiation, FTO expression progressively declines, whereas m6A methylation levels show a steady increase.[14] Observational studies on humans have suggested that the degree of physical activity may influence the effect of *FTO* SNPs on adiposity.[7] Notably, polymorphisms found in introns 1 (rs17817449, rs1421085, rs8050136, rs1558902, rs7206790, and rs1861868) and intron 8 (rs11644943) are strongly associated with waist circumference.[13] These associations underscore the genetic contribution of *FTO* polymorphisms to adiposity and metabolic traits, especially during early developmental stages.

It has been shown that FTO regulates important signaling pathways that control the differentiation of fat cells. Its deficiency inhibits adipogenesis in pre-adipocytes of both pig and mouse by suppressing the Janus kinase 2 (JAK2)–STAT3–CCAAT/enhancer-binding protein-beta (C/EBPβ) signaling cascade.[10] Early adipocyte differentiation depends on C/EBPβ transcription, which is mechanistically reduced in the absence of FTO due to decreased expression of JAK2 and impaired STAT3 phosphorylation. Under normal physiological conditions, skeletal muscle is the main peripheral organ involved in lipid metabolism.[23] Increased m6A methylation promotes lipid accumulation in skeletal muscle by upregulating genes linked to lipid synthase and downregulating genes linked to oxidation and lipolysis.[10,30] These results suggest FTO as a potential therapeutic target for the treatment of obesity and metabolic disorders.

In a high-fat diet mouse model, FTO overexpression leads to increased production of sterol regulatory element-binding protein 1 (SREBP1) and carbohydrate-responsive element-binding protein, promoting lipogenesis.[31] The result indicates that FTO acts as a transcriptional co-activator along with SREBP1c on the promoters of lipogenesis-related genes, thereby contributing to disease progression and severity.

3.4. *FTO* in metabolic dysfunction-associated steatotic liver disease

Nearly 25% of the adult population worldwide is affected by MASLD—the most common chronic liver disease, surpassing both T2DM and obesity in prevalence.[32] Although MASLD is highly prevalent worldwide, higher rates have been observed in countries with lower Human Development Index scores.[32,33] MASLD is also a major cause of liver-related mortality, yet no Food and Drug Administration (FDA)-approved treatment is currently

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NP_001350832.1      LHKEVQEAFLTLHKHGCLFRDLVRIQGKDLLTPVSRILIGNPGCTYKYLNTRLFTVPWPV   120
NP_001350828.1      LHKEVQEAFLTLHKHGCLFRDLVRIQGKDLLTPVSRILIGNPGCTYKYLNTRLFTVPWPV   120
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NP_001350829.1      LHKEVQEAFLTLHKHGCLFRDLVRIQGKDLLTPVSRILIGNPGCTYKYLNTRLFTVPWPV   120
NP_001350827.1      LHKEVQEAFLTLHKHGCLFRDLVRIQGKDLLTPVSRILIGNPGCTYKYLNTRLFTVPWPV   120
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NP_001350826.1      EGV----------------------TNAVLHEVKREGLPVEQRNEILTAILASLTARQNL   422
NP_001073901.1      EGV----------------------TNAVLHEVKREGLPVEQRNEILTAILASLTARQNL   448
NP_001350834.1      EGV----------------------TNAVLHEVKREGLPVEQRNEILTAILASLTARQNL   277
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Figure 6. Sequence homology of the human fat mass and obesity-associated (FTO) protein isoforms. Alignment of amino acid sequences of the 12 human FTO isoforms using the Clustal Omega (version 1.2.4; http://www.clustal.org/) multiple sequence alignment software. Gaps (--) were introduced to optimize the alignment of identical amino acid residues. All isoforms encode three conserved protein sequence motifs (highlighted). The N-terminal motifs "MSADFPRVGMGSSYNGQDEVDIKSRAAY NVTLLNFMDPQKMPYLKEEPYFGMGKMAVSWHHDENLVDRS" and "DLNATHQHCVLAGSQPRFSSTHRVA" are catalytic domains that exhibit demethylase activity. Sequences shown in bold are conserved in the FTO protein of all vertebrates (Figure 4). The C-terminal motif—EVEFEWLRQFWFQGNRYRKCTDWWCQPMAQLEALW KKMEGV—includes the EF-hand calcium-binding domain and cyclic adenosine monophosphate-dependent protein kinase phosphorylation site (shown in bold). The gene accession numbers for the 12 different FTO isoforms are: NP_001350823.1-FTO isoform 1 (526 aa), NP_001350820.1-FTO isoform 2 (515 aa), NP_001073901.1-FTO isoform 3 (505 aa), NP_001350825.1-FTO isoform 4 (499 aa), NP_001350826.1-FTO isoform 5 (479 aa), NP_001350827.1 -FTO isoform 6 (467 aa), NP_001350828.1-FTO isoform 7 (467 aa), NP_001350829.1-FTO isoform 8 (457 aa), NP_001350830.1-FTO isoform 9 (457 aa), NP_001350832.1-FTO isoform 10 (424 aa), NP_001350834.1-FTO isoform 11 (334 aa), and NP_001350917.1-FTO isoform 12 (482 aa).
Note: Data retrieved and modified from National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov).

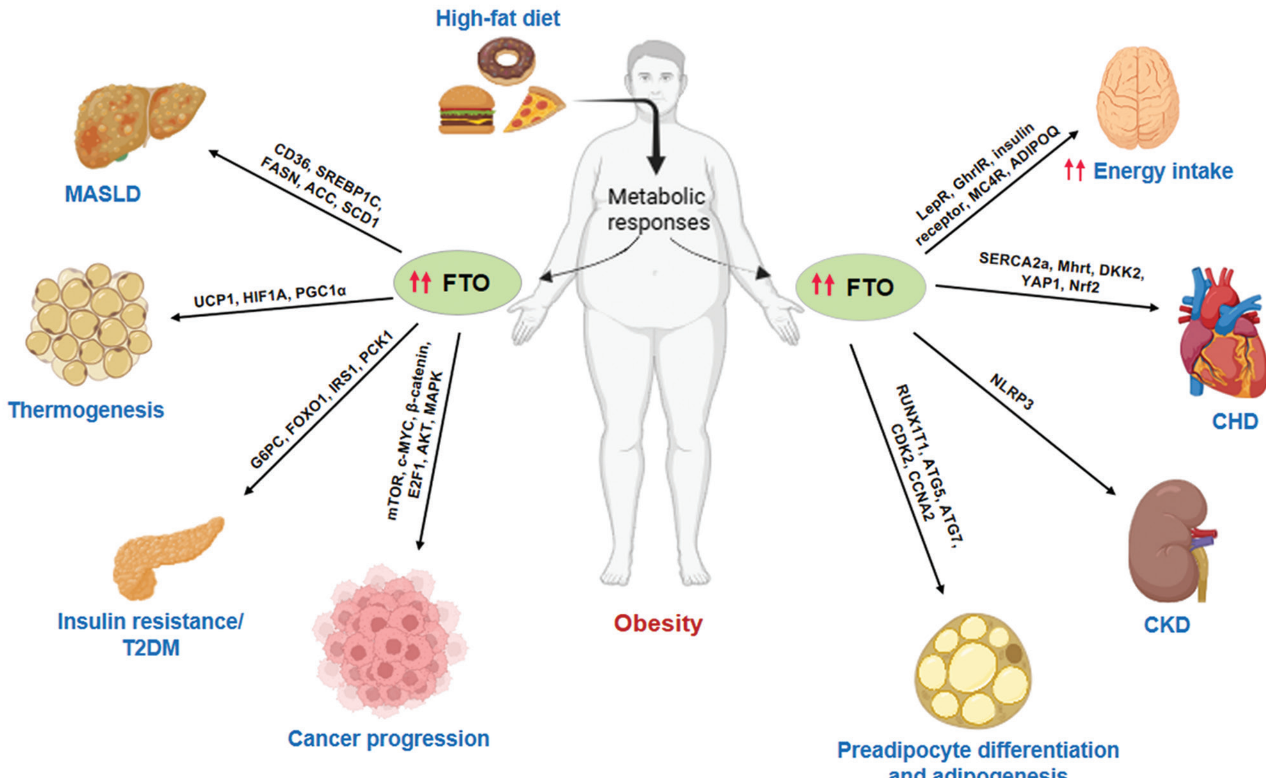

Figure 7. Dysregulation of FTO in obesity-associated diseases. The FTO protein plays a significant role in various physiological and pathological processes, with its altered expression influencing multiple metabolic functions. It is implicated in the regulation of energy balance and adipogenesis, mediated through pathways such as the leptin receptor, Ghrelin receptor, RUNX1 partner transcriptional co-repressor 1, and cyclin-dependent kinase 2/cyclin A2 signaling cascade. In addition, it contributes to the pathophysiology of insulin resistance and MASLD through modulation of the forkhead box protein O1 in gluconeogenesis and sterol regulatory element-binding protein-1c in lipogenesis. FTO also affects cardiac function by regulating sarcoplasmic/endoplasmic reticulum Ca 2+ ATPase 2a, myosin heavy chain-associated RNA transcript, and yes-associated protein 1. Moreover, it contributes to the pathophysiology of CKD through the modulation of NLR family pyrin domain containing 3. Furthermore, its oncogenic role has been associated with the activation of the phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin pathway and the c-MYC oncogene. The broad impacts of FTO on these diverse cellular processes underscore its importance in various disease conditions. Figure was created with BioRender.com (https://BioRender.com/pe6akt9).

Abbreviations: ADIPOQ: Adiponectin; CHD: Coronary heart disease; CKD: Chronic kidney disease; FTO: Fat mass and obesity-associated; MASLD: Metabolic dysfunction associated steatotic liver disease; T2DM: Type 2 diabetes mellitus.

available.[33] Despite extensive research, the mechanisms underlying MASLD pathogenesis are largely unknown. The defining characteristic of MASLD is increased hepatic triglyceride production, primarily due to increased activity of lipogenic enzyme regulators.[34-36]

Elevated FTO level plays its role in MASLD and metabolic dysfunction-associated steatohepatitis, potentially by altering m6A patterns, underscoring m6A's influence on liver disease progression.[7,15] In a glucocorticoid-induced chicken model, FTO was shown to contribute to the development of MASLD, specifically through glucocorticoid receptor-mediated m6A modifications of lipogenic mRNAs.[7] These findings suggest that targeting FTO may be a promising therapeutic approach for the treatment of MASLD.

3.5. *FTO* in CVD

According to a WHO report, CVD remains the foremost cause of death worldwide, accounting for nearly 18 million fatalities annually.[19] Among the main risk factors, obesity plays a pivotal role in promoting myocardial fat accumulation and fostering a pro-inflammatory and pro-thrombotic environment.[37] Heart failure is primarily driven by the loss of cardiomyocytes and their impaired contractility. Therefore, targeting the apoptotic pathways to mitigate cardiomyocyte loss may be a promising therapeutic strategy in the management of heart failure.[16]

The *FTO* gene has been identified as an important modulator of cardiovascular health.[10] Experimental studies have shown that FTO-mediated hypermethylation

Figure 8. Chemical structures of fat mass and obesity-associated protein inhibitors and their mode of action. Image created by the authors using standard chemical structures and Microsoft PowerPoint.

Abbreviations: CHTB: 4-Chloro-6-(6'-chloro-7'-hydroxy-2',4',4'-trimethyl-chroman-2'-yl)benzene-1,3-diol; N-CDPCB: N-(5-chloro-2,4-dihydroxyphenyl)-1-phenylcyclobutanecarboxamide; R-2HG: R-2-hydroxyglutarate.

plays a significant role in metabolic disturbances, leading to myocardial dysfunction, cardiomyocyte fibrosis, and apoptosis through the mitochondria-dependent apoptotic pathway.[10] Moreover, elevated FTO levels protect myocardial cells from apoptosis under hypoxic and reoxygenation conditions by modulating the m6A modification of myosin heavy chain-associated gene transcripts, ultimately improving heart failure outcomes.

Considering that obesity is a well-established contributor to CVDs, FTO is believed to play a crucial role in their onset and progression.[38] A meta-analysis evaluating the relationship between the *FTO* rs9939609 variant and CVD confirmed a significant association, independent of BMI, and other conventional CVD risk factors.[39] Moreover, this variant has been linked to both CVD and coronary heart disease, independent of established risk factors, and diabetes-related measures. These findings collectively indicate that FTO influences cardiovascular health through multiple molecular pathways, beyond its known role in obesity.

3.6. *FTO* in coronary artery disease (CAD)

CAD and its severe complication, myocardial infarction (MI), are major contributors to global morbidity and mortality, arising from a combination of environmental and genetic risk factors. Well-documented risk factors include smoking, excessive alcohol consumption, abnormal lipid profiles, obesity, hypertension, and T2DM.[40] Recently, FTO has been implicated in the pathogenesis of various CVDs, including atherosclerosis, CAD, and MI.[41] FTO protects against atherosclerosis by modulating lipid metabolism, inhibiting lipid uptake, increasing cholesterol efflux, and reducing the formation of macrophage-derived foam cells. Its upregulation improves cardiac contractility, lowers fibrosis, and promotes angiogenesis in ischemic myocardium.[40] Collectively, these results indicate that FTO plays a critical role in the pathophysiology of MI and CAD.

3.7. *FTO* in chronic kidney disease (CKD)

Nearly 20–40% of diabetic patients develop CKD.[42] It has become a major public health concern due to its increasing incidence, high medical expenses, and compromises on life expectancy and quality of life. While environmental and genetic factors play a key role, epigenetic modifications and genetic variations are also associated with the development and progression of CKD. Notably, a positive correlation between the FTO polymorphism rs7204609 and CKD has been established. Moreover, a reduced expression of FTO is observed in CKD patients, while its overexpression promotes cell growth and inhibits pyroptosis.[42] FTO shows protective effects on kidney function by modulating interleukin expression and pyroptosis-related protein levels in kidney tissue. Mechanistically, FTO influences CKD progression by modulating the expression of the *NLRP3* gene through m6A modifications and regulating the NACHT, LRR, and PYD domains-containing protein 3/Parkin signaling pathway.[43]

Apart from its role in metabolic diseases, FTO also plays an important role in pain sensitivity (hyperalgesia). It regulates the expression of P2X purinoceptor 3 receptors (P2X3Rs) through m6A modifications, key mediators of pain signaling.[44] In lumbar disc herniation, both FTO and P2X3R are upregulated in dorsal root ganglion neurons, contributing to increased neuronal excitability and hyperalgesia. Thus, targeting FTO or P2X3R presents a potential therapeutic strategy for alleviating pain sensitivity in lumbar disc herniation and related neuropathic conditions.

4. Inhibitors of FTO enzymatic activities

The active site of FTO includes three binding pockets—the 2OG binding site, which is crucial for its catalytic activity, as well as the Fe(II) binding site and substrate binding site, both of which offer a valuable framework for chemical intervention.[45] Therefore, the development of small molecules capable of targeting various functional domains of FTO and its expression holds considerable potential for developing new therapeutic strategies. Emerging studies on m6A regulation suggest a significant correlation between the extent of m6A modification and obesity-associated metabolic disorders, as well as between m6A modification and tumor development and progression.

The development of synthetic chemical inhibitors, as well as the identification of plant-derived molecules capable of targeting the m6A demethylase activity of FTO, represents a promising therapeutic approach for managing obesity and related metabolic disorders. Over the past decade, intensive research efforts on FTO inhibitors have led to the identification of several molecules that have shown promising results as anti-obesity and antitumor activities both *in vitro* and *in vivo* (Figure 8).

For example, rhein is a bioactive anthraquinone derivative isolated from rhubarb (*Rheum* species) and routinely used as a traditional Chinese medicinal herb. It is a strong FTO inhibitor (IC_{50} = 30 μM) that competitively binds to its catalytic domain and interferes with its interaction with single-stranded RNA substrates.[46]

Epigallocatechin gallate, a predominant catechin found in green tea, has been extensively studied for its potential anti-obesity and anti-adipogenic properties. It binds to the catalytic site of FTO and inhibits the demethylation of downstream targets cyclin-A2 and cyclin-dependent kinase 2, preventing the proliferation and differentiation of pre-adipocytes into mature adipocytes by effectively blocking mitotic clonal expansion and adipogenesis.[47]

Meclofenamic acid (MA), a non-steroidal anti-inflammatory molecule, was identified as a specific inhibitor of FTO demethylase activity, functioning through competitive binding at the catalytic site (IC_{50} = 8.6 μM).[48] Later, MA2 was developed as an ethyl ester derivative of MA, demonstrating enhanced FTO inhibition and increased m6A methylation of gene transcripts in a dose-dependent manner.[9] Based on the structural insights gained from MA, researchers have used structure-guided design strategies to develop more potent and selective FTO inhibitors.

A novel compound FB23 was synthesized using a dichloride-substituted benzene, resulting in an over 100-fold increase in its inhibitory potency (IC_{50} = 0.06 μM) compared to MA. Another potent FTO inhibitor, Dac51, was developed through structural optimization of FB23.[49] Dac51 showed enhanced inhibitory activity (IC_{50} = 0.4 μM) and maintained effective interactions within the FTO active site. Further chemical modifications of Dac51 led to the development of another FTO inhibitor, Dac85 (IC_{50} = 0.7 μM). In addition, another FTO inhibitor, ZLD115, was designed using a flexible alkaline side-chain-substituted benzoic acid structure derived from FB23. It showed improved pharmacological characteristics compared to earlier inhibitors, including FB23 and Dac85.

The therapeutic potential of the FDA-approved drug entacapone in metabolic disorders has been observed in a high-fat diet-induced obese mouse model, where it reduced body weight and blood glucose levels.[50] However, further research and comprehensive clinical validation are necessary to fully establish its efficacy and therapeutic value.

5. Conclusion

Obesity is a complex, multifactorial condition and a growing global epidemic linked to metabolic diseases, such as T2DM, CVDs, and certain cancers. While lifestyle and environmental factors are critical contributors to obesity, genetic predisposition also plays an equally important role. *FTO* encodes an m6A RNA demethylase that regulates gene expression through epitranscriptomic modifications essential for normal biological functions. Its reversible nature makes m6A a promising therapeutic target for FTO-related interventions. Understanding the FTO regulatory mechanisms may pave the way for the treatment of obesity, metabolic disorders, and cancer. Future research should focus on optimizing FTO modulators, exploring their structure–activity relationships, and assessing their clinical efficacy in managing complex diseases.

As illustrated in Figure 7, the dysregulation of FTO activity has been implicated in the development of obesity, metabolic disorders, and cancer. Therefore, targeting FTO using small-molecule inhibitors is a novel therapeutic

strategy. With its high expression in the brain, FTO influences energy intake and expenditure by mediating the demethylation of m6A RNA, thereby affecting the stability and translation of key genes involved in adipogenesis, energy balance, and metabolic homeostasis. In addition to its role in metabolic disorders, FTO is a crucial factor in cancer progression, making FTO a promising therapeutic target. Significant efforts have focused on developing small-molecule inhibitors that block FTO demethylase activity and modulate gene expression.

Some FTO inhibitors have shown promising antitumor effects in preclinical cancer models and have been shown to enhance insulin sensitivity and reduce adipogenesis in metabolic disorders. Despite their promise, further studies are required to evaluate the long-term safety, efficacy, and clinical relevance of FTO inhibitors.

Acknowledgments

None.

Funding

This work was supported in part by the J.C. Bose National Fellowship (Grant no. SR/S2/JCB-80/2012) from the Department of Science and Technology, Government of India, New Delhi (to V.K.). S.G. received a senior research fellowship from the University Grants Commission, India.

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: All authors
Visualization: All authors
Writing – original draft: Sunita Giri
Writing – review & editing: Vijay Kumar

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Not applicable.

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INNOSC Theranostics and Pharmacological Sciences

REVIEW ARTICLE

Therapeutic role of Nrf2/ARE pathway activation in chronic atrophic gastritis: Mechanisms, progress, and future perspectives

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Citation: Wu W, Chen S, Su Y, Chen Y, Lin T, Liu Y. Therapeutic role of Nrf2/ARE pathway activation in chronic atrophic gastritis: Mechanisms, progress, and future perspectives. *INNOSC Theranostics and Pharmacological Sciences.* 2025;8(4):16-24.
doi: 10.36922/ITPS025130016

Received: March 30, 2025

1st revised: September 4, 2025

2nd revised: September 15, 2025

Accepted: September 19, 2025

Published online: October 22, 2025

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Publisher's Note: AccScience Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract

Chronic atrophic gastritis (CAG) is a prevalent and progressive gastric disorder that can lead to significant gastric dysfunction and an increased risk of gastric cancer. Oxidative stress plays a central role in the pathogenesis of CAG, contributing to cellular damage and inflammation. The nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway is crucial in cellular defense against oxidative stress by regulating the expression of antioxidant and cytoprotective genes. Activation of this pathway has shown promise in mitigating oxidative damage and promoting mucosal healing in CAG. This review aims to explore the therapeutic potential of Nrf2/ARE pathway activation in the context of CAG. We discuss the mechanisms underlying the activation of Nrf2, its role in regulating antioxidant genes, and its impact on oxidative stress management in the gastric mucosa. Recent advancements in pharmacological agents that activate the Nrf2 pathway, such as rhein and certain traditional Chinese medicines, are also highlighted. These interventions have demonstrated protective effects against oxidative stress, reduced inflammation, and promoted gastric mucosal healing. We, further, explore the potential clinical applications of Nrf2 activators in CAG treatment and the challenges in translating these findings into clinical practice. In addition, we outline future research directions aimed at optimizing Nrf2 activation strategies and exploring combination therapies to enhance therapeutic outcomes in CAG patients. The findings underscore the importance of targeting the Nrf2/ARE pathway as a promising strategy for managing oxidative stress-related gastric diseases.

Keywords: Nrf2/ARE pathway; Chronic atrophic gastritis; Antioxidant genes; Oxidative stress

1. Introduction

Chronic atrophic gastritis (CAG) is an inflammatory gastric disorder involving progressive loss of gastric glandular cells, leading to mucosal atrophy and an increased

risk of gastric cancer. Key contributing factors include persistent *Helicobacter pylori* infection, autoimmune responses, and environmental influences. A central mechanism in CAG pathogenesis is oxidative stress, caused by an imbalance between reactive oxygen species (ROS) production and antioxidant defense. This imbalance promotes cellular damage, inflammation, and atrophy.

The nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway plays a critical role in combating oxidative stress. It regulates the expression of antioxidant and cytoprotective genes by binding to antioxidant response elements (AREs) in DNA. On activation, the Nrf2 proteins translocate to the nucleus and initiate the transcription of genes encoding enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, which collectively reduce oxidative damage and inflammation. Consequently, targeting the Nrf2/ARE pathway has emerged as a promising therapeutic strategy for CAG.

Compounds such as rhein have demonstrated protective effects in experimental models by activating Nrf2, reducing oxidative stress, and promoting mucosal healing.[1] Moreover, traditional Chinese medicine has also been explored for its efficacy in treating CAG, with various herbal formulations demonstrating potential in modulating the Nrf2/ARE pathway and improving clinical outcomes. This review aims to elucidate the latest research advancements regarding the involvement of the Nrf2/ARE signaling pathway in CAG, emphasizing its role in oxidative stress management and potential therapeutic strategies. By understanding the intricate relationship between oxidative stress, Nrf2 activation, and gastric mucosal integrity, we can better appreciate the therapeutic implications of targeting this pathway in the prevention and treatment of CAG and its associated complications.

2. Mechanistic basis of the Nrf2/ARE pathway

2.1. Activation sources and mechanisms of Nrf2

The Nrf2 protein is a pivotal transcription factor that orchestrates the cellular response to oxidative stress and electrophilic insults. Its activation is primarily regulated by the Kelch-like ECH-associated protein 1 (Keap1) complex, which serves as a sensor for oxidative stress. Under normal conditions, Nrf2 is maintained in the cytoplasm through its interaction with Keap1, leading to its ubiquitination and subsequent proteasomal degradation. However, on exposure to oxidative stressors, such as ROS and electrophiles, specific cysteine residues on Keap1 are modified, resulting in the dissociation of Nrf2 from Keap1. This release allows Nrf2 to translocate into the nucleus, where it binds to the ARE in the promoter regions of target genes, initiating the transcription of various cytoprotective and detoxifying enzymes, such as heme oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1). This classical activation mechanism and the interaction between Nrf2 and Keap1 are summarized in Figure 1. Recent studies have highlighted the role of additional regulatory proteins, such as p62, which can further enhance Nrf2 activation by promoting the degradation of Keap1 through selective autophagy, thereby establishing a positive feedback loop that amplifies the antioxidant response during prolonged oxidative stress exposure.[2,3]

2.2. Interaction between Nrf2 and ARE

The interaction between Nrf2 and ARE is crucial for the transcriptional activation of genes involved in cellular defense mechanisms. Nrf2 forms a heterodimer with small musculoaponeurotic fibrosarcoma proteins, which facilitates its binding to ARE sequences located in the promoter regions of target genes. This binding is essential for the recruitment of co-activators and the assembly of the transcriptional machinery necessary for gene expression. Once activated, Nrf2 not only promotes the expression of antioxidant enzymes but also regulates genes involved in cellular metabolism and inflammation, thus playing a comprehensive role in maintaining redox homeostasis. Furthermore, the Nrf2/ARE signaling pathway has been implicated in various pathophysiological conditions, including neurodegenerative diseases, where dysregulation of this pathway can lead to increased oxidative stress and neuronal damage.[3,4]

2.3. Regulation and expression of antioxidant genes

The Nrf2/ARE pathway serves as a central regulator of antioxidant gene expression, critical for cellular defense against oxidative stress. On activation, the Nrf2 proteins translocate to the nucleus and bind to AREs, initiating the transcription of key enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. These enzymes act synergistically to neutralize ROS and limit oxidative damage. Beyond enzymatic antioxidants, Nrf2 also upregulates phase II detoxifying enzymes that promote xenobiotic clearance. The regulation of this pathway is modulated through epigenetic mechanisms, including histone acetylation and methylation, as well as post-translational modifications of Nrf2, which fine-tune its transcriptional activity. This intricate regulation highlights the importance of the Nrf2/ARE system in redox homeostasis and its potential as a therapeutic target in oxidative stress-related diseases.[4-6]

doi: 10.36922/ITPS025130016

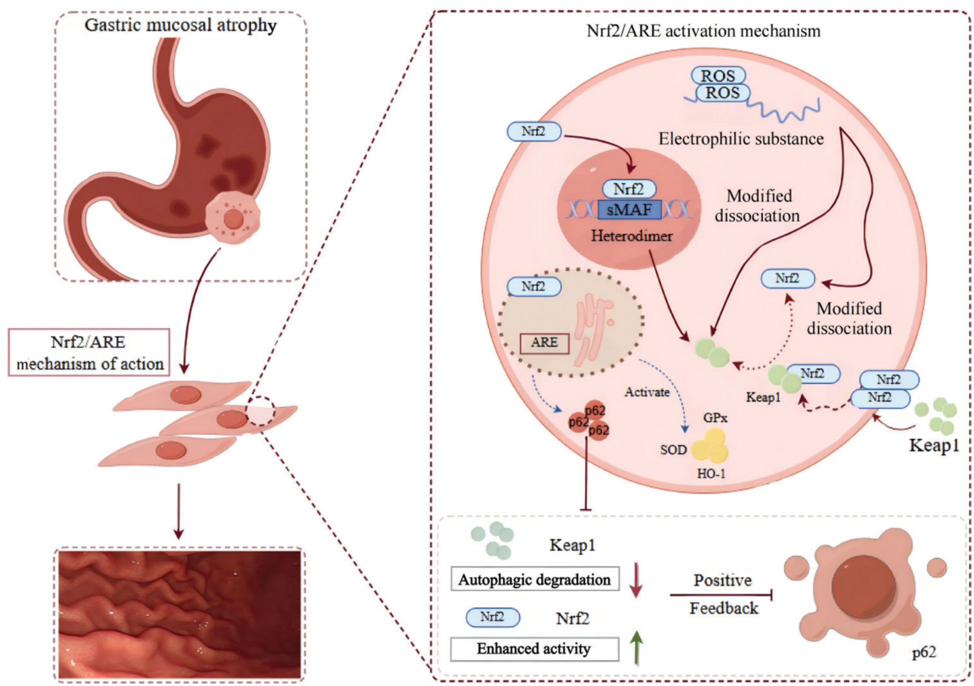

Figure 1. Schematic diagram illustrating the activation mechanism of the Nrf2/ARE pathway. Image created by the authors using Figdraw.com.
Abbreviations: ARE: Antioxidant response element; GPx: Glutathione peroxidase; HO-1: Heme oxygenase-1; Keap1: Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid 2-related factor 2; ROS: Reactive oxygen species; sMAF: Small musculoaponeurotic fibrosarcoma; SOD: Superoxide dismutase.

3. The impact of acute oxidative stress on the gastric mucosa

3.1. Sources and mechanisms of oxidative stress

Acute oxidative stress in the gastric mucosa can arise from various sources, including exogenous factors such as alcohol consumption, non-steroidal anti-inflammatory drugs (NSAIDs), and infections like *H. pylori* infection. These factors lead to the generation of ROS, which can overwhelm the antioxidant defenses of gastric epithelial cells. For example, ethanol exposure has been shown to induce oxidative stress by increasing the production of malondialdehyde and decreasing antioxidant enzyme activities, such as those of superoxide dismutase and glutathione peroxidase, in gastric tissues.[7] Furthermore, *H. pylori* infection is associated with a cascade of inflammatory responses that exacerbate oxidative stress, resulting in cellular apoptosis and impaired regeneration of the gastric mucosa.[8] The mechanisms through which oxidative stress affects gastric tissues include the activation of pro-inflammatory signaling pathways, such as the nuclear factor-kappa B (NF-κB) pathway, which leads to the upregulation of inflammatory cytokines, including tumor necrosis factor-alpha and interleukin 6, contributing to mucosal injury.[9] In addition, oxidative stress can trigger endoplasmic reticulum stress, further promoting apoptosis in gastric epithelial cells.[10] The central role of oxidative stress in gastric mucosal injury and its interplay with the Nrf2 pathway is summarized in Figure 2. Understanding these sources and mechanisms is crucial for developing therapeutic strategies to mitigate oxidative stress-related gastric injuries.

3.2. Cellular responses to oxidative stress exposure

When gastric epithelial cells are exposed to acute oxidative stress, they exhibit a range of cellular responses aimed at counteracting the damaging effects of ROS. These responses include the activation of antioxidant defense mechanisms and the initiation of repair pathways. For example, studies have demonstrated that treatment with protective agents, such as rebamipide, can significantly reduce apoptosis in gastric epithelial cells exposed to ethanol, primarily by inhibiting endoplasmic reticulum stress and enhancing autophagy-related pathways.[11] In addition, polysaccharides derived from natural sources, such as *Lachnum*, have been shown to improve oxidative stress responses by enhancing the activities of antioxidant enzymes and reducing levels of pro-inflammatory cytokines.[11] Moreover, the application of dietary antioxidants, such as those found in apple leaves, has been linked to decreased oxidative stress and enhanced

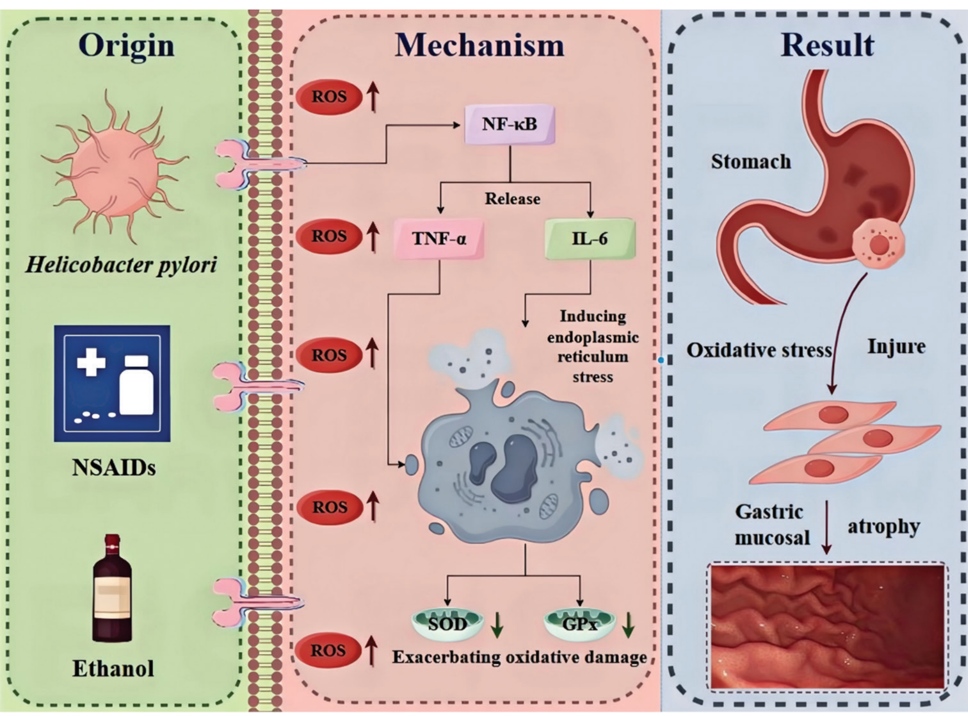

Figure 2. Relationship between oxidative stress and the Nrf2/ARE pathway in CAG. Image created by the authors using Figdraw.com.
Abbreviations: GPx: Glutathione peroxidase; IL-6: Interleukin 6; NF-κB: Nuclear factor-kappa B; NSAID: Non-steroidal anti-inflammatory drug; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TNF-α: Tumor necrosis factor-alpha.

healing of gastric mucosa following injury.[12] These cellular responses highlight the potential for pharmacological interventions to bolster the gastric mucosa's resilience against oxidative stress, thereby promoting mucosal integrity and healing. Furthermore, the interplay between oxidative stress and cellular signaling pathways, including the mitogen-activated protein kinase and NF-κB pathways, underscores the complexity of the gastric mucosal response to oxidative challenges.[13]

4. The role of the Nrf2/ARE pathway in CAG

4.1. The relationship between Nrf2 expression levels and disease progression

The Nrf2 protein is a pivotal transcription factor that regulates the expression of antioxidant and cytoprotective genes in response to oxidative stress. In CAG, the expression levels of Nrf2 can be indicative of disease progression. Studies suggest that reduced Nrf2 activity is associated with increased oxidative stress and inflammation, both of which are key contributors to the pathogenesis of CAG. For example, the activation of the Nrf2/ARE pathway has been shown to counteract oxidative damage and promote cell survival in various tissues, including the gastric mucosa. In patients with CAG, diminished Nrf2 levels correlate with heightened oxidative stress markers and inflammatory cytokines, suggesting a potential link between Nrf2 downregulation and disease severity. Furthermore, specific genetic or epigenetic modifications affecting the Nrf2 pathway may exacerbate the progression of gastritis by impairing the protective mechanisms normally conferred by Nrf2 activation.

Beyond the expression of Nrf2, the activity of the Nrf2 signaling pathway and the expression of its downstream target genes, such as *NQO1* and *HMOX1*, have emerged as promising biomarkers for assessing the risk of CAG progression and predicting therapeutic response. Research indicates that the expression levels of NQO1 and HO-1 are significantly correlated with patient prognosis. For example, in gastric cancer tissues, high expression of NQO1 serves as an independent risk factor predicting shorter overall survival and poorer progression-free survival.[14] In terms of treatment prediction, hyperactivation of Nrf2 signaling is closely associated with resistance to chemotherapeutic agents such as 5-fluorouracil.[15] This suggests that detecting Nrf2 signaling activity in tissues could help identify chemotherapy-insensitive populations and provides a rationale for combining Nrf2 inhibitors to reverse drug resistance.

This relationship underscores the importance of Nrf2 as a potential biomarker for assessing the severity of CAG

and highlights the need for therapeutic strategies aimed at enhancing Nrf2 signaling to mitigate disease progression and improve patient outcomes.

4.2. The impact of Nrf2-mediated antioxidant effects on gastric mucosal repair

The Nrf2/ARE signaling plays a key role in gastric mucosal repair, particularly in CAG. Activation of Nrf2 enhances the expression of cytoprotective genes, including *HMOX1*, glutathione S-transferases, and *NQO1*, which collectively alleviate oxidative damage and support mucosal healing. In experimental models of gastric injury, Nrf2 activation has been shown to reduce ROS levels, enhance mucosal integrity, and accelerate repair processes. Furthermore, several natural compounds that activate Nrf2 have demonstrated therapeutic potential in promoting gastric mucosal regeneration in CAG. Thus, targeting the Nrf2/ARE pathway represents a promising strategy for treating gastric mucosal injury and facilitating repair in conditions such as CAG.[16,17]

4.3. The dual-edged sword effect and potential risks of Nrf2 activation

Although Nrf2 activation plays a key role in the antioxidant protection in CAG, it must be recognized that its effects are not entirely beneficial. Extensive studies have shown that sustained and excessive activation of Nrf2 may promote tumor cell survival, proliferation, and drug resistance, acting as a "tumor-promoting factor." In gastric tissue, the Nrf2 pathway normally exerts cytoprotective effects by regulating antioxidant gene expression. However, during gastric carcinogenesis, persistent activation of this pathway, often due to Keap1 mutations or epigenetic alterations, instead enhances tumor cell survival, invasion, and chemoresistance by inhibiting apoptosis, promoting angiogenesis, and upregulating multidrug resistance proteins, highlighting the distinct dual-edged sword nature of Nrf2 in gastric cancer.[18,19] It is noteworthy that sustained activation of Nrf2 may promote tumorigenesis in certain contexts, particularly against a background of pre-existing immune-mediated damage in the gastric mucosa. For example, in autoimmune gastritis, chronic inflammation and epithelial remodeling create a microenvironment conducive to gastric carcinogenesis, and aberrant activation of Nrf2 could potentially exacerbate this process.[20]

5. Experimental and clinical research progress

5.1. Research on Nrf2 activation in animal models

The Nrf2 protein has emerged as a critical transcription factor in mediating cellular responses to oxidative stress. Research utilizing animal models has demonstrated the significant role of Nrf2 activation in various pathological conditions. For example, research has shown that Nrf2 activation can alleviate neuroinflammation and reduce tissue injury in neurological conditions, attenuate oxidative and inflammatory damages following acute injury, and protect against ischemia-reperfusion injury in transplantation models by inhibiting NF-κB activation.[21,22] These findings collectively underscore the importance of Nrf2 as a modulator of oxidative stress and inflammation across various experimental models, providing a robust foundation for its exploration in clinical settings.

5.2. Clinical cases and the efficacy of Nrf2 regulation

Clinical research has increasingly focused on the therapeutic implications of Nrf2 modulation in various diseases. In patients with coronary heart disease, studies have shown that treatment with nicorandil combined with trimetazidine resulted in decreased levels of serum miR-223-3p and Nrf2, correlating with improved clinical outcomes.[23] In addition, in the context of autoimmune diseases, the activation of the Nrf2 pathway has been associated with reduced inflammation and oxidative stress, suggesting that Nrf2 activators may serve as potential therapeutic agents.[24] In the realm of cancer, particularly hepatocellular carcinoma, overexpression of Nrf2 has been linked to poor prognosis, indicating that while Nrf2 activation can confer protective effects against oxidative stress, its dysregulation may facilitate tumor progression.[25] Moreover, recent insights into the role of Nrf2 in modulating the immune response during COVID-19 highlight its potential as a therapeutic target in managing hyperinflammation associated with the disease.[26]

Although Nrf2 activators show promise in monotherapy, their interactions in combination therapies, which are particularly significant for patients with CAG who often require polypharmacy, remain unclear. For example, NSAIDs are common triggers of gastric mucosal injury. Although Nrf2 activators could theoretically mitigate such damage, their metabolic interactions with NSAIDs and the long-term safety of combination therapy require further investigation.[27] On the other hand, Nrf2-mediated HO-1 induction enhances the protective effect of proton-pump inhibitors against NSAID-induced gastric injury, extending beyond acid suppression alone.[28] However, the hypoacidic environment caused by proton-pump inhibitors may also affect the bioavailability of certain Nrf2 activators, potentially leading to a conflict with their mucosal protective goals, a matter warranting further exploration. Risk–benefit assessment is especially critical for CAG patients who require long-term NSAID use due to cardiovascular disease or arthritis. Future studies should employ more sophisticated preclinical models and well-designed clinical trials to thoroughly investigate

the pharmacokinetic and pharmacodynamic interactions between Nrf2 activators and commonly used drugs, thereby facilitating the development of safe and effective individualized treatment strategies.

Collectively, these clinical findings illustrate the dual nature of Nrf2 regulation, emphasizing the need for a nuanced approach in targeting this pathway for therapeutic interventions across a spectrum of diseases.

5.3. Challenges and limitations in translational medicine

There exists a significant "translational gap" in current research. Translating the remarkable efficacy observed in animal models into clinical benefits for human patients faces multiple challenges. First, the complexity and heterogeneity of human diseases are far greater than those of experimental animal models. Second, natural compounds, such as sulforaphane and curcumin, generally suffer from low bioavailability.[29,30] Determining their optimal effective dosage, administration routes, and therapeutic windows in humans is crucial, yet remains insufficiently studied. Finally, the dual role of Nrf2 necessitates long-term and rigorous safety evaluations of its activators, especially in populations at risk for gastric cancer. Most existing clinical studies are small-scale and short-term observations, lacking large-scale, randomized, double-blind, and placebo-controlled clinical trials to confirm their efficacy and safety.

6. Future research directions

6.1. Development and application of Nrf2 agonists

The Nrf2 pathway is a pivotal regulator of cellular defense against oxidative stress, making it a promising therapeutic target for conditions driven by such mechanisms, including CAG. Recent studies have highlighted the potential of both natural and synthetic Nrf2 agonists to enhance antioxidant responses and mitigate cellular damage. For example, compounds such as sulforaphane and bardoxolone methyl have shown efficacy in activating Nrf2, increasing the expression of downstream antioxidant enzymes, and improving cellular resilience against oxidative stress.[31,32] Critically, despite a strong mechanistic rationale, robust clinical evidence supporting the use of Nrf2-targeting therapies in CAG patients is still lacking. This highlights a significant gap in translational medicine. Future efforts must prioritize the development of highly specific and safe Nrf2 agonists and rigorously evaluate their efficacy in dedicated *in vivo* models of CAG and early-phase clinical trials. Particular attention should be paid to optimizing dosing regimens and developing novel delivery systems, such as nanoparticle-based systems, thereby enhancing the bioavailability and effectiveness of Nrf2 activators in clinical settings.[33] Furthermore, given the context-dependent role of Nrf2, especially its ambiguous function in carcinogenesis,[34] future studies must carefully assess the long-term safety and disease-stage-specific therapeutic window of these agents in the context of CAG and its progression to gastric cancer.

6.2. Exploration of combination therapy strategies

The complexity of many diseases, particularly chronic conditions such as metabolic syndromes, cancers, and neurodegenerative diseases, has prompted increasing interest in combination therapy strategies. These strategies aim to enhance therapeutic efficacy by targeting multiple pathways simultaneously, thus overcoming limitations associated with monotherapy, such as drug resistance and insufficient treatment response.[32,35] For example, combining Nrf2 agonists (Table 1) with other pharmacological agents

Table 1. Summary of Nrf2 activators and potential therapeutic applications

Nrf2 activator	Source	Potential therapeutic applications	Mechanism of action	Stage of development	Limitations/ considerations
Sulforaphane	Natural compound (cruciferous vegetables)	• Cardiovascular diseases • Neurodegenerative disorders • Cancer prevention	• Direct activation of Nrf2 • Increases antioxidant enzyme expression	Clinical trials	Dosage and bioavailability challenges
Bardoxolone methyl	Synthetic compound	• Chronic kidney disease • Inflammatory conditions	• Potent Nrf2 pathway activation • Reduces oxidative stress	Approved	Potential pro-inflammatory effects in some contexts
Rhein	Natural compound (traditional Chinese medicine)	• Chronic atrophic gastritis • Anti-inflammatory treatments	• Modulates Nrf2 pathway • Reduces oxidative stress in the gastric mucosa	Preclinical	Limited long-term safety data
Curcumin	Natural compound (turmeric)	• Neurological disorders • Metabolic syndrome • Cancer prevention	• Indirect Nrf2 activation • Suppresses inflammatory pathways	Preclinical	Poor bioavailability
Polysaccharides	Natural compounds	• Gastric mucosal protection • Antioxidant therapy	• Enhances antioxidant enzyme activities • Reduces pro-inflammatory cytokines	Preclinical	Variability in extraction and potency

Abbreviation: Nrf2: Nuclear factor erythroid 2-related factor 2.

has shown promise in preclinical studies, particularly in enhancing antioxidant defenses and reducing inflammation in various models of disease.[33,34] Moreover, the integration of Nrf2 agonists with immunotherapies has the potential to improve outcomes in cancer treatment by modulating the tumor microenvironment and enhancing immune responses.[31] Future research should prioritize the identification of synergistic drug combinations through rigorous preclinical and clinical trials, focusing on understanding the underlying mechanisms of action and the potential for adverse interactions among combined therapies. The development of personalized medicine approaches, which tailor combination strategies based on individual patient profiles and disease characteristics, represents a promising avenue for improving treatment outcomes and minimizing side effects.

7. Conclusion

The Nrf2/ARE signaling pathway has gained considerable interest as a promising therapeutic target for CAG. Activation of this pathway enhances the expression of antioxidant genes, thereby alleviating oxidative stress and its damaging effects on the gastric mucosa. While the protective role of Nrf2 activators against oxidative damage is well-established, existing studies present variability in the choice of agents, dosages, and clinical applications. These inconsistencies underline the need for more standardized research approaches to better evaluate their therapeutic potential. Future efforts should focus on translating promising preclinical results into clinical practice, addressing not only efficacy but also safety, side effects, and drug interactions. Research is also needed to clarify the mechanisms behind Nrf2-mediated protection and to identify predictive biomarkers for patient response. Such insights will help in developing personalized treatments for CAG. The therapeutic potential of the Nrf2/ARE pathway in CAG is evident, yet it requires further exploration to fully realize its clinical benefits. By addressing the gaps in current research and fostering collaboration among scientists, clinicians, and pharmaceutical developers, we can pave the way for innovative treatments that significantly improve patient outcomes in this challenging condition.

Acknowledgments

None.

Funding

This study was funded by the Nanhai Xinxing Medical and Health Talent Platform Project of Hainan Province (NHXX-WJW-2023030) and the Hainan Province Science and Technology Special Fund of China (ZDYF2022SHFZ304).

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Not applicable.

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INNOSC Theranostics and Pharmacological Sciences

REVIEW ARTICLE

Nanomedicine in bone healing: A review of innovative silver nanoparticle therapies and theranostic advances

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Citation: Messina PV. Nanomedicine in bone healing: A review of innovative silver nanoparticle therapies and theranostic advances. *INNOSC Theranostics and Pharmacological Sciences*. 2025;8(4):25-52.
doi: 10.36922/ITPS025260039

Received: June 27, 2025

Revised: November 22, 2025

Accepted: November 28, 2025

Published online: December 16, 2025

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Abstract

Nanomedicine, at the convergence of nanotechnology and medicine, holds transformative potential for bone healing through advanced diagnostic and therapeutic strategies. Among various nanomaterials, silver nanoparticles (AgNPs) have gained attention due to their broad-spectrum antimicrobial, anti-inflammatory, and anticancer properties. This review examines the principles of nanomedicine and theranostics, AgNP synthesis methods and physicochemical characteristics, and their application in bone regeneration for infection control, scaffold design, osteoinduction, and angiogenesis. The integration of AgNPs into theranostic platforms for simultaneous therapy and imaging is critically evaluated, highlighting modalities such as micro-computed tomography, magnetic resonance imaging, and photoacoustic imaging. The preclinical and early clinical evidence is analyzed, addressing critical limitations including toxicity, biocompatibility, pharmacokinetics, manufacturing reproducibility, and regulatory hurdles. This review outlines future trends, such as green synthesis, stimuli-responsive systems, and personalized scaffolds. A comprehensive understanding of these multidimensional facets is crucial for advancing AgNP-based nanomedicine from the bench to the bedside in bone healing applications.

Keywords: Bone defects; Tissue imaging; Personalized medicine; Antimicrobial scaffolds; Photothermal therapy; Bone regeneration

1. Introduction

Bone is a highly specialized and dynamic connective tissue that plays essential structural, metabolic, and protective roles in the human body. Under normal physiological conditions, it possesses a remarkable capacity for self-repair and remodeling through coordinated actions of osteoblasts, osteoclasts, and osteocytes.[1,2] However, this intrinsic regenerative ability is often insufficient when confronting large-scale bone defects.[3] Such critical-size defects may arise from a variety of clinical scenarios, including high-energy trauma, chronic infections such as osteomyelitis, surgical resection of bone tumors, or developmental anomalies. In these cases, the defect exceeds the regenerative threshold of the body, necessitating medical intervention to restore bone integrity and function. Conventional strategies for bone defect repair primarily rely on bone grafting

techniques, which include autografts (the patient's own bone), allografts (donor bone from another human), and synthetic grafts composed of bioceramics or polymers. Each of these approaches, while valuable, is accompanied by significant drawbacks.[4] Autografts remain the gold standard due to their osteoconductive, osteoinductive, and osteogenic properties, but they are limited by donor site morbidity, pain, and volume constraints.[5] Allografts and xenografts introduce risks of immunogenic rejection and disease transmission, and often lack sufficient biological activity to fully support regeneration. Synthetic materials, though customizable and scalable, frequently suffer from poor integration, limited vascularization, and susceptibility to infection, especially in compromised patients.[6]

In response to these limitations, nanomedicine has emerged as a promising interdisciplinary field at the intersection of nanotechnology, biology, and medicine. It enables the engineering of nanoscale materials and systems designed to interact precisely with cellular and molecular processes involved in bone regeneration.[7] Nanoscale platforms offer unique advantages, including increased surface area for protein adsorption and cell attachment, enhanced control over drug and ion release, and the capacity to integrate therapeutic and diagnostic functions into a single device. One of the most innovative directions in this field is the development of theranostic systems, nanoscale constructs that simultaneously deliver therapeutic agents and provide diagnostic feedback.[8,9] In the context of bone repair, theranostic nanomaterials enable real-time tracking of healing progression, early detection of infection or inflammation, and site-specific delivery of drugs, growth factors, or antimicrobial agents. These capabilities align well with the goals of personalized medicine, which seeks to tailor treatments to individual patients based on dynamic physiological cues.[10-12]

Within the array of nanomaterials under investigation, silver nanoparticles (AgNPs) have garnered significant attention due to their multifaceted therapeutic properties.[13] They are well known for broad-spectrum antimicrobial activity, including efficacy against multidrug-resistant bacteria, a feature particularly valuable in orthopedic settings where infection poses a major threat to implant success. Beyond their antimicrobial role, AgNPs have been shown to modulate local immune responses, reducing excessive inflammation while supporting tissue healing. Emerging evidence also points to their potential in inhibiting tumor cell proliferation, making them attractive candidates for post-oncologic bone reconstruction. Importantly, AgNPs can be readily incorporated into various delivery platforms, including porous scaffolds, hydrogels, bone cements, and injectable nanocomposites, allowing flexible application across diverse clinical scenarios.[14-17] Together, these features position AgNP-based nanomedicine as a frontier technology for the regeneration of complex bone defects, addressing limitations of current therapies while opening new avenues for integrated, responsive, and effective clinical solutions.[15,18] Accordingly, this review addresses the following aspects:

(i) The core principles of nanomedicine and theranostic technology
(ii) AgNP synthesis, surface engineering, and mechanism of action
(iii) Application of AgNPs in bone repair for antimicrobial defense, osteoinduction, angiogenesis, and bone imaging
(iv) Integration into multifunctional platforms merging therapy and diagnosis
(v) Preclinical and early clinical insights
(vi) Key challenges in safety, production, and regulatory compliance
(vii) Future directions emphasizing customized, responsive nanoplatforms for bone regeneration.

By synthesizing existing knowledge and pinpointing gaps, this review aims to chart a roadmap for translating AgNP-based theranostic systems into practical, clinical bone-healing solutions. It synthesizes current evidence, considers often-overlooked practical factors, and incorporates perspectives from nanomedicine, theranostics, and bone-repair engineering. It also highlights important knowledge gaps that need to be addressed to transform AgNP-based theranostic systems into effective clinical solutions for bone healing.

2. Fundamentals of nanomedicine

2.1. Definition and historical evolution

Nanomedicine is defined as the medical application of nanotechnology, specifically, the use of materials and systems at the nanoscale, typically within the range of 1–100 nm, to diagnose, monitor, treat, and prevent disease.[19,20] This emerging field represents a paradigm shift in the way biomedical challenges are addressed, offering unprecedented precision, control, and multifunctionality at the molecular and cellular levels. The nanoscale dimension enables materials to interact with biological structures, such as proteins, DNA, and cell membranes, in unique ways that are not possible with conventional macroscale or microscale tools.[19,21,22] These interactions enable novel approaches for targeted drug delivery, non-invasive imaging, real-time diagnostics, regenerative therapies, and combined therapeutic and diagnostic (theranostic) applications. The origins of nanomedicine can be traced back several decades to early research on drug delivery systems, particularly liposomal formulations. In the 1990s, major clinical milestones

were achieved with the approval of Doxil®, the first Food and Drug Administration-approved polyethylene glycol (PEG)-coated liposomal formulation of doxorubicin, used for treating Kaposi's sarcoma and various solid tumors.[23] Doxil demonstrated how nanoparticle encapsulation could improve pharmacokinetics, reduce toxicity, and enhance drug accumulation at tumor sites through the enhanced permeability and retention effect. Another landmark was the development of Abraxane®, an albumin-bound nanoparticle form of paclitaxel, which enabled solvent-free formulation and improved bioavailability of a poorly soluble chemotherapeutic agent.[24] These early successes marked the beginning of clinical nanomedicine and underscored the potential of nanoscale materials to overcome longstanding limitations in drug solubility, biodistribution, and systemic toxicity.

Over time, the scope of nanomedicine has expanded far beyond liposomes to encompass a wide variety of engineered nanostructures, including polymeric nanoparticles, dendrimers, solid lipid nanoparticles, metallic and metal oxide nanomaterials, quantum dots, and nanogels.[25,26] More recent developments have incorporated biologically inspired and biomimetic nanodevices, such as cell membrane-coated nanoparticles that evade immune detection and DNA origami structures capable of logic-gated drug release.[27,28] The evolution of nanomedicine has also paralleled advances in nanofabrication techniques, surface functionalization strategies, and high-resolution imaging technologies.[17,21,29-32] Today, nanomedicine represents a cornerstone of precision medicine. It plays a pivotal role in oncology, cardiovascular therapy, neurology, infectious diseases, and regenerative medicine. As regulatory frameworks evolve and more nanoparticle-based formulations enter clinical trials or receive market approval, nanomedicine continues to transform both diagnostic and therapeutic paradigms, opening pathways toward safer, more effective, and patient-specific healthcare solutions.[33]

2.2. Classification of nanoparticles

Nanoparticles exhibit a broad spectrum of physical, chemical, and biological properties, making them highly versatile tools in nanomedicine.[34] However, not all nanoparticles behave alike. Their composition, morphology, and surface chemistry significantly influence their interaction with biological systems. Differences in material type—whether metallic, polymeric, or biomimetic—translate into varied capabilities in drug loading, release kinetics, cellular uptake, immune response modulation, and biodistribution. Therefore, understanding the distinct characteristics of each nanoparticle class is crucial for designing effective therapeutic and diagnostic systems tailored to specific clinical applications. These distinctions are particularly important in the context of bone regeneration and disorders affecting mineralized tissues, where a delicate balance between antimicrobial protection, inflammation control, osteogenesis, and angiogenesis must be achieved. The local microenvironment in bone injuries or pathologies such as osteomyelitis, osteosarcoma, or osteoporotic fractures presents unique challenges. Here, nanoparticle-based systems can be engineered to meet specific regenerative and therapeutic needs, for example, by delivering antibiotics directly to infected bone tissue, releasing osteoinductive factors to promote bone formation, or enabling localized chemotherapeutic activity in bone malignancies. Within this context, nanoparticles can be broadly classified into several categories.[34-36]

2.2.1. Inorganic nanoparticles

This group includes metal-based nanoparticles,[37,38] such as silver, gold, and copper, as well as metal oxide nanoparticles like zinc oxide and titanium dioxide,[39] and silica-based[40] systems. In the context of bone healing, AgNPs are widely used in bone scaffolds and coatings for orthopedic implants due to their potent antibacterial properties, which help prevent or treat bone infections, such as osteomyelitis.[41,42] Zinc oxide nanoparticles have been shown to stimulate osteoblast proliferation and mineralization,[43] making them valuable in bone tissue engineering. Similarly, gold nanoparticles have been explored for their anti-inflammatory and pro-osteogenic effects,[44] and their use in photothermal therapy for osteosarcoma[45] is under active investigation. Ceramic[46] and hydroxyapatite[47-51] nanoparticles play a pivotal role in bone therapies due to their excellent biocompatibility, bioactivity, and structural similarity to natural bone mineral. These nanoparticles support osteoconduction and promote bone regeneration by serving as scaffolds that enhance cell adhesion, proliferation, and differentiation. Hydroxyapatite, in particular, is widely used to coat implants or deliver therapeutic agents, improving integration with surrounding bone tissue and accelerating healing in cases of fractures, defects, or orthopedic surgeries. Their tunable surface properties also enable functionalization with drugs or growth factors, allowing for targeted and sustained therapeutic effects.[47-51] Recent advances in inorganic nanomaterials for bone cements include the addition of ceramic and carbon-based materials. These additives have shown significant improvements in mechanical performance and functionality. Ceramic components, such as zirconia, hydroxyapatite, and bioactive glass, can enhance compressive strength, radiopacity, and interfacial bonding. They also support bone growth responses. Carbon-based additives, such as carbon nanotubes,

graphene, and carbon fibers, significantly strengthen the cement matrix by increasing toughness, crack resistance, and load distribution. The series "Effect of Various Admixtures on Selected Mechanical Properties of Medium Viscosity Bone Cements: Parts 1–3" provides solid evidence on how these ceramic and carbon materials affect key properties.[52-54] These properties include bending strength, impact resistance, elastic modulus, and fatigue behavior. This research highlights how these additives can improve cement formulations beyond traditional fillers. These findings enhance our understanding of how inorganic nanostructures can be utilized to develop next-generation bone cements with improved mechanical reliability and multifunctional properties.

2.2.2. Polymeric and liposomal nanoparticles

These biodegradable systems (e.g., based on poly[lactic-co-glycolic acid] [PLGA], PEG, or chitosan) are widely used to deliver osteoinductive agents such as bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), or antiresorptive drugs like bisphosphonates.[55-57] For example, PLGA nanoparticles loaded with BMP-2 have been used to enhance bone formation in critical-size defects,[58] while liposomal formulations have been designed to encapsulate antibiotics or anti-inflammatory drugs, allowing for targeted and sustained release in infected or inflamed bone tissue.[59,60]

2.2.3. Biomimetic nanoparticles

Biomimetic nanoparticles include lipoprotein-based carriers,[61] exosome-like vesicles,[62] or nanoparticles cloaked with bone cell-derived membranes.[63] Their surface composition mimics native biological structures, enhancing compatibility and enabling targeted delivery to bone tissue. For instance, exosome-mimetic nanovesicles derived from mesenchymal stem cells (MSCs) have shown promise in promoting osteogenic differentiation and angiogenesis,[64,65] key processes in bone regeneration. In addition, bone-targeting peptides can be conjugated to nanoparticle surfaces to enhance accumulation at sites of demineralization or fracture.[66,67]

2.2.4. Hybrid composite nanoparticles

These systems combine the mechanical or imaging advantages of inorganic cores with the biocompatibility and versatility of polymeric shells. A common example is hydroxyapatite-coated polymeric nanoparticles, which offer both osteoconductivity and drug delivery capacity.[49,68,69] Hybrid systems have also been developed to co-deliver antibiotics and growth factors,[70] targeting infected bone defects with simultaneous regeneration and antimicrobial functions. Critical physicochemical features such as size, shape, surface charge, and surface functionalization govern the *in vivo* behavior of these nanoparticles. For bone-related applications, nanoparticles in the 50–200 nm range are often preferred, as they strike a balance between efficient cellular uptake and systemic circulation time. Surface modifications, such as conjugation with alendronate[71] or other bone-targeting ligands,[49,72,73] enhance selective accumulation in calcified tissues, while neutral or slightly negative charges are typically favored to minimize rapid clearance and immune activation.[74]

Optimizing these parameters is essential for enhancing the therapeutic efficacy and safety of nanoparticle-based strategies in bone repair and calcified tissue disorders. These systems represent a critical innovation in regenerative and precision medicine, offering multifunctional platforms that can diagnose, monitor, and treat complex bone pathologies with high specificity and reduced systemic side effects.

2.3. Diagnostic and therapeutic roles

One of the most transformative advantages of nanomedicine lies in its capacity to integrate diagnostic and therapeutic functions within a single platform.[11,20,30,50,59,61,62,73] Nanoparticles can be engineered not only to deliver therapeutic agents precisely where they are needed but also to enhance diagnostic imaging, monitor disease progression, and provide real-time feedback on treatment efficacy. This convergence of functionalities is central to the concept of theranostics, which seeks to bridge the gap between therapy and diagnosis, particularly valuable in complex, dynamic conditions such as bone infections, tumors, or regenerative therapies. Targeted drug delivery is a cornerstone of nanoparticle-based therapy. Nanoparticles can exploit the enhanced permeability and retention effect in inflamed or tumorous tissues, allowing passive accumulation at pathological sites.[75] Moreover, active targeting strategies, involving the functionalization of nanoparticle surfaces with ligands (e.g., peptides, antibodies, aptamers) that bind specifically to cellular markers (e.g., osteoblasts, tumor cells, or pathogens), further refine tissue selectivity.[71,73] In bone regeneration, this approach has been used to direct osteoinductive molecules such as BMP-2[76,77] or anti-resorptive drugs like alendronate[71] to sites of injury or disease, minimizing systemic side effects. In parallel, nanoparticles significantly enhance diagnostic imaging. Iron oxide nanoparticles, for example, serve as contrast agents in magnetic resonance imaging (MRI),[78] enabling sensitive detection of inflammatory or neoplastic bone lesions. Gold nano shells and nanorods offer strong absorption in the near-infrared range, making them suitable for photoacoustic and computed tomography (CT) imaging, as well as photothermal therapy.[79] Quantum dots, with their bright

and stable fluorescence, are used for high-resolution tracking of cellular processes and scaffold integration in bone tissue engineering studies.[80]

Nanoparticles also support physical therapy modalities. Magnetic nanoparticles can be used to generate localized hyperthermia under alternating magnetic fields, effectively damaging tumor cells in bone malignancies.[81,82] Similarly, gold nanoparticles can induce photothermal ablation when irradiated with near-infrared light, while photodynamic therapy is facilitated by nanoparticles that generate reactive oxygen species (ROS) on light activation, offering localized tumor control with minimal invasiveness.[83-85] Importantly, several nanoparticles exert intrinsic antimicrobial and anti-inflammatory effects. AgNPs and zinc oxide nanoparticles are particularly noteworthy in the context of bone healing, where bacterial infection (e.g., osteomyelitis) can severely compromise regeneration.[13-16,41-43] These nanoparticles can eliminate multidrug-resistant pathogens, modulate the immune response, and support osteogenic activity, making them especially suitable for incorporation into bone scaffolds or injectable hydrogels. Taken together, these multifaceted capabilities are converging into theranostic systems—multifunctional platforms capable of simultaneous drug delivery, imaging enhancement, and real-time monitoring of therapeutic outcomes.[50] In bone-related applications, such systems are being developed to monitor infection status, assess bone regeneration progress through imaging, and adapt drug release profiles in response to local stimuli (e.g., pH, enzymatic activity, or temperature changes). The integration of diagnostic and therapeutic roles in nanoparticle systems represents a paradigm shift in how bone diseases and injuries are managed, moving from static, one-size-fits-all approaches to adaptive, personalized interventions.

3. Theranostics in bone healing

3.1. Concept and rationale

Theranostics refers to the integration of therapeutic and diagnostic functionalities within a single nanoplatform, offering a highly synergistic approach for bone healing applications.[86] In the context of orthopedic regeneration, this dual functionality is particularly advantageous for real-time, *in situ* tracking of healing processes, detection of complications such as infection or delayed union, and localized therapeutic delivery. The rationale behind this approach lies in its ability to provide clinicians with continuous feedback on treatment efficacy, allowing for dynamic adjustment of therapies. By combining diagnostic imaging with therapeutic modalities, theranostic systems enhance precision medicine and reduce systemic side effects. Key advantages of theranostics in bone healing include:

(i) Longitudinal monitoring of scaffold integration and bone regeneration over time, enabling clinicians to assess healing progression or failure without repeated invasive procedures
(ii) Early detection of complications, such as bacterial infection, inflammation, or non-union, through responsive imaging signals
(iii) Localized and controlled drug delivery, which ensures the therapeutic agents act precisely where needed, while imaging signals verify the success of the delivery in real time
(iv) Simultaneous therapeutic feedback allows the clinician to monitor both anatomical and functional outcomes during treatment.

3.2. Imaging modalities

Several imaging techniques have been integrated into theranostic platforms for bone healing, each offering specific advantages based on resolution, depth penetration, and biocompatibility.[87,88]

Micro-CT offers high-resolution, three-dimensional visualization of bone architecture, mineral density, and scaffold integration. Its performance is significantly enhanced by the use of metallic nanoparticles, such as silver or gold, which act as radiopaque contrast agents. These nanoparticles enhance imaging clarity, enabling a detailed assessment of mineralized tissue regeneration.[89,90]

Magnetic resonance imaging provides excellent soft-tissue contrast and is especially useful for assessing inflammatory responses and soft-tissue integration around bone scaffolds.[78] Superparamagnetic nanoparticles, including those doped with gadolinium or iron oxide, are commonly used. AgNPs co-functionalized with these magnetic elements have demonstrated the ability to support both MRI and CT imaging, enabling multimodal diagnostic capabilities.[91,92]

In addition, photoacoustic imaging utilizes the plasmonic resonance of metallic nanostructures such as AgNPs or gold–silver alloy nanoparticles, combining optical and ultrasonic modalities for high-contrast, deep-tissue imaging.[93,94] It offers excellent resolution at significant depths, which is valuable for non-invasive monitoring of scaffold behavior and infection sites.[95,96]

Although less commonly used in bone tissue applications, fluorescence and near-infrared II imaging with rare-earth-doped nanoparticles have shown potential for visualizing inflammatory responses within the bone marrow.[97] This modality allows for sub-millimeter resolution without exposing the tissue to ionizing radiation, making it a promising tool for long-term studies.[17,32,98]

3.3. Dual-function nanosystems

Advanced theranostic systems often rely on AgNPs due to their intrinsic antimicrobial properties and imaging compatibility.[99,100] These platforms can be further functionalized with polymers, peptides, or other biocompatible coatings to enhance targeting and responsiveness to environmental stimuli such as pH shifts or ROS, which are common in infected or inflamed bone tissues.[101,102] A notable example includes silver–strontium mesoporous bioactive glass nanoparticles,[103] which serve multiple functions: they enhance CT imaging due to their high atomic number, release therapeutic silver ions to combat infection, and promote osteogenic differentiation, thereby supporting bone regeneration. Moreover, multifunctional scaffolds that combine antimicrobial and imaging capabilities have been validated in preclinical models.[104] In small animal studies, such scaffolds have demonstrated not only effective bone tissue formation but also visible suppression of infection through real-time imaging feedback.[105-107] These integrated systems exemplify the potential of theranostic platforms to revolutionize bone healing by bridging therapeutic intervention with continuous diagnostic monitoring.

4. Synthesis, properties, and mechanisms of AgNPs

4.1. Synthesis methods

The synthesis of AgNPs can be achieved through various chemical, physical, and biological approaches, each offering unique advantages and limitations regarding particle size, shape, purity, and environmental impact.[108]

4.1.1. Chemical methods

Chemical methods are the most commonly used strategy for AgNP synthesis. They typically involve the reduction of silver ions (Ag^+), often derived from silver nitrate, using chemical reducing agents such as sodium borohydride or trisodium citrate.[109] These methods enable the formation of well-dispersed colloidal nanoparticles with controlled sizes ranging from approximately 5 to 100 nm. While highly reproducible and tunable, a key drawback is the potential presence of toxic chemical residues from the reducing or stabilizing agents, which can limit biomedical applications unless extensive purification is carried out.

4.1.2. Physical methods

Physical methods include top-down techniques such as laser ablation,[110] evaporation–condensation, and ball milling. For instance, laser ablation, for instance, involves irradiating a silver target submerged in a liquid medium, producing nanoparticles with minimal chemical contamination.[110] These methods often yield AgNPs with high purity and good crystallinity, although they frequently exhibit a broad size distribution (polydispersity) and are limited in terms of scalability and cost-efficiency.[111]

4.1.3. Green synthesis

In response to concerns regarding toxicity and environmental sustainability, green synthesis approaches have gained prominence. These leverage natural reducing and capping agents such as plant extracts (rich in polyphenols, terpenoids, or alkaloids), microorganisms, or biomolecules like proteins.[112,113] Recent advances include the use of extracellular matrix proteins, such as fibronectin[114] or silk fibroin,[115] which can simultaneously reduce silver ions and stabilize the resulting nanoparticles. These biomimetic approaches yield biocompatible AgNPs with applications in tissue engineering and regenerative medicine, particularly as osteoinductive surface coatings that support cell adhesion and proliferation.[116]

4.2. Physicochemical properties

The functional performance of AgNPs in biomedical or environmental applications is closely linked to their physicochemical characteristics, which can be tailored through synthesis conditions and post-synthesis modifications.[17,101,102,108]

4.2.1. Size and shape

The size of AgNPs plays a critical role in determining their biological activity.[22,117] Smaller nanoparticles, particularly those under 10 nm, exhibit an enhanced ability to penetrate bacterial biofilms and cellular membranes due to their high surface area-to-volume ratio.[118] However, these same properties also increase their reactivity and potential cytotoxicity toward mammalian cells. Conversely, AgNPs in the range of 20–50 nm strike a balance between antimicrobial efficacy and reduced cytotoxicity, making them more favorable for clinical use.[119] In addition to size, shape (spherical, rod-like, triangular) influences their surface plasmon resonance and interaction with biological interfaces.[22,120]

4.2.2. Surface charge

The zeta potential of AgNPs governs their colloidal stability and interaction with cell membranes.[121,122] Positively charged nanoparticles exhibit stronger electrostatic interactions with negatively charged bacterial membranes, enhancing antimicrobial action.[123] However, they also have a higher propensity for internalization by eukaryotic cells, which can lead to unintended cytotoxic effects. To mitigate these issues, surface modification with neutral or slightly negative coatings such as PEG can be employed to modulate cellular uptake and prolong systemic circulation.[124]

4.2.3. Surface coatings and functionalization

Stabilizing ligands or coatings are crucial for maintaining nanoparticle dispersion and conferring specific biological functions.[108,125] Common capping agents include citrate, PEG, polyvinylpyrrolidone, and dextran, each influencing solubility, immune recognition, and biodistribution.[124] Furthermore, bioactive molecules such as BMP-2,[76] fibronectin,[114] or peptides[61,102] can be conjugated to AgNP surfaces to enhance cellular targeting, osteoinduction, and integration with host tissues, particularly in the context of implantable scaffolds or drug delivery systems.

4.3. Biological mechanisms of action

AgNPs exhibit multifaceted biological activities, making them attractive for a wide range of biomedical applications, including antimicrobial therapies, tissue engineering, and oncology.[126-128]

4.3.1. Antimicrobial activity

AgNPs are known for their potent, broad-spectrum antimicrobial properties.[129] They act through several mechanisms: disruption of bacterial cell wall and membrane integrity, induction of oxidative stress through ROS generation, and sustained release of Ag^+, which interferes with microbial DNA replication and protein synthesis. Minimum inhibitory concentrations for AgNPs are typically below 4 mg/L, and silver-impregnated materials, such as wound dressings or bone grafts, have demonstrated high efficacy against pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*.

4.3.2. Anti-biofilm effects

One of the most challenging aspects of bacterial infections is the formation of biofilms, which provides pathogens with resistance to antibiotics and host immune responses.[129] Immobilized AgNPs, particularly when integrated into scaffolds or coatings, can inhibit early-stage microbial adhesion and reduce surface colonization. These effects prevent the maturation of biofilms, making AgNP-based materials especially useful in medical device coatings and surgical implants.

4.3.3. Osteogenic and angiogenic stimulation

Beyond antimicrobial action, AgNPs also influence cellular pathways related to bone regeneration and vascularization.[15] They can modulate MSC behavior by activating the hypoxia-inducible factor 1-alpha pathway through ROS-mediated signaling, leading to increased expression of VEGF and osteogenic markers such as runt-related transcription factor 2 (Runx2) and osteocalcin (OCN). *In vitro* studies have shown enhanced alkaline phosphatase (ALP) activity and mineral deposition in cultures treated with AgNPs, supporting their potential in bone tissue engineering.

4.3.4. Anti-inflammatory and anticancer potential

At controlled doses (typically below 100 μg/mL), AgNPs have been shown to reduce the expression of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6, which may aid in controlling chronic inflammation associated with implant rejection or autoimmune conditions.[130,131] In addition, preliminary *in vitro* studies indicate that AgNPs can inhibit the proliferation of certain bone cancer cells, such as osteosarcoma, by inducing apoptosis and oxidative damage. However, further research is necessary to fully elucidate the therapeutic window and long-term safety of AgNPs in oncological applications.

5. Bone healing and the role of AgNPs

5.1. Bone healing: Inflammatory, reparative, and remodeling phases

Bone healing is a tightly regulated, multi-phase biological process that involves the coordination of cellular and molecular events aimed at restoring skeletal integrity and function after injury. This process unfolds in three overlapping phases: Inflammation, repair, and remodeling.[18,132]

Immediately following a bone injury, a local inflammatory response is initiated. Neutrophils are the first responders, clearing microbial contaminants and necrotic tissue, followed by the recruitment of monocytes that differentiate into macrophages. These immune cells release a cascade of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which serve to recruit and activate MSCs to the site of the defect.[133]

Recruited MSCs undergo osteogenic differentiation under the influence of local growth factors (e.g., BMP-2, transforming growth factor beta). These osteoprogenitor cells deposit a collagen-rich extracellular matrix, which subsequently mineralizes, forming immature woven bone. Concurrently, angiogenesis is triggered by hypoxia-induced expression of VEGF, ensuring an adequate supply of oxygen, nutrients, and osteogenic cells to support tissue regeneration.

Over time, immature bone is remodeled into mature lamellar bone through the coordinated activity of osteoblasts and osteoclasts. This phase restores the structural and mechanical properties of the original bone tissue.

However, this intricate process can be severely compromised in the presence of infection, extensive tissue

damage, or chronic inflammation—conditions frequently encountered in critical-sized bone defects or trauma-related injuries. These clinical challenges highlight the limitations of traditional bone grafts, which often lack the capacity to simultaneously support bone regeneration, promote vascular ingrowth, and prevent or control infection. AgNPs, due to their multifunctional biological activities, are being explored as a novel solution to address these limitations.[18]

5.2. AgNP-based systems in bone therapy

Among the diverse nanomaterials employed in theranostic strategies, AgNPs occupy a prominent position due to their potent antimicrobial properties, biocompatibility at controlled concentrations, and emerging roles in immunomodulation and osteogenesis (Figure 1).[14-17,21,58,67,102] These characteristics make them particularly attractive for applications in bone healing and repair, where the need to simultaneously control infection, reduce inflammation, and support tissue regeneration presents a significant clinical challenge. AgNPs demonstrate broad-spectrum antimicrobial efficacy, including against multidrug-resistant bacteria such as *S. aureus*, *E. coli*, and *P. aeruginosa*, which are often implicated in osteomyelitis, peri-implantitis, and post-operative infections. Their bactericidal activity, which involves membrane disruption, the generation of ROS, and interference with DNA replication, does not rely on classical antibiotic pathways, making them especially valuable in an era of growing antimicrobial resistance.

When incorporated into bone scaffolds, implant coatings, or injectable hydrogels, AgNPs can create an antimicrobial microenvironment at the injury site, preventing bacterial colonization and biofilm formation. Beyond infection control, AgNPs have shown potential in modulating inflammatory responses, which are critical to both the initiation and resolution phases of bone healing. Studies have demonstrated their ability to downregulate proinflammatory cytokines, such as TNF-α and IL-6, while also modulating macrophage polarization toward the M2 phenotype, which is associated with tissue repair.[134-136] This dual role—antibacterial and anti-inflammatory—makes AgNPs uniquely suited for multifunctional regenerative platforms. In addition, emerging evidence suggests that AgNPs can directly influence the behavior of bone cells. At optimized concentrations, AgNPs have been

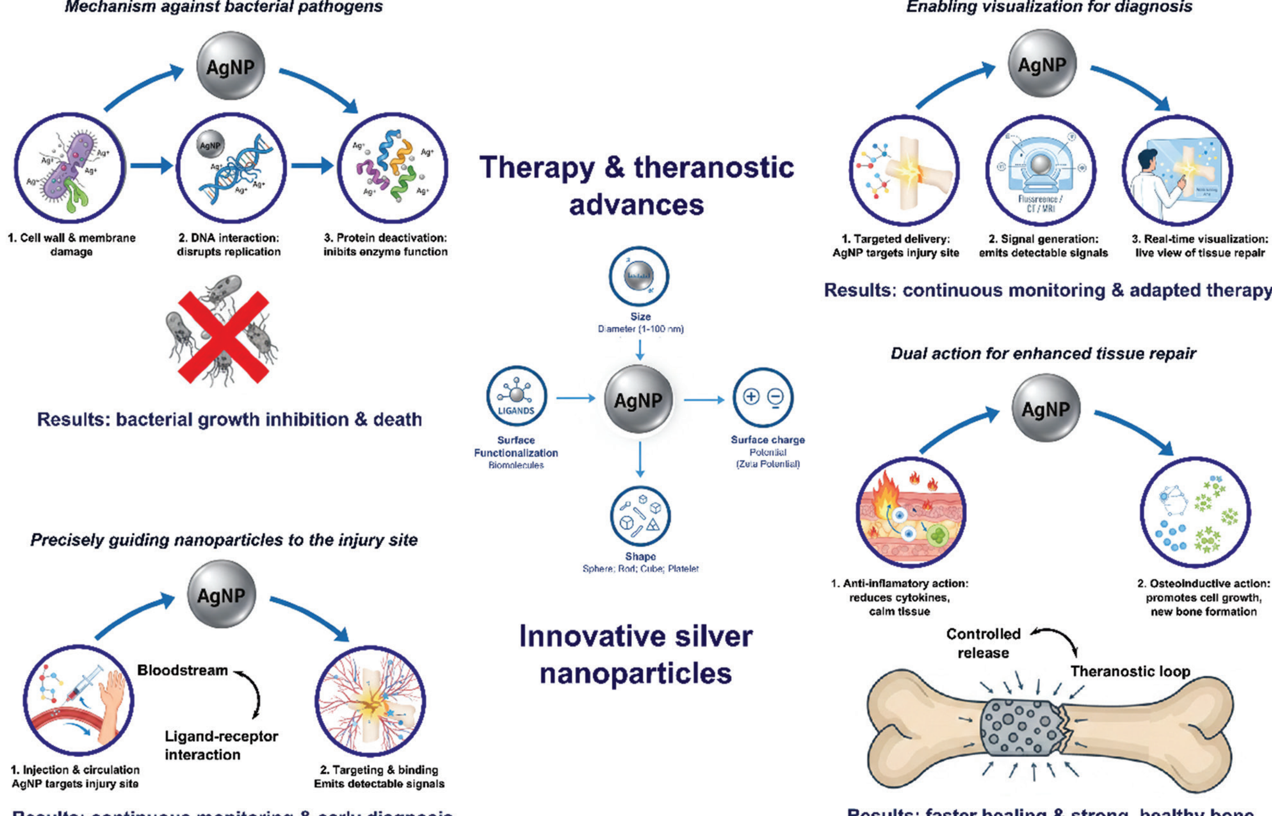

Figure 1. Silver nanoparticle-driven theranostic approaches in bone tissue regeneration

reported to promote osteoblast adhesion, proliferation, and differentiation, while avoiding cytotoxic effects. This is particularly promising when AgNPs are used in synergy with osteoconductive or osteoinductive materials such as hydroxyapatite, collagen, or bioactive glass. Hybrid scaffolds incorporating AgNPs not only offer mechanical support and structural mimicry of bone extracellular matrix but also enhance local tissue integration while deterring infection. Theranostically, AgNPs are increasingly being integrated into stimuli-responsive systems, where their release or activity is triggered by environmental changes such as pH shifts, enzymatic activity, or oxidative stress—conditions commonly present in inflamed or infected bone tissue. Moreover, the plasmonic and conductive properties of AgNPs have potential applications in biosensing and photo-responsive therapies, such as photothermal ablation of osteosarcoma or real-time monitoring of infection resolution. In summary, AgNP-based systems represent a versatile and powerful class of theranostic agents for bone therapy. Their ability to simultaneously disinfect, modulate inflammation, and support bone regeneration, while also being adaptable for diagnostic integration, aligns closely with the goals of precision and regenerative medicine. Continued advances in nanoparticle engineering, surface functionalization, and dose optimization will be essential to fully unlock their clinical potential in complex orthopedic and maxillofacial scenarios.

5.3. Antimicrobial scaffolds and grafts

Theranostic platforms combining therapeutic and diagnostic capabilities are an emerging frontier in bone tissue engineering.[11] As noted earlier, AgNPs, with their potent antimicrobial properties and compatibility with various imaging agents, are central to several innovative dual-function systems. These platforms are designed to enhance bone regeneration, prevent infection, and enable real-time monitoring of treatment progress. A growing body of preclinical evidence demonstrates that scaffolds and bone graft substitutes functionalized with AgNPs possess a dual therapeutic function: providing antimicrobial protection while also supporting osteogenesis.

5.3.1. Rabbit cranial 6-mm defect model

In a controlled study, AgNP-loaded scaffolds composed of either bovine bone xenografts or gelatin methacryloyl (GelMA) hydrogel matrices were implanted in rabbit cranial defects. Approximately 100 μg of AgNPs per scaffold was sufficient to inhibit bacterial proliferation without eliciting any signs of local tissue necrosis or inflammation. Histological analysis revealed enhanced osteoblast proliferation and mineral deposition, confirming both the safety and efficacy of this approach for bone regeneration applications.[137]

5.3.2. Bovine cancellous bone granules functionalized with AgNPs (~50 nm)

These porous bone substitutes exhibited minimum inhibitory concentrations ranging from 0.25 to 4 mg/L against a spectrum of clinically relevant pathogens, including *S. aureus*, *P. aeruginosa*, *E. coli*, *Enterococcus faecalis*, and *C. albicans*. In a murine calvarial defect model, the AgNP-functionalized granules maintained excellent radiolucency, biocompatibility, and structural integrity with reduced resorption rates.[138]

5.3.3. Strontium–silver-coated titanium implants

These multi-metallic implants demonstrated synergistic effects in both *in vitro* and *in vivo* systems. In rat femoral defect models, the implants significantly enhanced MSC osteogenic differentiation markers, including ALP and Runx2. Simultaneously, antibacterial efficacy was demonstrated against *S. aureus* and *E. coli*, indicating their suitability for use in infected or high-risk surgical sites.[139]

5.3.4. Silver–strontium mesoporous bioactive glass nanoparticles

These nanoparticles combine silver and strontium within a mesoporous silica matrix, allowing for the sustained release of therapeutic ions. Silver offers antimicrobial effects, while strontium supports osteoblast activity and bone matrix mineralization. Furthermore, the presence of both metals enhances X-ray attenuation, providing improved contrast in CT imaging. This integration supports non-invasive monitoring of graft incorporation and infection control.[103]

Together, these studies suggest that AgNP-loaded bone constructs can effectively integrate antimicrobial protection with osteoconductive and osteoinductive properties, making them promising candidates for managing contaminated or compromised bone defects.

5.4. AgNP–polymer composites and hydrogels

The incorporation of AgNPs into biodegradable and biocompatible polymer matrices has emerged as a powerful strategy to engineer multifunctional bone scaffolds.

Commonly used materials include PLGA,[58,68] silk fibroin,[101,102,115] chitosan,[100] and GelMA.[137,140] These polymers provide a supportive framework with tunable mechanical properties, controlled porosity, and inherent bioactivity, which is conducive to bone tissue engineering.

AgNP–polymer composites are designed to release silver ions within a therapeutic window (typically 5–30 μg/mL), sufficient to exert antimicrobial effects without inducing

cytotoxicity in host cells. Sustained release profiles have been achieved through nanoparticle encapsulation or surface functionalization strategies.[141,142]

These composites facilitate the adhesion, spreading, proliferation, and differentiation of MSCs. Injectable formulations, particularly GelMA-based hydrogels loaded with AgNPs, have demonstrated effectiveness in addressing irregular or critical-sized defects, such as rodent femoral fractures.[137,140,143] Preclinical outcomes show accelerated bone regeneration, improved vascularization, and reduced infection rates compared to control scaffolds. This approach offers a versatile platform for delivering AgNPs in a spatially and temporally controlled manner, maximizing therapeutic benefit while minimizing systemic exposure.

5.5. Nanoparticle–cell interactions in bone tissue engineering

Nanoparticles engage dynamically with bone-related cells, including osteoblasts, osteoclasts, and MSCs, influencing proliferation, differentiation, and remodeling pathways.[144,145] Osteoblast-like lineages (e.g., MC3T3-E1, Saos-2) internalize nanoparticles via endocytosis; their fate depends on the size, charge, and surface chemistry of the nanoparticles. The review by Zhang *et al.*[145] highlighted that compositional nuances (e.g., metal oxide vs. polymeric) and surface modifications modulate bone cell behavior through signaling cascades such as ERK/MAPK and Wnt/β-catenin.[145] Notably, AgNPs at low concentrations (~5–30 μg/mL) have induced osteogenic gene expression (*RUNX2*, *ALP*, *OCN*) and stimulated angiogenic factors like VEGF in MSC cultures, though higher doses may impair proliferation.[146-148] Concerning therapeutic intent, nanoparticle scaffolds must strike a balance between antimicrobial efficacy and osteogenesis. Researchers have fine-tuned the kinetics of ion release, for example, by doping bone grafts with silver and strontium, to promote osteoblast differentiation while reducing bacterial colonization.[149] Multifunctional composites such as silk fibroin–silver–nanohydroxyapatite achieved excellent osteoblast adhesion while resisting *S. aureus* biofilm formation. Nonetheless, precise dosing and kinetic profiles are crucial; overdose risks ROS overproduction and apoptosis.[150]

5.6. Osteoinduction and angiogenesis

One of the most promising aspects of AgNPs' use in bone regeneration is their ability to simultaneously promote osteogenesis and angiogenesis, provided that dosing is carefully optimized.[15]

At sub-toxic concentrations, AgNPs induce a moderate level of intracellular ROS, which in turn activates the hypoxia-inducible factor 1-alpha pathway. This leads to the upregulation of VEGF and other pro-angiogenic and osteogenic signals. Enhanced ALP activity, collagen Type I synthesis, and extracellular matrix mineralization have all been observed in MSC cultures treated with AgNPs.[151]

The biological activity of AgNPs is highly dose-dependent. Concentrations between 5 and 30 μg/mL are generally considered optimal for stimulating osteogenic differentiation without compromising cell viability. However, at higher concentrations (>50 μg/mL), excessive ROS production may lead to oxidative stress, apoptosis, or necrosis in both progenitor and mature cells, emphasizing the importance of precise dose control in scaffold design.[15,152]

The integration of AgNPs into bone tissue engineering platforms thus provides a dual-function system—combining antimicrobial activity with pro-regenerative signaling. This multifunctionality is particularly advantageous in treating infected bone defects, where traditional grafts may fail due to the hostile inflammatory environment.[153]

5.7. Combined drug delivery and targeting

In addition to their antimicrobial and imaging functions, AgNP-based systems are increasingly designed for combinatorial therapies. These include the localized, controlled delivery of secondary therapeutic agents and active targeting to bone-specific sites.

AgNPs–BMP-2–PLGA composites[154] integrate BMP-2, a potent osteoinductive agent, within a PLGA matrix containing AgNPs. The composite ensures the sustained release of BMP-2, stimulating osteogenesis, while the AgNPs provide continuous antimicrobial protection—a dual action particularly beneficial for infected bone defects or post-surgical implant sites.

Furthermore, AgNPs can be embedded in pH-sensitive or ROS-responsive matrices that trigger the release of drugs under pathological conditions such as infection or inflammation.[155] These "smart" systems offer site-specific and condition-triggered release of antibiotics or anti-inflammatory agents, thereby minimizing systemic toxicity and maximizing local therapeutic effects.

Surface modification of AgNPs with bone-seeking peptides, such as AspSerSer, enhances their affinity for hydroxyapatite-rich regions.[156] This targeting strategy improves the retention of therapeutic agents in the defect zone, thereby optimizing efficacy and minimizing off-target effects.

By integrating antimicrobial, imaging, and therapeutic functionalities within a single platform, these advanced

systems represent a major step toward personalized, image-guided regenerative therapies. Such platforms are particularly promising for treating complex conditions such as osteomyelitis, peri-implant infections, and bone tumors, where localized and multifunctional approaches are essential.

5.8. Preclinical and clinical advances in AgNP-based bone therapies

The growing interest in AgNP-based systems for bone regeneration and infection control has translated into a rapidly expanding body of preclinical research and a smaller but promising number of early clinical studies.[157,158] These investigations aim to validate the therapeutic efficacy, biosafety, and multifunctionality of AgNP-containing platforms in models of bone injury, osteomyelitis, and implant-associated complications. In preclinical settings, AgNPs have been integrated into a wide range of biomaterials designed to mimic the structure and function of native bone.[159] For example, hydroxyapatite–AgNP composites have shown superior antibacterial properties and enhanced osteointegration in rabbit tibial defect models, compared to hydroxyapatite alone.[157,160] Similarly, AgNP-loaded collagen and chitosan scaffolds have demonstrated accelerated bone healing and reduced inflammation in rat calvarial and femoral defect models, confirming the dual osteoconductive and antimicrobial properties of AgNPs.[161-163] The inclusion of growth factors (e.g., BMP-2) or MSCs into AgNP-containing matrices further amplifies regenerative outcomes, supporting a synergistic approach that addresses both biological and infectious barriers to bone repair.[164] Studies have also examined dose-dependent effects and nanoparticle characteristics, such as size, shape, and surface coating, on both efficacy and cytotoxicity. Smaller AgNPs (<20 nm) typically exhibit stronger antimicrobial activity but may pose a greater risk of oxidative stress or mitochondrial dysfunction at high concentrations.[49,70,71,86,137,165] Thus, surface modification strategies (e.g., PEGylation, silica coating, or peptide conjugation) are employed to improve biocompatibility, prolong circulation, and enable targeted delivery *in vivo*.[73,124] In implant-associated models, AgNP coatings on titanium and stainless-steel surfaces have significantly reduced bacterial adhesion and biofilm formation, while maintaining compatibility with osteoblasts.[17,32,102,139,162] These findings are particularly relevant to orthopedic and dental implants, where infection and aseptic loosening remain major causes of failure. While clinical translation is still in early stages, several products incorporating AgNPs for wound healing and infection prevention are already in clinical use (e.g., silver dressings, bone cement additives).[166,167] A few pilot clinical studies have reported the use of AgNP-modified bone grafts or implants in maxillofacial and orthopedic surgeries, showing favorable outcomes in terms of infection control, graft stability, and early tissue integration.[168] However, large-scale, controlled clinical trials are still lacking, and regulatory concerns (particularly related to nanoparticle toxicity, long-term accumulation, and manufacturing reproducibility) remain key hurdles for broader clinical acceptance.

Nonetheless, the consistent preclinical evidence and growing clinical interest suggest that AgNP-based nanotherapeutics are approaching clinical readiness, especially when incorporated into multifunctional, scaffold-integrated platforms designed for complex bone pathologies, as shown in Table 1. Future work will need to focus on standardizing formulations, optimizing dose–response profiles, and designing theranostic protocols that integrate real-time imaging and adaptive drug release for personalized regenerative therapies.

6. Toxicity, biocompatibility, and pharmacokinetics

The clinical translation of AgNP-based platforms in bone regeneration critically depends on their safety profile. While AgNPs are effective antimicrobials, their biological interactions at the cellular and systemic levels must be carefully balanced to avoid adverse effects. Understanding their cytotoxicity thresholds, biodistribution patterns, and immunological interactions is essential for designing safe and effective theranostic systems; results are discussed below and summarized in Table 2.

6.1. *In vitro* cytotoxicity

Cell-based assays consistently demonstrate a dose-dependent cytotoxic response to AgNPs, which varies according to particle size, coating, concentration, and exposure duration. MSCs and pre-osteoblast MC3T3-E1 cells tolerate AgNP concentrations up to

Table 1. Preclinical evidence of silver nanoparticle-based strategies in bone repair and implant integration

Study type	Model/system	Main outcomes
Preclinical (Rabbit) [160,169]	Tibial defect/Hydroxyapatite–AgNPs scaffold	Enhanced osteointegration, infection prevention
Preclinical (Rat) [170]	Calvarial defect/Collagen–AgNPs composite	Accelerated bone healing, reduced inflammation
Preclinical (Mouse) [171]	Femoral fracture/AgNPs	Improved callus formation, synergistic osteoinduction
Implant model (Rat) [172,173]	Titanium implant coated with AgNPs	Reduced bacterial adhesion, biocompatible interface

Abbreviation: AgNP: Silver nanoparticle.

Table 2. Comparative summary of toxicity, biocompatibility, and pharmacokinetics of silver nanoparticle-based systems

Characteristic	Key findings	Determinants/variables	Bone-regenerative applications
In vitro cytotoxicity[15,108,174-178]	• MSCs and MC3T3-E1 cells remain viable up to~30 μg/mL • Toxicity increases sharply above 50–100 μg/mL due to ROS accumulation and mitochondrial dysfunction	• Particle size and coating (polyethylene glycol, polyvinylpyrrolidone, chitosan, silk fibroin) • Concentration and exposure duration • Release kinetics of Ag^+ions (e.g., poly[lactic-co-glycolic acid])/AgNP composites)	• Surface engineering reduces non-specific protein adsorption and ROS • Controlled release supports MSC proliferation, osteogenic differentiation, and matrix mineralization • Highlights the need for precise dose control to balance antimicrobial effects with cytocompatibility
In vivo biodistribution and accumulation[148,179-182]	• Implanted AgNP-loaded scaffolds retain most nanoparticles locally • Small amounts of Ag^+may reach the liver, spleen, and lymph nodes—mainly early after implantation. • No organ damage reported at therapeutic doses in rabbit defect models • Long-term (>8 weeks) biodistribution is still poorly understood	• Administration route (localized scaffold vs. systemic) • Particle dissolution rate and ion release • Implant location and vascularization	• Localized delivery minimizes systemic exposure and toxicity • Need for long-term studies on clearance, metabolism, and excretion • Essential for evaluating repeated or high-dose applications
Dose-dependency and safety thresholds[14,59,101,102,126,129,130,150,153,155,161]	• Strong antibacterial activity at 0.25–4 mg/L (below cytotoxic range) • Cytotoxicity generally occurs above 50–100 μg/mL • HepG2 IC_{50}≈250 μg/mL • Localized slow-release systems have wide safety margins; systemic and topical routes have narrower ones	• Delivery route (local vs. systemic) • Carrier systems (scaffolds, gels, injections) • Exposure frequency and cumulative dose	• Clear therapeutic window for localized delivery • Necessity for pharmacokinetic optimization in systemic applications • Supports development of slow-release bone scaffolds for safer dosing
Immunogenicity and inflammatory responses[186-189]	• Low–moderate doses trigger mild oxidative stress that may support angiogenesis and early repair • High concentrations (>100 μg/mL) induce proinflammatory cytokine (tumor necrosis factor alpha, interleukin 6) • *In vivo* grafts with 100 μg AgNPs show minimal leukocyte infiltration and normal healing	• Concentration and exposure time • Local microenvironment (infection, immune status) • Particle chemistry and surface modifications	• Mild inflammation may enhance regenerative cascades • High-dose inflammatory risk underscores tight dose control • Need for further study in immune-compromised or infection-prone models

Abbreviations: Ag^+: Silver ions; AgNP: Silver nanoparticle; MSC: Mesenchymal stem cell; ROS: Reactive oxygen species.

approximately 30 μg/mL without significant loss of viability.[15,174] At levels above 50–100 μg/mL, elevated intracellular levels of ROS and mitochondrial dysfunction lead to apoptosis or necrosis.[175,176]

Poly(lactic-co-glycolic acid)/AgNP composites, commonly used in scaffold fabrication, are engineered to release Ag^+ ions gradually over time. This controlled release strategy helps maintain silver concentrations below toxic thresholds, supporting sustained MSC proliferation, osteogenic differentiation, and matrix mineralization. Surface modifications play a crucial role in mitigating the toxicity of AgNPs. Coatings such as PEG, polyvinylpyrrolidone, chitosan, and natural biopolymers like silk fibroin help reduce non-specific protein adsorption, improve colloidal stability, and reduce ROS-related cellular stress.[108,177] Moreover, these coatings can facilitate targeted delivery to osteoprogenitor or immune-modulating cells, enhancing therapeutic specificity. These *in vitro* findings underline the importance of dose control and surface engineering in ensuring biocompatibility while preserving antimicrobial functionality.[178]

6.2. *In vivo* biodistribution and accumulation

Animal models have provided insight into the spatial and temporal distribution of AgNPs following localized or systemic administration. When AgNPs are incorporated into bone scaffolds and implanted at defect sites, they tend to remain primarily localized within the implant region, exerting localized antimicrobial and osteoregenerative effects.[179,180] This spatial confinement minimizes systemic exposure. However, trace amounts of silver, mainly in ionic form (Ag^+), may enter circulation and accumulate transiently

in the liver, spleen, and lymphatic tissues, especially during the early post-implantation period.[148] At the same time, therapeutic doses used in rabbit cranial defect models have not been associated with histologically observable organ damage or signs of systemic toxicity. Despite promising short-term findings, comprehensive studies on long-term biodistribution, metabolism, and excretion of AgNPs remain scarce, particularly beyond 6–8 weeks post-implantation. Understanding whether AgNPs are fully cleared or persist in organs is essential for risk assessment, especially for repeated or high-dose applications.[181,182]

6.3. Dose-dependency and safety thresholds

Preclinical evaluations have helped delineate a therapeutic window where AgNPs are both effective and safe. Antibacterial activity is generally observed at concentrations as low as 0.25 mg/L, with biofilm disruption occurring in the 1–4 mg/L range against pathogens such as *S. aureus*, *E. coli*, and *P. aeruginosa*. These concentrations are typically lower than those required to induce cytotoxic effects in mammalian cells.[14,59,101,102,126,129,130,150,153,155,161]

Cytotoxic thresholds for mammalian cells, including osteoblasts, fibroblasts, and hepatocytes, are generally higher, typically exceeding approximately 50–100 μg/mL, indicating a feasible therapeutic window when AgNPs are delivered locally and in a controlled manner (e.g., embedded in slow-release scaffolds). In contrast, topical or systemic delivery systems (e.g., AgNP gels, injections) pose a narrower safety margin. For example, in HepG2 liver cells, the half-maximal inhibitory concentration of AgNPs is approximately 250 μg/mL. Therefore, systemic delivery must be approached with caution, emphasizing the need for pharmacokinetic optimization and refinement of the delivery route.[43,73,122,129,138,151-153,165,166,173,175,181,183-185]

6.4. Immunogenicity and inflammatory responses

AgNPs not only interact with bone and stem cells but also influence various components of the immune system. Their immunomodulatory properties can be beneficial or detrimental depending on the dose and exposure context. At low to moderate concentrations, AgNPs induce mild oxidative stress, which can activate signaling pathways that promote angiogenesis, immune cell recruitment, and early-stage tissue repair. This mild proinflammatory response may be advantageous in bone healing. At high concentrations (>100 μg/mL), AgNPs trigger the release of proinflammatory cytokines, including TNF-α and IL-6, which can lead to excessive inflammation and tissue damage if not properly controlled. In *in vivo* rabbit studies, grafts containing 100 μg of AgNPs did not elicit significant leukocyte infiltration, necrosis, or chronic inflammatory reactions. Histological analyses typically reveal normal healing patterns with minimal immune cell activation, suggesting a favorable immunological profile under therapeutic dosing conditions.[186-189] Future research should aim to further characterize the dose-dependent immunological effects of AgNPs, particularly in immune-compromised or infection-prone environments, to ensure safe clinical translation.

In summary, despite their well-documented antimicrobial and regenerative potential, AgNPs have several important limitations and adverse effects that must be critically discussed when considering clinical translation. At the cellular level, AgNP toxicity is primarily mediated by oxidative stress, mitochondrial dysfunction, and DNA damage, resulting in dose- and size-dependent cytotoxicity and genotoxic risk in various cell types.[190] Neurotoxic outcomes have been reported in multiple models and are associated with oxidative inflammation and disrupted autophagy pathways, raising specific concerns for systemic exposure or accumulation in neural tissues.[191] *In vivo* biodistribution studies show that although locally implanted AgNPs often remain concentrated at the defect site, Ag^+ and small particulates can translocate to the liver, spleen, kidneys, and brain, with clearance kinetics that are incompletely characterized beyond early post-implantation windows; this persistence poses long-term safety uncertainties, including organ accumulation and functional impairment.[192] Mechanistically, many of these adverse effects stem from ROS overproduction and mitochondrial damage (reported across cell and animal studies), which can cause inflammation, cell death, and impaired tissue function if exposure is excessive or prolonged.[193] Finally, although "green" biosynthesis of AgNPs may reduce reagent costs and environmental impact, such methods often yield variable particle size, surface chemistry, and batch reproducibility that complicate Good Manufacturing Practice (GMP)-compliant scale-up and quality control, factors that directly affect both safety and efficacy.[194] Taken together, these limitations suggest a conservative, evidence-based approach to clinical use. Rigorous dose-finding studies, long-term biodistribution and clearance experiments, standardized particle characterization, and the employment of mitigation strategies (e.g., robust surface coatings, controlled-release matrices, targeted delivery) should be required to minimize systemic exposure while preserving antimicrobial efficacy.

7. Manufacturing, scaling, and regulatory considerations

7.1. Reproducibility in synthesis

Reliable and scalable synthesis of AgNPs is fundamental to clinical translation.[195] Reproducibility in terms of

particle size, shape, surface charge, crystallinity, and functionalization is crucial, as these factors significantly influence both biological activity and safety. Chemical reduction methods, such as those using sodium citrate or sodium borohydride, provide fine control over nanoparticle size and monodispersity. However, residual reducing agents or capping chemicals can pose biocompatibility issues, necessitating the use of extensive purification protocols. Physical techniques, such as laser ablation or evaporation–condensation, produce high-purity AgNPs with minimal contamination. However, they often result in broader size distributions and lower yields, which can complicate downstream applications. Green synthesis, leveraging biological reducing and capping agents (e.g., plant polyphenols, microbial enzymes, silk fibroin, or fibronectin), generates biocompatible AgNPs with inherent functional coatings. These methods also reduce environmental impact and reagent toxicity. However, batch-to-batch variability due to natural source heterogeneity and seasonal or geographic differences in raw materials remains a significant limitation for industrial scalability. Future strategies to improve synthesis reproducibility include automated microfluidic reactors,[17,22,113] real-time monitoring with ultraviolet–visible spectroscopy and dynamic light scattering, and artificial intelligence-based process control systems that optimize reaction parameters dynamically.

7.2. Quality control and standardization

Quality control is crucial for ensuring the consistency, safety, and regulatory compliance of AgNP-containing biomaterials.[196] Standard quality control parameters should include:

(i) Particle characterization:[167] Dynamic light scattering for hydrodynamic size, zeta potential for surface charge, transmission electron microscopy for morphology, X-ray diffraction for crystallinity, and surface composition analysis via Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy
(ii) Ion release profiling: Quantification of Ag^+ ion release over time in physiological conditions using inductively coupled plasma mass spectrometry or atomic absorption spectroscopy, with emphasis on achieving therapeutic yet non-cytotoxic levels
(iii) Scaffold evaluation: In composite systems (e.g., AgNP/PLGA foams or hydrogels), mechanical integrity (e.g., compressive strength ≥18 MPa), porosity (>70%), biodegradation rate (tailored to defect healing timeline), and sustained antimicrobial function must be validated
(iv) Sterility and endotoxin testing, per International Organization for Standardization and United States Pharmacopeia standards, is also required for clinical applications.[197]

Establishing inter-laboratory standard operating procedures and adopting international nanomaterial reference standards would significantly advance reproducibility and global standardization.

7.3. Regulatory pathways

AgNP-based bone repair platforms are generally classified as medical devices or combination products, depending on their primary mode of action. Regulatory approval typically requires:[19,166,198] (i) Preclinical studies adhering to ISO 10993 standards to evaluate cytotoxicity,[199] sensitization, irritation, systemic toxicity, genotoxicity, and implantation responses;[200,201] (ii) *In vivo* validation using standard animal models of bone defects, such as critical-size rabbit cranial or rat femoral defects, to demonstrate both biocompatibility and efficacy in promoting bone regeneration and infection control; (iii) Chemistry, manufacturing, and controls documentation that details nanoparticle synthesis, purification, batch reproducibility, and stability; and (iv) Clinical trials designed to confirm scaffold integration, functional bone recovery, absence of systemic silver accumulation, and lack of adverse effects such as chronic inflammation or allergic reactions.

Given the dual-function nature of theranostic AgNP platforms, regulators may impose additional requirements to address long-term biocompatibility, nano-bio interactions, and potential environmental toxicity or nanoparticle leaching. Interdisciplinary collaboration and early engagement with regulatory agencies (e.g., pre-investigational new drug application or pre-submission meetings) are crucial for successful translation.

8. Challenges and clinical translation

8.1. Resistance development

While AgNPs possess a broad-spectrum, multimodal mechanism of antimicrobial action, including membrane disruption, ROS generation, and DNA interference, concerns over microbial adaptation are rising. Emerging reports suggest that bacteria chronically exposed to sub-lethal AgNP doses can develop adaptive mechanisms, including the upregulation of efflux pumps, altered membrane permeability, and enhanced biofilm formation.[202] Mitigation strategies include alternating nanoparticle coatings, co-delivery of silver with other antimicrobials or enzymes (e.g., lysozyme), and responsive release systems that minimize prolonged sub-therapeutic exposure. Monitoring microbial resistance patterns during preclinical and clinical use will be critical for ensuring sustained efficacy and informing future design.[203-205]

A critical comparison with current clinical standards is important to understand the potential of AgNP-based systems in bone repair. Although antibiotic-loaded bone cements remain the standard choice for treating infected or high-risk defects, their effectiveness faces challenges.[206] These challenges include an increase in antimicrobial resistance, limited penetration into biofilms, and the gradual loss of mechanical strength that comes with antibiotic release. Recent studies by Karpiński *et al.*,[52-54] particularly their work on how different mixtures affect the mechanical properties of medium-viscosity bone cements, demonstrate that even well-optimized traditional cements are susceptible to mixing inconsistencies, porosity, and decreased long-term stability. These issues hinder their overall clinical performance. In contrast, AgNPs offer broad-spectrum antimicrobial activity, prolonged effectiveness against biofilm-forming pathogens, and the potential for integrating therapeutic and diagnostic functions into multifunctional scaffolds.[108] However, their production costs are higher due to nanoparticle synthesis, surface treatment, and sterilization requirements.[207] Overall, current evidence suggests that AgNP-based platforms could be most beneficial in situations with a high risk of infection or when conventional antibiotic-enhanced cements offer limited effectiveness. Still, further cost–benefit analyses and direct comparative studies are necessary before these can be widely used in clinical practice.

8.2. Long-term effects

Although short-term *in vivo* studies (up to 12 weeks) generally demonstrate favorable outcomes, long-term exposure scenarios are still poorly characterized. Ag^+ and particles may accumulate in bone, liver, or lymphatic tissues, and over time, may be converted into less soluble compounds, such as silver sulfide, which could disrupt the balance between osteoblasts and osteoclasts or trigger chronic inflammation. Potential downstream effects include altered bone remodeling, delayed healing, or immunogenic reactions. These risks underscore the importance of extended-duration studies (>6–12 months) in large animal models, including toxicokinetics and organ histopathology. Understanding the clearance kinetics, degradation pathways, and long-term retention of AgNPs will be essential for establishing safe chronic-use protocols.[208,209]

8.3. Cost-effectiveness

Cost remains one of the major barriers to the widespread clinical adoption of AgNP-based platforms for bone repair. Although green synthesis routes offer a more sustainable alternative and can reduce dependence on expensive chemical reagents, they often suffer from batch-to-batch inconsistency, variability in particle size and surface chemistry, and difficulties in reproducing results at an industrial scale. These limitations significantly restrict their commercial feasibility, especially in medical applications where reproducibility and regulatory compliance are essential. Moreover, the incorporation of high-value biomolecules, such as BMP-2, VEGF, antimicrobial peptides, or cell-targeting ligands, substantially increases production costs. These biologically active components require rigorously controlled, sterile, and GMP-compliant synthesis environments, which add layers of cost and regulatory oversight to an already complex manufacturing pipeline.

The urgency of cost control is underscored by foundational work in traditional bone cement systems. Karpiński *et al.*[52-54] have demonstrated that even well-established polymethylmethacrylate (PMMA) cements are subject to significant manufacturing challenges. For example, contamination by physiological fluids, such as blood or saline, significantly degrades mechanical performance.[210] Their studies[210,211] demonstrate how porosity, reduced strength, and instability arise when cement is processed under non-ideal conditions. Moreover, their more recent research on the incorporation of glassy carbon in PMMA cements[212] reveals that adding functional fillers is not a trivial solution. Particle size, concentration, and distribution have profound impacts on the final material's performance, which in turn demands more stringent quality control and potentially higher manufacturing costs.

Emerging technologies offer promising avenues to offset these economic challenges. Three-dimensional printing and modular scaffold fabrication enable precise control over architecture and material composition, reducing material waste and enabling the production of patient-specific constructs without the need for large inventories.[213] Likewise, point-of-care biomanufacturing units, designed to operate directly in hospital settings, could decentralize scaffold production and significantly reduce transportation, storage, and sterilization costs.[214,215] These advances support a "just-in-time" fabrication model that aligns well with personalized medicine and may help close the cost gap between nanotechnology-enhanced scaffolds and conventional alternatives.[216]

To justify clinical integration, robust cost–benefit analyses are needed that compare AgNP-based systems with current treatment standards such as antibiotic-laden PMMA cements, autografts, or allografts. While traditional therapies are generally less expensive and benefit from extensive clinical experience, they suffer from limitations,

including antibiotic resistance, donor site morbidity, incomplete regeneration, and inadequate biofilm control, which AgNP-based systems may help overcome. A systematic comparison of long-term outcomes, infection recurrence rates, implant longevity, and overall economic impact will be essential to demonstrate whether the added technological complexity of AgNP-based constructs translates into measurable clinical and economic advantages.

8.4. Clinical protocol integration

For successful clinical implementation, AgNP scaffolds must integrate seamlessly into existing surgical workflows. Materials should exhibit high moldability, sterilization compatibility (e.g., resistance to gamma irradiation or autoclaving), and mechanical robustness during implantation. Compatibility with orthopedic hardware, such as plates or screws, and co-administration with systemic antibiotics or anti-inflammatories should be evaluated to avoid cross-reactivity or interference. Tools for patient monitoring, such as non-invasive Ag^+ detection in urine or serum, could allow real-time tracking of systemic exposure. The development of pre-packaged, off-the-shelf scaffold kits tailored to specific bone defects (e.g., cranial, mandibular, or long-bone injuries) would simplify clinical adoption and improve procedural consistency.[105,143,158,195]

9. Emerging trends and future directions

9.1. Green synthesis and advanced surface engineering

Green synthesis strategies utilizing natural reducers (e.g., microbial metabolites, seaweed extracts, biopolymers) are gaining traction for producing AgNPs with minimal environmental impact and bioactive coatings.[43,61,112,126,130,184,195] Surface engineering advances allow for the functionalization of AgNPs with bone-homing peptides (e.g., AspSerSer), tumor-targeting ligands, or immunomodulatory proteins. Coatings incorporating stimuli-responsive polymers (e.g., pH- or ROS-sensitive hydrogels) provide smart targeting, releasing silver only under pathological conditions such as infection or inflammation.

9.2. Stimuli-responsive and smart scaffolds

Next-generation AgNP-based scaffolds incorporate smart release mechanisms: pH-responsive hydrogels,[155] ROS-degradable linkers, and enzyme-sensitive matrices ensure that silver and co-delivered therapeutics are only released when needed. Light-responsive systems, such as those triggered by near-infrared irradiation, enable spatially and temporally controlled Ag^+ release, minimizing systemic toxicity and maximizing local efficacy. These innovations may be particularly useful in oncological or chronically infected bone sites, where precise control of therapeutic release is critical.

9.3. Multifunctional nanoparticles

Multicomponent AgNP systems are being developed to deliver therapeutic, diagnostic, and regenerative functionalities in a single platform. Silver–gold hybrid nanoparticles enable photoacoustic or CT imaging, while simultaneously providing plasmonic heating and antimicrobial action.[217,218] Silver–iron oxide composites allow for magnetic targeting and MRI visualization.[219] Strontium-incorporated AgNPs enhance osteoinductive activity while preserving antibacterial properties.[103] These multifunctional platforms are ideal candidates for personalized, image-guided bone therapies.

9.4. Personalized bone repair

Three-dimensional printing and computer-aided design technologies enable the fabrication of patient-specific scaffolds tailored to the defect's size, shape, and anatomical constraints. Electrically conductive AgNP–polycaprolactone scaffolds have been demonstrated to enhance osteogenic differentiation through electromechanical stimulation, with potential applications in bioelectronic sensing and stimulation systems.[220,221] Point-of-care additive manufacturing, using portable bioprinters or preloaded scaffold cartridges, may revolutionize emergency bone repair in trauma centers or military field hospitals.[222-225]

10. Conclusion

AgNP-based materials represent a new frontier in regenerative medicine, combining antimicrobial protection, stimulation of bone growth and blood vessel formation, imaging capabilities, and controlled release of therapies in a single multifunctional platform. In various preclinical models, AgNP–enabled constructs have shown strong potential to tackle the many challenges involved in bone repair, especially in cases complicated by infection, reduced blood flow, or chronic inflammation. This review offers an integrated perspective on AgNP-based bone therapies through a theranostic lens. We present, in a unified analysis, how surface engineering methods, interactions between nanomaterials and cells, immune system modulation, imaging compatibility, and smart release mechanisms work together to create next-generation platforms with combined therapeutic effects. In addition, this review connects fundamental concepts in nanomedicine with practical considerations, including manufacturing processes, regulatory pathways, cost assessments, and comparisons with current clinical

standards, such as antibiotic-loaded cements. We also highlight several important gaps in knowledge that need to be filled for successful clinical application, such as (i) The development of consistent, standardized, and GMP-compliant synthesis methods, (ii) Long-term studies on how these materials spread in the body, accumulate, and trigger immune responses, (iii) Optimization of safety profiles based on dosage, (iv) Techniques to reduce antimicrobial resistance through smart, multifunctional designs, and (v) Alignment of regulatory frameworks for complex nanotheranostic systems.

Advancing in these areas requires coordinated efforts among materials scientists, clinicians, nanotechnologists, regulatory bodies, and industry. Only through this collaborative approach can breakthroughs in the lab become reliable technologies in clinical settings. By combining insights into mechanisms, technological developments, translational challenges, and future research priorities, this review offers a roadmap for guiding the next generation of AgNP-based theranostic scaffolds toward practical use in clinical settings. In doing so, it positions AgNP-enabled platforms as potential key elements in the new approach to precision bone regeneration.

Acknowledgments

None.

Funding

The author acknowledges the financial support of the National University of the South (PGI 24/Q131), the National Scientific and Technical Research Council (CONICET; PIP 11220210100126CO), and the National Agency for the Promotion of Science and Technology (PICT-2021-I-A-00108). P.V.M. is a principal researcher of CONICET.

Conflict of interest

The author declares no conflict of interest.

Author contributions

This is a single-authored article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Not applicable.

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INNOSC Theranostics and Pharmacological Sciences

ORIGINAL RESEARCH ARTICLE

Pharmaceutical formulation and evaluation of *Xylopia aethiopica* fruit-based topical ointment and cream

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Abstract

For centuries, humans have long sought plant-based remedies derived from barks, seeds, and fruits in their enduring battle against illness. Over time, traditional medicines have evolved to become more effective and safer through pharmacological validation. One key area where these remedies have shown promise is in treating skin infections, as the skin is often the first point of contact for microorganisms and particularly vulnerable to infections caused by bacteria, fungi, viruses, and parasites. Currently, topical infections are primarily treated using ointments and creams, which deliver high concentrations of antimicrobials directly to the affected area. Studies reveal that ethanolic extracts have potent antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus* species. This effect is ascribed to moderate to high concentrations of saponins, known for their antibacterial qualities. The present study investigates the antibacterial properties of ethanolic and aqueous extracts of *Xylopia aethiopica* for the formulation of a topical ointment. To determine their minimum inhibitory concentrations (MICs), cultures of *Staphylococcus aureus* and *P. aeruginosa* were tested against the extracts, which exhibited MIC values ranging from 2.5 mg/mL to 10 mg/mL for both the ethanolic and aqueous forms. The ointments and creams formulated from these extracts demonstrated notable antibacterial activity, alongside excellent physicochemical properties, including a smooth texture, appealing appearance, uniform consistency with no phase separation, characteristic brown color, excellent spreadability, stability, and suitable pH values (6.3 for the ointment and 7 for the cream), as well as non-irratating effects on the skin. The findings confirm that *X. aethiopica* possesses effective antibacterial properties against *S. aureus* and *P. aeruginosa*, supporting its potential for further *in vivo* studies and broader therapeutic applications.

Keywords: Traditional medicine; Microorganisms; Pharmacological; Xylopia aethiopica; Antibacterial; Staphylococcus aureu; Pseudomonas aeruginosa

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Citation: Osei-Asare C, Aboagye D, Akambase E, Quarshie A, Asiama DM, Owusu FWA. Pharmaceutical formulation and evaluation of *Xylopia aethiopica* fruit-based topical ointment and cream. *INNOSC Theranostics and Pharmacological Sciences.* 2025;8(4):53-61.
doi: 10.36922/itps.8155

Received: December 24, 2024

Revised: June 14, 2025

Accepted: July 3, 2025

Published online: August 8, 2025

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1. Introduction

The quest for effective and natural remedies has been an enduring aspect of human civilization. Since ancient times, humans have sought medications from the bark, seeds, fruits, and other parts of plants to combat various illnesses.[1-4] *Xylopia aethiopica* (Figure 1), commonly known as the African pepper, has traditionally been used for medicinal purposes. This plant is native to West Africa[5] and has a rich historical use in traditional medicine, particularly for its antibacterial, antifungal, and anti-inflammatory properties.[6] *X. aethiopica* is an angiosperm belonging to the family Annonaceae that thrives in the evergreen rainforests of tropical and subtropical Africa.[7] The plant's name originates from the Greek words "*xylon pikron*," meaning "bitter wood." The second part of the plant's binomial name, *aethiopica*, refers to its origin, Ethiopia;[8] however, it currently grows most prominently as a crop in Ghana.[9] The plant has several local names. In Ghana, it is known as "*hwenteaa*" in Akan, "*etso*" in Ewe, "*so*" in Ga, and "*samaamdabile*" by the Waala people in the Upper West Region of Ghana.

In contemporary medicine, skin infections remain a prevalent issue, often caused by various pathogens, including bacteria, fungi, viruses, and parasites.[10,11] The skin, which is the largest organ and the body's first line of defense, is frequently exposed to these microorganisms. Topical infections can lead to a range of conditions, ranging from mild irritation to severe dermatological issues.[11,12] Currently, the most used treatments for such infections are topical formulations, such as ointments and creams. These formulations deliver high concentrations of antimicrobial agents directly to the site of infection, thereby ensuring effective treatment.[13-15]

The present study aims to explore the potential of *X. aethiopica* in the formulation of ointments and creams. Specifically, it investigates the antibacterial properties of aqueous and ethanolic extracts of *X. aethiopica* fruit and their efficacy when incorporated into topical formulations. The choice of *X. aethiopica* is influenced by its historical usage and the presence of bioactive compounds, such as alkaloids, glycosides, saponins, tannins, and flavonoids, which are known for their therapeutic benefits.[16] Phytochemical analysis of *X. aethiopica* revealed the presence of these compounds, which contribute to its antibacterial activity. Saponins have been linked to potent antibacterial effects. This study performed phytochemical tests on aqueous and ethanolic extracts of *X. aethiopica* to identify bioactive compounds.[17] The antibacterial efficacy of the extracts was tested against common skin pathogens, including *Staphylococcus aureus and P. aeruginosa*, through minimum inhibitory concentration (MIC) assays.[18] These pathogens are known to cause significant skin infections and represent the bacterial targets that an effective topical formulation must address.[19,20]

Figure 1. Dried fruits of *Xylopia aethiopica*

The formulation process involves incorporating the aqueous and ethanolic extracts into ointments and cream bases, followed by a comprehensive evaluation of these formulations.[21,22] The evaluation criteria included assessing the texture, appearance, homogeneity, phase stability, and color of the formulations.[23] In addition, the antibacterial activity of the formulated ointments and creams was tested against selected microorganisms to confirm their efficacy.[24,25]

The significance of this study lies in its potential to provide a natural and effective alternative for treating skin infections. By utilizing the medicinal properties of *X. aethiopica*, this study aimed to develop formulations that are not only effective but also have minimal side effects compared to synthetic antibiotics. Furthermore, this research sought to validate traditional medicinal knowledge through scientific methods, bridging the gap between traditional practices and modern medicine.[26] In summary, this study represents a promising step toward the development of natural antimicrobial treatments. These findings could pave the way for further research into the *in vivo* efficacy of *X. aethiopica* and its potential applications in dermatology and beyond.

2. Materials and methods

2.1. Collection of plant materials

Fresh *X. aethiopica* fruits were obtained from the Agbogbloshie market in Accra. The fruits were dried and ground into a powder, which was then examined and authenticated by Mr. Peter Boateng—a Botanist at the Center for Plant Medicine Research, Mampong, Ghana—using the herbarium library at the Center for Plant Medicine Research.

2.2. Materials, chemicals, and reagents

Glacial acetic acid, watermelon seeds, Fehling's A and B solutions, picric paper, hydrochloric acid, hard paraffin, cetostearyl alcohol, filter paper, ferric chloride, methanol, Mueller–Hinton agar, white paraffin, concentrated sulfuric acid, wool fat, dilute ammonia, and 20% sodium hydroxide were obtained from the Chemistry and Pharmacology Laboratory of the School of Pharmacy, Central University.

2.3. Equipment and instrumentation

An analytical balance (Model ALE-223; US-Solid, USA), test tubes, beakers, funnels, wash bottles, measuring cylinders, cotton wool, spatula, Petri dishes, a hot air oven (Model DGT-G500-X; Narang medical limited, India), stirring rods, a DLC electric blender (Model-411; Dongguan Jinsen Craft Technology Co. Ltd, China), a water bath (Model-WB10C11B; Cole-Palmer, United States), an autoclave (Model CJ-18LDJ), Whatman no.1 filter papers, and a pH meter (Model – 6177M/6177EU; Jenco Instruments, United States) were used in this study.

2.4. Drying and preparation of crude extracts

The fruits obtained were weighed and dried under direct sunlight for 5 days. The weight was measured until a constant weight was achieved. The fruits were ground into a fine powder using an electric blender. The resulting powder was weighed and stored under suitable conditions. A total of 200 g of the pulverized extract was weighed and dissolved in 2 L of a water and 100% ethanol mixture (ratio of 1:1) and was allowed to stand for 48 h. Subsequently, the solution was filtered using a Whatman no. 1 filter paper, and the filtrates were evaporated in an oven at 65°C for 6 h.[27,28]

2.5. Phytochemical analysis

Qualitative phytochemical analyses were conducted on both the ethanolic and aqueous extracts of *X. aethiopica* fruits according to standard procedures as previously described.[16,27,29-31] Samples were screened for saponins, tannins, flavonoids, alkaloids, glycosides, and reducing sugars.

2.6. Test organisms

The test organisms, *S. aureus* and *Pseudomonas aeruginosa*, were obtained from the Microbiology Laboratory of Central University. Cultures of these test organisms were purified. These bacteria were re-cultured in a newly prepared nutrient broth by dissolving 2.6 g of nutrient broth powder in a total of 300 mL of distilled water—initially using a small portion of the water to mix the powder, followed by the addition of the remaining volume. The prepared nutrients were distributed evenly into test tubes and were autoclaved at 121°C for 20 min. After 20 min of heating, the broth was allowed to cool before introducing the bacteria, followed by incubation at 37°C for 24 h.

2.7. Sterilization of materials

All apparatus was sterilized using the autoclave at 121°C for 15 min. The workbench was disinfected with 70% ethanol before and after use. A total of 400 mL of distilled water was autoclaved to ensure sterility. Micropipette tips were also sterilized using the autoclave.

2.8. Determination of the MIC of the plant extracts

The MIC of the extracts was determined using the tube dilution method as described. A 0.1 mL aliquot of the prepared bacterial suspension for each test organism was added to eight sterile test tubes containing nutrient broth with two-fold serial dilutions of the extracts at concentrations of 80, 40, 20, 10, 5, 2.5, 1.25, and 0.625 mg/mL. Test tubes containing an antibiotic (ciprofloxacin) with the growth medium, the growth medium with the bacterial suspension, and nutrient broth alone were used as controls. All tubes were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration that prevented visible bacterial growth (turbidity) compared to the control tubes.[18,28,32]

2.9. Preparation of topical cream and ointment of *X. aethiopica*

The ointment of *X. aethiopica* was prepared according to the master formula presented in Table 1.

2.9.1. Preparation of 10g of a 10%w/w X. aethiopica ointment

The herbal ointment was prepared by carefully blending 1 g of *X. aethiopica* ethanolic extract with the ointment base (Table 1) using the levigation method. The process involved first forming a smooth paste by mixing the extract with two or three times its weight in the base. An additional base was then gradually incorporated until a uniform ointment was obtained. The final product was then placed in an appropriate container and properly sealed. This same process was repeated for the aqueous extract.[22]

2.9.2. Preparation of 10 g of a 10%w/w X. aethiopica cream

The master formula for the cream formulation is presented in Table 2. In one porcelain dish, 1.3 g of stearic acid, 0.5 g of potassium hydroxide, and 0.5 g of sodium carbonate were melted together at 70°C. In another dish, 0.6 g of glycerin, 7.1 g of water, and 1 g of ethanolic extract of *X. aethiopica* were heated to 70°C. The aqueous mixture was then added to the oil mixture while stirring continuously at 70°C. After the transfer was complete, the mixture was allowed to cool

to room temperature while being continuously stirred. It was then immediately poured into a container, capped, labeled, and stored until future use. This same process was repeated for the aqueous extract.[26]

2.10. Antimicrobial sensitivity test on formulated X. aethiopica creams and ointments

The susceptibility of microbial clinical strains against the prepared aqueous and ethanolic creams and ointments, as well as standard antibiotics, was evaluated using the agar well diffusion method.[32,33] A 0.1 mL sample of the standard culture of *S. aureus* and *P. aeruginosa* was added to sterile Mueller–Hinton agar. The mixtures were thoroughly combined, left at room temperature for 3 h, and then poured into sterile Petri dishes to solidify. Wells were created in the seeded plates using a sterile 6 mm diameter cork borer and subsequently filled with 1.2 g of the cream and ointment samples, along with antibiotics of known concentrations. The plates were incubated at 35°C for 24 h, after which the zones of inhibition were measured.[34]

2.11. Quality evaluation tests on formulated *X. Aethiopica* creams and ointments

Several evaluation tests were conducted on the formulated aqueous and ethanolic ointments and creams to determine their consistency, durability, comfort, and acceptability. These tests included assessments of physical appearance, color, odor, texture, phase separation, stability, pH, skin irritability, and homogeneity.[25] A random selection of students from Central University performed these tests. They visually inspected the ointment, applied a small amount to their skin, and assessed the texture by rubbing it between their thumb and index finger to determine if it was rough or smooth.

Table 1. Master formula for ointment preparation

Ingredient	Weight (g)
Extracts	1
Wool fat	0.5
Hard paraffin	0.5
Cetostearyl alcohol	0.5
White paraffin	7.5

Table 2. The master formula for cream preparation

Phase	Ingredient	Weight (g)
Aqueous	Extracts	1
	Glycerin	0.6
	Water	7.1
Oil	Stearic acid	1.3
	Potassium hydroxide	0.5
	Sodium carbonate	0.5

2.12. Statistical analysis

The data obtained were analyzed using one-way analysis of variance on a completely randomized design using an Excel spreadsheet (Microsoft Office Excel 2021). Statistical significance was set at $p<0.05$.

3. Results and discussion

3.1. Phytochemical screening of *X. aethiopica* fruit extracts

The phytochemical screening result of the *X. aethiopica* fruit extracts is shown in Table 3. The ethanolic extracts possessed a rich presence of steroids, tannins, glycosides, and reducing sugars. The aqueous extracts contained saponins, tannins, and reducing sugars, but glycosides and alkaloids were absent.

3.2. The MICs of the *X. aethiopica* fruit extracts

The MICs of the fruit extracts against the microorganisms are shown in Table 4. MIC refers to the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism. The ethanol extract had stronger antibacterial activity against *S. aureus* and *P. aeruginosa* compared to the aqueous fraction. The MICs for all active extracts of *X. aethiopica* ranged between 2.5 mg/mL to 10 mg/mL.

3.3. Antimicrobial sensitivity of *X. aethiopica* formulated creams and ointments

The sensitivity pattern of the bacterial isolates against the different formulated dosage forms was compared with ciprofloxacin (1 mg/mL), a standard antibiotic (Table 5). The sensitivity tests indicated that ciprofloxacin was highly effective against both Gram-positive and Gram-negative bacteria, which supports its use as a broad-spectrum

Table 3. Phytochemical analysis of *Xylopia aethiopica* fruit extracts

Phytochemicals	Extract	
	Ethanolic	Aqueous
Saponins	+	+
Tannins	+	+
Glycosides	+	–
Alkaloids	+	–
Reducing sugars	+	+

Notes: + Indicates present; – Indicates absence.

Table 4. Minimum inhibitory concentration of ethanolic and aqueous extracts of *Xylopia aethiopica* fruits

Test organism	Extract	Minimum inhibitory concentration (mg/mL)								
		80	40	20	10	5	2.5	1.25	0.625	Ciprofloxacin (1 mg/mL)
Staphylococcus aureus	Ethanolic	–	–	–	–	–	MIC	++	++	–
	Aqueous	–	–	–	MIC	++	++	++	++	–
Pseudomonas aeruginosa	Ethanolic	–	–	–	–	–	MIC	++	++	–
	Aqueous	–	–	–	MIC	++	++	++	++	–

Notes: + Indicates growth (turbid); – Indicates no growth.
Abbreviation: MIC: Minimum inhibitory concentration.

Table 5. The sensitivity pattern of formulated *Xylopia aethiopica* creams and ointments against test bacterial isolates

Test organism	Zone of inhibition (mm)				
	Ointments		Creams		Ciprofloxacin
	Ethanolic	Aqueous	Ethanolic	Aqueous	
Staphylococcus aureus	31	20	25	11	40
Pseudomonas aeruginosa	25	14	19	8	52

antibiotic. The formulated creams and ointments showed antimicrobial activity against all tested microorganisms. This was demonstrated by the observed zones of inhibition (Figure 2).

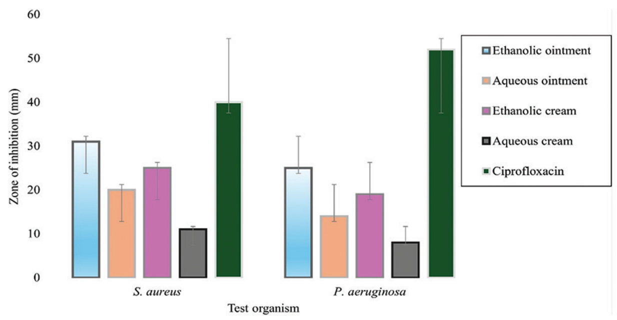

Figure 2. Antibacterial activities of the formulated *Xylopia aethiopica* creams and ointments against test bacterial isolates.

3.4. Physicochemical evaluation of formulated ointments and creams

The quality of the prepared *X. aethiopica* ointment and cream was determined based on organoleptic properties, including physical appearance, color, texture, phase separation, uniformity, and texture.[35-39] The formulated ointments and creams were found to have a smooth texture, pale brown color for ointment and a dark brown color for cream, an elegant appearance with no phase separation, as shown in Table 6.

The presence of phytochemicals in *X. aethiopica* was identified using established phytochemical methods.[16] The tested phytochemicals included tannins, saponins, glycosides, and alkaloids, each playing a vital role in wound healing and medicinal applications.[36,37] Tannins aid in wound healing through their astringent properties, promoting tissue contraction and reducing bleeding, and possess antioxidant properties that protect tissues from oxidative damage.[38,39] Saponins offer antimicrobial and anti-inflammatory benefits, aid in the management of wound infections and inflammation, and act as surfactants, enhancing the solubility of other active compounds.[40,41] Glycosides, convertible into active forms in the body, exhibit anti-inflammatory, analgesic, and antioxidant properties, contributing to pain relief and inflammation reduction in wound healing.[42,43] Alkaloids provide analgesic properties and can impact the central nervous system, aiding in pain relief and patient well-being during the healing process.[44,45] The results from two extraction methods (aqueous and ethanolic) showed that the ethanolic extraction possessed more phytochemicals, highlighting the impact of the solvent on the plant's phytochemical content.[17,46] Further research is needed to uncover valuable data *on X. aethiopica* fruit extracts.

The results showed that the extract significantly inhibited the growth of *S. aureus* and *P. aeruginosa* (which are common bacteria causing wound infections[47]) at a certain concentration,[48] with varying degrees of inhibition. It was observed that the antibacterial effect of the extract depended on the concentration and the solvent used in the extraction,[49,50] which was evident in the MIC test performed. The MICs of the extracts recorded ranged from 2.5 mg/mL to 10 mg/mL (Table 4). Standard references used included ciprofloxacin, a broad-spectrum antibiotic that inhibits the growth of both Gram-positive and Gram-negative organisms.

Table 6. Physicochemical evaluation of formulations of *Xylopia aethiopica* ointments and creams

Physicochemical parameters	Observation	
	Ointments	Creams
Physical appearance	Opaque	Opaque
Color	Pale brown	Dark brown
Texture	Fine	Fine
Odor	Characteristic	Characteristic
Phase separation	No phase separation	No phase separation
Homogeneity	Positive	Positive
Consistency	Smooth on skin	Smooth on skin
pH	6.3	7
Spreadability (s)	5	4
Irritancy	Non-irritant	Non-irritant
Stability	Stable	Stable

There were clear zones observed around the boreholes in the agar where the ointments and creams were seeded, indicating bacterial growth inhibition. The zone of inhibition of the ethanolic ointments against *S. aureus* and *P. aeruginosa* was 31 mm and 25 mm, respectively (Table 5). The aqueous ointments produced inhibition zones of 20 mm and 14 mm against *S. aureus* and *P. aeruginosa*, respectively, as shown in Table 5. The ethanolic and aqueous creams yielded similar results to those of the ointments. The zone of inhibition for the ethanolic cream against *S. aureus and P. aeruginosa* was 25 mm and 19 mm, respectively, whereas the aqueous cream exhibited zones of 11 mm and 8 mm, respectively. Overall, the ethanolic formulations demonstrated greater antibacterial activity than their aqueous counterparts, with the ethanolic ointment showing the highest efficacy (Figure 2). A larger zone of inhibition was observed around the ciprofloxacin-containing disk, indicating the test organism's susceptibility to the antibiotic. Comparing the cream and the ointment, the ointment showed a greater zone of inhibition than the cream against the test organisms used.

With the aim of using *X. aethiopica* as a basis for pharmaceutical creams and ointments for wound healing, this study explored the potential of the plant's phytochemicals, such as tannins, saponins, glycosides, and alkaloids. Each of these phytochemicals plays a distinct and important role in promoting wound healing and holds promise for alternative medicine applications.[51] This diverse phytochemical composition provides a comprehensive approach to wound healing by supporting both physical tissue repair and psychological well-being. The MIC obtained shows the effectiveness of the extract at low concentrations, representing a practical advantage, such as reduced product usage and a lower likelihood of adverse reactions/side effects. These findings suggest strong potential for both product safety and commercial viability.

In summary, this research indicates a promising future for *X. aethiopica*-based pharmaceutical products in wound healing. The synergistic phytochemical combination, precise manufacturing processes, and favorable MIC results provide a strong foundation for further research. This work encourages ongoing exploration of natural remedies and innovative medical solutions.

4. Conclusion

In conclusion, the results have shown that both ethanolic and aqueous extracts of *X. aethiopica* fruits have antibacterial properties and can be used as a potential source of natural antimicrobial compounds. This research validates the historical use of *X. aethiopica* in traditional medicine by demonstrating its significant antibacterial properties against *S. aureus* and *P. aeruginosa*. Both ethanolic and aqueous extracts of the plant showed effective antibacterial activity with MIC values ranging from 2.5 mg/mL to 10 mg/mL. The development of topical ointments and creams from these extracts resulted in formulations with excellent organoleptic properties and antibacterial efficacy. These findings not only underscore the potential of *X. aethiopica* as a viable source of antimicrobial agents but also set the stage for further research into its broader therapeutic applications.

Acknowledgments

The authors are grateful to the technical staff of the Department of Pharmaceutical Sciences, Central University, for their assistance in the laboratory.

Funding

None.

Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

This study was approved by the Central University Institutional Review Board (CU-IRB), with approval number CU-IRB/PHARM/2024/012. All participants involved in the product evaluation provided verbal informed consent before participation. The study was conducted in accordance with the ethical standards outlined in the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data

The datasets used during the current study are available from the corresponding author upon request.

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INNOSC Theranostics and Pharmacological Sciences

ORIGINAL RESEARCH ARTICLE

Phytochemical characterization of *Alcea rosea* seeds using gas chromatography-mass spectrometry: A preliminary investigation of bioactive compounds

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Abstract

The growing demand for natural therapeutic agents has intensified interest in plant-based compounds with potential pharmacological applications. *Alcea rosea*, traditionally employed in various ethnomedical systems, particularly for inflammatory and respiratory conditions, remains underexplored at the phytochemical level, especially concerning its seeds. The current study aims to bridge this gap by evaluating the chemical constituents of *A. rosea* seeds and assessing their potential bioactive significance using advanced analytical techniques. Ethyl acetate extraction was chosen for its efficiency in isolating semi-polar compounds, followed by gas chromatography-mass spectrometry to identify the chemical content of the extract. This method enabled the detection of over 50 distinct molecular entities, among which 15 were identified as major components based on peak abundance and database matching using the National Institute of Standards and Technology mass spectral library and the Wiley Registry of Mass Spectral Data. The chemical landscape of the extract included a range of fatty acids, esters, and complex organic molecules, many of which are known to possess anti-inflammatory, antioxidant, antimicrobial, and cytotoxic properties. Notable among these were linoleic acid, oleic acid, and palmitic acid compounds, which are widely recognized for their contributions to cellular signaling, oxidative stress modulation, and tumor suppression. The identification of bis(2-ethylhexyl) phthalate and A13-09519 (chemically ethyl N-methylcarbamate) also points to lesser-known or unconventional phytoconstituents that may hold biomedical interest, although their origin and activity warrant further validation. This investigation reveals *A. rosea* seeds as a chemically rich botanical source with a diverse array of compounds that could support therapeutic applications. The study sets a precedent for future bioactivity-driven analyses and highlights the value of integrating traditional knowledge with modern phytochemical screening to uncover novel drug leads from botanical sources.

Keywords: *Alcea rosea*; Ornamental; Ethyl acetate; Gas chromatography mass–spectrometry analysis; Phytoconstituents

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Citation: Mir GJ, Dar BA, Ganie SA, Hamid R. Phytochemical characterization of *Alcea rosea* seeds using gas chromatography–mass spectrometry: A preliminary investigation of bioactive compounds. *INNOSC Theranostics and Pharmacological Sciences.* 2025;8(4):62-72.
doi: 10.36922/ITPS025170030

Received: April 24, 2025

Revised: June 23, 2025

Accepted: August 11, 2025

Published online: September 2, 2025

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1. Introduction

Alcea rosea, widely recognized as hollyhock and a member of the Malvaceae family, is not only admired for its ornamental appeal but also revered for its long-standing role in traditional medicinal practices. This herbaceous plant has been cultivated and utilized in folk medicine across various geographical regions, including Iran, Central and South Asia, and several parts of Europe, with a notable ethnomedicinal presence in the Kashmir Valley.[1-3] Conventionally, multiple parts of the plant, particularly its roots, have been employed to manage a range of ailments, such as respiratory disorders (bronchitis and cough), gastrointestinal disturbances (ulcers and dyspepsia), and various inflammatory conditions. The continued use of *A. rosea* in these settings underscores its cultural and pharmacological importance across generations. Scientific inquiry into the medicinal potential of *A. rosea* has validated many of these traditional uses. Pharmacological investigations have attributed a wide spectrum of bioactivities to this plant, with specific studies demonstrating its anti-inflammatory activity,[4] antibacterial properties,[5] antiurolithiatic activity,[6] analgesic effects,[7] and immunomodulatory activity.[8] These therapeutic attributes are largely attributed to the plant's rich phytochemical reservoir, which includes a diverse assortment of flavonoids, phenolic acids, polysaccharides, and essential oils.[9] These secondary metabolites are well known for their roles in modulating oxidative stress, microbial growth, immune responses, and inflammatory pathways, thus providing a scientific basis for the plant's traditional applications.

In a prior collaborative study conducted by our team,[10] the anticancer properties of *A. rosea* seed extracts were investigated. In particular, ethyl acetate-derived fractions of the seed extract were shown to exhibit significant cytotoxic activity against colorectal cancer (CRC) cells. The extract was observed to suppress cell proliferation, arrest the cell cycle at the G0/G1 phase, and induce apoptotic pathways in CRC cell lines. Notably, the extract exhibited the capacity to target CRC stem cells, a subpopulation of cells associated with tumor initiation, therapy resistance, and recurrence. This was evidenced by a marked downregulation of pivotal cancer stem cell surface markers, including aldehyde dehydrogenase 1, CD133, CD44, CD166, CD24, and doublecortin-like kinase 1, which play crucial roles in maintaining stemness and self-renewal capabilities in colorectal tumors. In addition, the extract's anticancer mechanism appeared to involve the inhibition of major oncogenic signaling cascades, particularly the Wnt/β-catenin and PI3K/Akt pathways, which are frequently activated in CRC and known to regulate cell survival, proliferation, and stemness.[10] Furthermore, the treatment was found to modulate epigenetic regulators, notably through the downregulation of the enhancer of zeste homolog 2, a histone methyltransferase implicated in cancer progression and stem cell maintenance. Simultaneously, an upregulation of tumor suppressor proteins, such as E-cadherin, was observed, indicating a reversal of epithelial-to-mesenchymal transition, a key process in metastasis.[10]

These findings collectively underscore the multifactorial anticancer potential of *A. rosea* seed extracts, highlighting the plant's rich phytochemical diversity and its capacity to influence a range of molecular and cellular targets implicated in CRC progression. The ability of the extract to inhibit cell proliferation, arrest the cell cycle, induce apoptosis, suppress oncogenic signaling, and downregulate cancer stem cell markers presents a compelling case for its further investigation as a multitargeted therapeutic agent. Importantly, these effects are not attributable to a single mechanism but rather suggest a coordinated modulation of various intracellular pathways, which may enhance therapeutic efficacy and reduce the risk of resistance development. Such a holistic pharmacological profile not only supports its use in traditional medicine but also provides a scientifically robust foundation for modern drug discovery initiatives. As such, the existing body of evidence strongly advocates for expanded preclinical research, mechanistic elucidation, and chemical characterization to unlock its full therapeutic potential. Motivated by these earlier findings, the present study was conceptualized to advance this line of inquiry by aiming to isolate, purify, and characterize individual bioactive compounds from the ethyl acetate crude extract of *A. rosea* seeds. While prior work convincingly demonstrated that the crude extract exhibits potent anticancer activity, the specific chemical entities responsible for these effects remained unidentified. The complexity of the extract necessitates detailed phytochemical investigation to distinguish the active constituents from inactive or potentially synergistic compounds. This step is essential to facilitate a structure activity relationship (SAR) analysis, optimize bioactivity, and enable further preclinical validation of the most promising candidates. In addition to direct cytotoxic and antiproliferative actions, the antioxidant and anti-inflammatory activities of *A. rosea* play a crucial role in its chemopreventive potential, especially relevant in the context of CRC. Oxidative stress and chronic inflammation are well-established drivers of colorectal tumor initiation and progression, often creating a microenvironment that supports genetic mutations, aberrant signaling, and immune evasion.[11,12] By mitigating these processes, the phytochemicals in A.rosea may not only inhibit tumor growth but also prevent tumorigenesis

at an early stage. Thus, the plant's dual chemotherapeutic and chemopreventive capabilities merit focused research to elucidate their underlying biochemical mechanisms and therapeutic implications. Despite these promising observations, the full therapeutic landscape of *A. rosea* seeds remains largely underexplored, particularly regarding their utility in CRC. Most available studies have concentrated on general pharmacological properties or the effects of crude extracts, with limited attention given to the identification and validation of individual active molecules. This represents a significant knowledge gap, especially when considering the plant's historical usage and initial scientific evidence suggesting strong anticancer properties.

Accordingly, the present study aims to address this gap by undertaking a comprehensive chemical fingerprinting of *A. rosea* seed extracts using advanced analytical techniques. By establishing a detailed profile of the constituent phytochemicals, this work seeks to provide a scientific platform for targeted pharmacological evaluations. Ultimately, these efforts aim to support the integration of *A. rosea* into evidence-based, plant-derived anticancer strategies, offering a natural, multipronged alternative for CRC management and potentially other malignancies.

2. Materials and methods

2.1. Collection and identification of plant material

The entire plant of *A. rosea* was meticulously collected from Malhar Hi-Tech Nursery, a recognized horticultural center situated in the Ganderbal district of Kashmir, India. This region is geographically located at a latitude of 34°13'35"N and a longitude of 74°47'34"E, with an elevation of 1,732.77 m above sea level, offering a temperate climate and suitable edaphic conditions conducive to the growth of medicinal and ornamental flora. The selection of this site was deliberate, as the natural habitat of the plant is known to influence its phytochemical profile, and high-altitude locations are often associated with increased secondary metabolite production due to enhanced exposure to environmental stressors.

To ensure the highest quality of the plant material and preserve the integrity of its bioactive constituents, sampling was strategically carried out during September, which corresponds to the late flowering and early seed maturation phase of *A. rosea*. This period is considered optimal for collecting seeds and aerial parts intended for pharmacological and phytochemical analyses, as it coincides with peak metabolite accumulation in many medicinal plants. Great care was taken during harvesting to avoid contamination, physical damage, or degradation of the plant material, thereby maintaining its therapeutic potential for subsequent laboratory analysis.

For accurate botanical classification, the plant material underwent taxonomic identification and authentication at the center of plant taxonomy (COPT), Department of Botany, University of Kashmir, which is a reputable institution for regional floristic studies. The identification process was conducted by qualified plant taxonomists, employing both macroscopic and microscopic diagnostic characteristics in accordance with standard botanical keys and herbarium references. To facilitate reproducibility of the study and allow for future taxonomic verification by other researchers, a voucher specimen (No. 2289-KASH, dated September 25, 2017) was formally prepared and deposited in the herbarium of COPT.

2.2. Extraction

The extraction of phytochemicals from *A. rosea* seeds was conducted using a carefully optimized and standardized protocol to ensure maximum recovery and integrity of bioactive constituents. Initially, the seeds were meticulously sorted and cleaned by gentle rinsing, followed by air-drying to eliminate any adhering dust particles and superficial surface contaminants. This pre-cleaning step was essential to prevent interference in subsequent extraction steps and analytical procedures. After cleaning, the seeds were shade-dried under aseptic conditions at ambient temperature (not exceeding 30°C) to preserve thermolabile and photosensitive compounds, which are often degraded under direct sunlight or elevated temperatures. The drying process continued for approximately seven days until a constant weight was achieved, indicating the removal of residual moisture.

Once completely dried, the seeds were subjected to coarse grinding using a stainless-steel mechanical grinder, which minimized metal contamination and thermal friction. The powdered seed material was then sieved through a No. 50 mesh (Retsch test sieve, 90 μm, stainless steel, 200 mm × 50 mm, DIN ISO 3310/1) to ensure uniform particle size distribution, an essential criterion that facilitates homogeneous solvent penetration and enhances extraction efficiency. A consistent powder texture contributes to the reproducibility of phytochemical extraction and is particularly critical when comparing extracts across solvents or replicates.

To selectively remove non-polar components and enhance the subsequent extraction of pharmacologically relevant semi-polar compounds, a two-step extraction approach was employed. In the first step, 2.3 kg of the sieved seed powder underwent defatting using analytical-grade *n*-hexane (ACS reagent, Sigma-Aldrich, assay

≥96.0%, CAS No. 110-54-3), a non-polar solvent widely used to dissolve and eliminate lipids, waxes, sterols, and other hydrophobic impurities. The powder was macerated with *n*-hexane for 12 h at room temperature with occasional agitation to ensure complete solvent contact. The hexane extract, which predominantly contained non-polar interfering substances, such as triglycerides and chlorophyll derivatives, was subsequently discarded. The residual defatted biomass, now enriched in semi-polar phytochemicals, was dried and prepared for further solvent extraction.

In the second step, the defatted seed material was subjected to exhaustive maceration using analytical-grade ethyl acetate (ACS reagent, Sigma-Aldrich, assay ≥ 99.5 %, CAS No. 141-78-6), a mid-polarity solvent ideal for extracting a wide range of semi-polar compounds. These include flavonoids, phenolic acids, coumarins, alkaloids, and certain terpenoids that are often responsible for the therapeutic activities attributed to medicinal plants. The maceration was performed in a multistep manner (5 × 5 L) over a total duration of 72 h, with the solvent refreshed at each interval to ensure comprehensive extraction. The use of ethyl acetate was guided by its high selectivity, relatively low toxicity, and minimal co-extraction of undesirable polymeric or pigment-based contaminants.

The pooled ethyl acetate extracts were filtered using filter paper (Whatman No. 1, 11 μm pore size, 180 μm thickness, ash ≤ 0.06%, Sigma-Aldrich, Whatman 1001-045, dia. 45 mm) to remove coarse particles and plant debris, and then concentrated using a rotary evaporator (Büchi Rotavapor R-100, Sigma-Aldrich, model Z741982) under reduced pressure at 35°C. This gentle concentration method preserved the chemical integrity of heat-sensitive compounds while efficiently removing the solvent. The process ultimately yielded 107 g of dark-brown, semi-solid crude extract, corresponding to a 4.6% (w/w) yield relative to the starting seed mass of 2.3 kg.

To support the rational selection of ethyl acetate as the principal extraction solvent, a preliminary solvent screening study was conducted. This screening compared ethyl acetate with methanol, ethanol, and chloroform under identical maceration conditions. The solvents were assessed based on four key parameters: (i) Total extractive yield, (ii) total phenolic and flavonoid content, (iii) qualitative phytochemical profile by thin-layer chromatography (TLC), and (iv) antiproliferative activity against HCT116 CRC cells using a standard MTT-based cytotoxicity assay.

Among all tested solvents, ethyl acetate demonstrated the most favorable profile, achieving a balanced combination of extraction yield, phytochemical richness, and biological activity. It efficiently extracted a broad spectrum of bioactive compounds, exhibited comparatively higher antiproliferative potential, and minimized the co-extraction of unwanted pigments and polymeric substances. Furthermore, the TLC chromatograms revealed better separation and resolution of phytoconstituents in ethyl acetate compared to the other solvents.

To ensure reproducibility and process reliability, the optimized extraction procedure was repeated in triplicate, yielding consistent results with a variation of less than ± 0.3% in the final extract weight. This low variability affirms the robustness and repeatability of the protocol. The final ethyl acetate crude extract was transferred into amber glass bottles, which shield the sample from light-induced degradation, and stored at 4°C in a desiccator to prevent moisture uptake and preserve its stability until further use.

This rigorously standardized and analytically validated extraction process not only ensures optimal recovery of pharmacologically relevant phytoconstituents from *A. rosea* seeds but also provides a reproducible platform for subsequent steps, involving fractionation, compound isolation, phytochemical characterization, and biological evaluation. The methodological rigor adopted here sets a strong foundation for advancing the therapeutic exploration of *A. rosea* in cancer and other disease models.

2.3. Gas chromatography–mass spectrometry (GC-MS) analysis

To elucidate the phytochemical composition of the ethyl acetate extract of *A. rosea* seeds, analysis was performed using GC-MS (QP2010 Ultra system, Shimadzu, Japan), an advanced analytical platform known for its high sensitivity and resolution in identifying complex organic compounds. This technique was chosen for its capability to separate, detect, and characterize volatile and semi-volatile constituents, which are typically abundant in plant extracts and often responsible for key pharmacological properties.

The gas chromatographic conditions were carefully optimized to ensure maximal resolution of the analytes. The ethyl acetate extract of *A. Rosea* seeds was analyzed using a 5% phenyl-methylpolysiloxane stationary phase column (Hewlett-Packard, Agilent Technologies, USA). The column oven temperature was initially set at 80°C, providing a stable baseline for the early eluting compounds. The injector temperature was maintained at 250°C to facilitate complete vaporization of the sample without thermal degradation. The injection mode was configured to split, which helps reduce the sample load entering the column and improves peak shape and reproducibility. A flow control mode with linear velocity was selected, coupled with a column flow rate set at 1 mL/min, ensuring

consistent carrier gas movement through the column. The purge flow was maintained at 3 mL/min, effectively eliminating non-volatile residues and preventing contamination. A split ratio of 5 was employed to balance sample dilution with analytical sensitivity.

The temperature programming was structured in a two-stage ramp. Initially, the temperature was increased from 80°C at a rate of 3°C/min until it reached 200°C, allowing for effective separation of low to mid-boiling compounds. This was followed by a further increment of 10°C, which was then held at 200°C for 5 min to facilitate elution of higher boiling-point components. This gradient-based temperature programming enabled comprehensive profiling of a wide range of phytochemicals within the sample, spanning various polarity and volatility ranges.

For the mass spectrometric detection, the ion source temperature was precisely maintained at 240°C, allowing for efficient ionization of eluting analytes. The interface temperature was stabilized at 250°C, ensuring thermal consistency between the gas chromatography and mass spectrophotometry units. A solvent cut time of 2 min was programmed to prevent early solvent peaks from interfering with the detector response. The mass scan range was configured from 45 to 900 m/z, accommodating a broad spectrum of molecular weights and enabling detection of both small and large molecular species. The event time was set to 0.30 seconds, providing a good balance between spectral resolution and scan speed. Data acquisition was initiated at 3.5 min and concluded at 51 min, covering the complete elution profile of the sample.

These carefully calibrated parameters created optimal chromatographic and mass spectrometric conditions, allowing for the accurate separation, identification, and quantification of the chemical constituents present in the ethyl acetate extract of *A. rosea* seeds. The analytical procedure enabled a detailed chemical fingerprinting, providing critical insights into the phytochemical diversity and potential bioactivity of the extract. This comprehensive approach contributes significantly to the standardization and quality assessment of plant-derived extracts intended for pharmacological evaluation.

2.4. Identification of components

The GC-MS analysis of the ethyl acetate extract of *A. rosea* seeds was performed over a total runtime of 51 min, during which the complete chromatographic elution of compounds was observed. The resulting chromatogram, as depicted in Figure 1, presents a clear distribution of peaks corresponding to the various volatile and semi-volatile constituents detected in the sample. Each peak represents a distinct compound or a class of structurally related compounds that were subsequently analyzed for identification.

The mass spectral data obtained from the analysis were subjected to comparative interpretation using established spectral libraries, specifically the National Institute of Standards and Technology (NIST) database and the Wiley Registry of mass spectral data (Wiley library). These spectral databases are widely accepted and commonly used for high-confidence compound identification in natural product research and metabolomic profiling. The NIST library, in particular, is known for its extensive catalog of mass spectra for organic and inorganic compounds. On the other hand, the Wiley library provides complementary coverage of bioactive and naturally occurring substances.

For those chromatographic peaks corresponding to unidentified components, the mass spectra were matched against reference spectra of known compounds stored in the NIST and Wiley libraries. This comparative matching was based on fragmentation patterns, base peaks, molecular ion values, and relative abundance profiles. Spectral similarities and matching scores were carefully assessed to ensure a high degree of accuracy in compound identification. For ambiguous or partially matched components, further comparison with structurally similar compounds aided in approximating molecular frameworks, functional groups, and possible analogs.

Through this rigorous spectral comparison process, a range of bioactive phytochemicals present in the *A. rosea* seed extract were successfully identified. These included compounds with well-characterized molecular weights, structural moieties, and known or predicted pharmacological activities. The application of such a robust and validated method enabled a comprehensive chemical characterization of the extract, enabling researchers to draw informed correlations between chemical composition and potential biological functions.

3. Results

The GC-MS analysis of the *A. rosea* seed extract identified 15 major compounds along with 113 minor compounds, which were further characterized. These compounds were discerned based on their elution order. Table 1 presents the major compounds together with their corresponding peaks, retention times, areas, and area percentages. In addition, Figure 1 illustrates the gas chromatogram of the ethyl acetate extract, and Figure 2 provides a pie chart representation of the area percentages covered by the major compounds identified in the extract. Among the identified major compounds, six were pharmacologically important due to their significant concentrations and potential medicinal properties. These include:

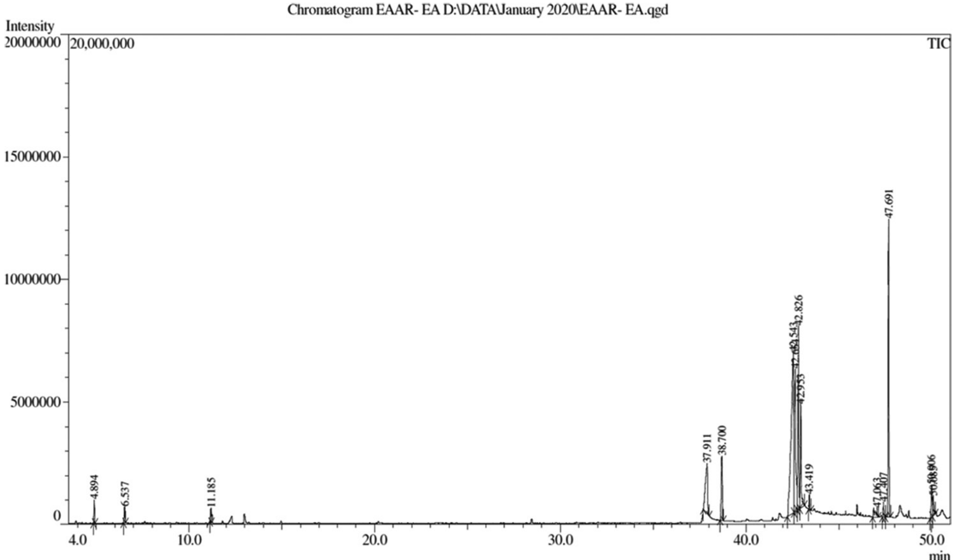

Figure 1. Gas chromatography–mass spectrometry chromatogram of ethyl acetate extract of *Alcea Rosea* seeds
Abbreviation: TIC: Total ion chromatogram.

Table 1. Phytocompounds identified in the ethyl acetate extract of *Alcea Rosea* seeds

Peak	Retention time (min)	Area	Area %	Name of compound	Molecular formula	Molecular weight
1	4.894	1741042	0.83	1-decyne	$C_{10}H_{18}$	138
2	6.537	1974686	0.94	*D*-mannitol	$C_6H_{14}O_6$	182
3	11.185	2101064	1.00	A13-09519	C_4H_9NO	103
4	37.911	16031218	7.67	n-hexadecenoic acid	$C_{16}H_{30}O_2$	254
5	38.700	9704774	4.64	Hexadecanoic acid	$C_{16}H_{32}O_2$	256
6	42.543	62456355	29.87	9,12-octadecadienoic acid (*Z, Z*)	$C_{18}H_{32}O_2$	280
7	42.651	31113346	14.88	*Cis*-9-octadecenoic acid (*E*)	$C1_8H_{34}O_2$	282
8	42.826	21555589	10.31	Linoleic acid ethyl ester	$C_{20}H_{34}O_2$	306
9	42.953	14520846	6.94	(*E*)-9-octadecenoic acid ethyl ester	$C2_0H_{38}O_2$	310
10	43.419	1354562	0.65	Octadecanoic acid	$C_{18}H_{36}O_2$	284
11	47.063	3022341	1.45	Bis (2-ethylhexyl) phthalate	$C_6H_4(COOC_8H_{17})_2$	390
12	47.407	1935050	0.93	Hexadecanoic acid	$C_{16}H_{32}O_2$	254
13	47.691	33674102	16.10	Bis (2-ethylhexylexyl) phthalate	$C_6H_4(COOC_8H_{17})_2$	390
14	50.006	4651105	2.22	9,12-octadecadienoic acid (*Z, Z*)-2,3 dihydroxypropyl ester	$C2_1H_{38}O_4$	354
15	50.085	3291589	1.57	9-octadecenoic acid (*Z*)-2,3-dihydroxy propyl ester	$C2_1H_{34}O_4$	356
Total		209127669	100.00			

(i) 9,12-Octadecadienoic acid (29.87%)
(ii) Bis(2-ethylhexyl) phthalate (DEHP) (16.10%)
(iii) *Cis*-9-octadecenoic acid (14.88%)
(iv) Linoleic acid ethyl ester (10.31%)
(v) n-hexadecenoic acid (7.67%)
(vi) A13-09519 (ethyl N-methylcarbamate, currently under clinical trials for anti-HIV treatment) (1%).

4. Discussion

In our current investigation, the GC-MS analysis of the ethyl acetate extract from *A. rosea* seeds revealed a highly diverse and complex phytochemical profile, reflecting the plant's rich metabolic output. The chromatographic analysis detected over 50 distinct peaks, each representing different volatile or semi-volatile compounds, as shown

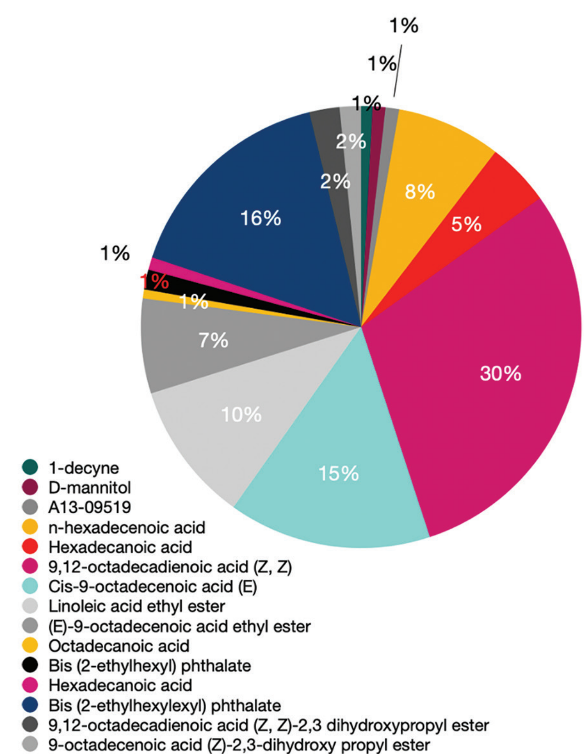

Figure 2. Pie chart showing the major compounds in the ethyl acetate extract of *Alcea Rosea* seeds

in Figure 1. Among these, 15 major compounds were selected for detailed evaluation based on their relative abundance and pharmacological relevance, as presented in Table 1. Compound identification was accomplished through meticulous spectral interpretation using both the NIST databases and Wiley libraries, which are well-established resources for the structural elucidation of organic molecules. These results, supplemented by detailed structural confirmation, are further supported in the Supplementary File, which contains full-spectrum and structural annotations. The findings indicate that *A. rosea* seeds contain an extensive array of bioactive small molecules, many of which exhibit therapeutic potential, particularly in the context of inflammation, cancer, and metabolic regulation.

Among the constituents identified, 9,12-octadecadienoic acid, a polyunsaturated omega-6 fatty acid, also known as linoleic acid, emerged as the dominant compound, accounting for 29.87% of the total area under the chromatogram. This compound has been extensively documented in the literature for its broad spectrum of biological activities, including anticancer,[13,14] cardioprotective,[15] anti-inflammatory,[16] antioxidant,[17] and neuroprotective properties.[18] In addition, 9,12-octadecadienoic acid has demonstrated antiproliferative effects in several cancer cell lines, such as KPL,[19] Caco-2,[20] MAC16,[21] DU145,[22] BT74, and A-549,[23] indicating its potential in multicancer chemoprevention and therapy. Its structural characteristics enable it to modulate lipid signaling pathways, influence membrane dynamics, and alter intracellular redox states, mechanisms often exploited in oncological interventions. Another abundant compound detected was DEHP, which contributed approximately 16.10% to the total area. While this compound is widely recognized as a synthetic plasticizer, it has also been isolated from biological specimens and plant extracts under specific conditions. Reports suggest its anti-tumor activity in various preclinical settings.[24] However, given its ubiquitous presence in plastic laboratory consumables and solvents, the identification of DEHP in this study remains provisional. Acknowledging its potential as a GC-MS artifact, we emphasize the importance of future validation using instrumental blanks, glass-only extraction protocols, and orthogonal analytical methods, such as liquid chromatography-mass spectrometry/tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR) spectroscopy, to confirm its true origin and rule out contamination.

Another compound, *cis*-9-octadecenoic acid, more commonly known as oleic acid, was also identified in significant concentration, representing 14.88% of the extract. Oleic acid, a monounsaturated omega-9 fatty acid, is widely acknowledged for its health-promoting properties. It has been shown to exert antiproliferative effects by upregulating critical tumor suppressor genes, including p53, p21, and p27, especially in esophageal cancer cells.[25] Furthermore, it has been reported to modulate apoptotic and autophagic signaling pathways, thereby contributing to the inhibition of tongue squamous cell carcinoma progression.[14] In addition, oleic acid demonstrates the capacity to suppress Her-2/neu overexpression, a key oncogenic driver in breast cancer, making it a compound of high interest in targeted therapies.[26] In the cardiovascular context, oleic acid contributes to reduced low-density lipoprotein cholesterol levels and oxidative stress, thereby enhancing endothelial function. These properties align with its established role in promoting cardiovascular health, particularly in the context of Mediterranean dietary patterns.[15] Furthermore, oleic acid possesses significant anti-inflammatory potential, mediated by the downregulation of pro-inflammatory cytokines, such as interleukin-6 and tumor necrosis factor-α, as well as the inhibition of cyclooxygenase-2 expression.[16] It also acts as an effective antioxidant, scavenging free radicals and stabilizing lipid membranes, thereby mitigating oxidative damage.[17] In addition, emerging studies suggest that oleic acid plays a neuroprotective role, potentially due to its

anti-inflammatory and antioxidant properties.[18] These multifaceted pharmacological attributes warrant further investigation of oleic acid as a key bioactive constituent of *A. rosea* seed extracts. The accurate International Union of Pure and Applied Chemistry chemical structure of oleic acid, along with other pharmacologically relevant compounds, is illustrated in Figure 3, developed based on validated data from chemical databases, such as PubChem and ChemSpider.

Although present in a smaller proportion, linoleic acid ethyl ester (0.31%) is also noteworthy. This compound has been documented to possess anti-inflammatory,[27] anti-acne,[28] and antidiabetic activities.[29] Its relevance in dermatological and metabolic applications is supported by studies showing modulation of lipid metabolism, insulin sensitivity, and inflammatory mediator production, even at low concentrations. Similarly, hexadecanoic acid (palmitic acid), contributing 7.67% of the extract, has been linked to anticancer activity through its ability to inhibit β-catenin expression in HT29 colon cancer cells, particularly when conjugated with dicer-substrate small interfering RNA.[30] Since β-catenin overexpression is a hallmark of colorectal tumorigenesis, its downregulation by hexadecanoic acid may present an important therapeutic avenue. In addition, this compound also exhibits anti-androgenic effects,[31] supporting its broader role in hormonal regulation and cancer treatment.

Among the unique constituents identified, A13-09519, chemically classified as ethyl N-methylcarbamate, was also present. Although less frequently reported in natural products literature, data from databases, such as PubChem and DrugBank, suggest that A13-09519 has biological activity targeting Shiga-like toxin 1 subunit B, a pathogenic factor that binds to globotriaosylceramide receptors on human intestinal microvilli.[32-34] Given its potential implication in toxin neutralization and microbial interactions, it warrants further investigation. However, due to its structural class and occurrence pattern, A13-09519 is also under consideration as a possible instrument- or solvent-derived contaminant, necessitating follow-up validation through high-resolution mass spectrometry and comparison with a reference compound.

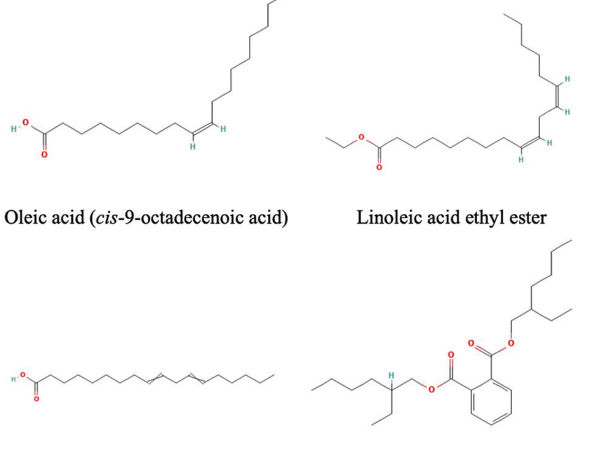

Figure 3. Chemical structure of pharmacologically important compounds identified in the ethyl acetate extract of *Alcea Rosea* seeds

Collectively, these findings reflect the therapeutic promise of several compounds identified within the ethyl acetate extract of *A. rosea* seeds. However, it is important to note that GC-MS analysis is inherently limited to detecting volatile and thermally stable compounds, potentially overlooking a vast range of non-volatile phytoconstituents, such as alkaloids, glycosides, flavonoids, and tannins, which are commonly found in medicinal plants. To obtain a comprehensive metabolic profile, future studies will incorporate advanced analytical platforms, such as LC-MS, ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry, and NMR spectroscopy. These approaches will enable the identification of polar, thermolabile, and high-molecular-weight constituents that are inaccessible via GC-MS. While the current study draws upon existing literature to suggest potential bioactivities of the identified compounds, it is recognized that these SARs are preliminary and correlative. Hence, moving forward, we aim to conduct bioassay-guided fractionation, *in vitro* cytotoxicity evaluations, and *in vivo* pharmacokinetic and toxicity assessments. These investigations will not only help confirm the biological relevance and safety of the crude extract and its constituents but also contribute to the rational development of phototherapeutics derived from *A. rosea*.

5. Conclusion

The current investigation offers a foundational insight into the phytochemical architecture of *A. rosea* seeds, shedding light on their potential as a reservoir of biologically active natural compounds. The study employed GC-MS, a sensitive and robust analytical tool, to characterize the ethyl acetate extract. This led to the successful identification of several high-abundance and pharmacologically significant compounds, validating the traditional medicinal use of *A. rosea* and supporting its consideration in modern drug discovery pipelines. The predominance of linoleic acid among the identified constituents highlights the relevance of this polyunsaturated fatty acid in the seed's pharmacodynamic landscape. Known for its anti-inflammatory and antioxidant properties, linoleic acid is also implicated in cellular signaling pathways that govern proliferation and apoptosis. Its significant representation within the extract provides

a biochemical rationale for the potential anticancer and cardioprotective effects traditionally attributed to *A. rosea*. Similarly, the detection of oleic acid contributes to the therapeutic properties of the extract, which modulate the expression of tumor suppressor genes and interfere with oncogenic signaling networks. Its reported effects on cell cycle regulation and apoptosis, particularly in various epithelial malignancies, strengthen the argument for further exploration of *A. rosea* in oncology-focused phytotherapy research. A nuanced aspect of the findings involves the identification of DEHP, a compound typically associated with synthetic sources but increasingly reported in plant-derived matrices. While its pharmacological attributes, such as antimicrobial and cytotoxic potential, warrant consideration, its dual identity as a potential laboratory contaminant emphasizes the importance of confirmatory analyses in natural products research.

Future studies should incorporate stringent control measures and utilize orthogonal techniques, such as LC-MS/MS and NMR, to verify the origin of compounds and authenticity. The presence of other structurally and pharmacologically diverse molecules, including palmitic acid and linoleic acid ethyl ester, suggests a synergistic matrix of fatty acids within *A. rosea* seeds that could collectively influence biological systems. These compounds, even at modest concentrations, are known to participate in lipid signaling, immune modulation, and metabolic regulation areas highly relevant to chronic diseases, including diabetes and cancer. Importantly, the identification of A13-09519, a compound with a lesser-known natural occurrence but suggested involvement in microbial toxin interactions, opens an exciting avenue for exploration in antimicrobial and antiviral therapeutics. While the biological role of this compound remains to be validated, its detection in this context merits further pharmacological and structural studies.

While GC-MS served as an efficient tool in profiling volatile and semi-volatile constituents, it is acknowledged that this method is inherently restricted in its scope. Non-volatile classes of compounds, such as flavonoids, alkaloids, and glycosides, which are common in medicinal plants, may have been overlooked. As such, future research should incorporate complementary techniques, including liquid chromatography and high-resolution mass spectrometry, to capture a fuller phytochemical spectrum. In addition, while this study offers meaningful insights into the chemical identity of *A. rosea* seed constituents, the therapeutic implications of these compounds require empirical validation. The proposed bioactivities, although supported by existing literature, must be confirmed through well-designed pharmacological assays, including *in vitro* cytotoxicity, mechanistic pathway analysis, and *in vivo* efficacy and toxicity studies.

In conclusion, the findings of this study lay a solid groundwork for the scientific validation of *A. rosea* seeds as a source of multifunctional bioactive compounds. The chemical diversity observed suggests considerable potential in addressing inflammatory, neoplastic, and microbial conditions. By integrating phytochemical data with biological testing and formulation science, *A. rosea* may be developed into evidence-based therapeutics, reinforcing the relevance of traditional plant knowledge in the advancement of modern pharmacology.

Acknowledgments

We extend our sincere gratitude to the Departments of Biochemistry, Clinical Biochemistry, and Nanotechnology at the University of Kashmir, and the Department of Chemistry at Government Degree College, Uri, for their crucial support with space, instrumentation, and facilities, significantly advancing our study.

Funding

None.

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Gh Jeelani Mir
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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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INNOSC Theranostics and Pharmacological Sciences

ORIGINAL RESEARCH ARTICLE

Treadmill exercise promotes melatonin regulation of TXNIP/NLRP3 and inhibits pyroptosis-mediated osteoarthritis progression in a DMM rat model

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Citation: Altahla R, Alshorman J, Chaoyi L, Umar M, Hourani MM, Wang Y. Treadmill exercise promotes melatonin regulation of TXNIP/NLRP3 and inhibits pyroptosis-mediated osteoarthritis progression in a DMM rat model. *INNOSC Theranostics and Pharmacological Sciences*. 2025;8(4):73-84. doi: 10.36922/ITPS025360046

Received: September 2, 2025

Revised: September 25, 2025

Accepted: October 10, 2025

Published online: November 18, 2025

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Abstract

Osteoarthritis (OA) is characterized by the activation of nucleotide-binding and oligomerization domain-like receptor protein 3 (NLRP3) by thioredoxin-interacting protein (TXNIP), which promotes inflammation and pyroptosis. This study investigates whether treadmill exercise (TE) enhances melatonin-mediated regulation of the TXNIP/NLRP3 pathway and attenuates OA progression by modulating pyroptosis in a destabilization of the medial meniscus (DMM) rat model. A total of 32 male Sprague–Dawley rats (6 weeks old; 220 ± 20 g) were randomly assigned to four groups: Sham, DMM, DMM + melatonin, and DMM + melatonin + TE. The intervention lasted for 8 weeks. Morphological staining, immunofluorescence (IF), microcomputed tomography with three-dimensional reconstruction, Western blot, quantitative real-time polymerase chain reaction, and enzyme-linked immunosorbent assay were used to assess protein and gene expression. Compared with the DMM + melatonin group, the DMM + melatonin + TE group showed greater reductions in cartilage–subchondral bone damage and OA progression through modulation of the pyroptosis pathway. IF staining revealed that TXNIP protein expression was significantly reduced in the DMM + melatonin + TE group. Both the DMM + melatonin and combination treatment groups significantly regulated TXNIP/NLRP3 signaling and inhibited OA progression through pyroptosis. Furthermore, combining TE with melatonin significantly reduced the expression of pyroptotic cytokines compared with the DMM + melatonin group. This study suggests a potential therapeutic approach for managing OA by combining melatonin treatment with moderate TE.

Keywords: Osteoarthritis; Exercise; Melatonin; Thioredoxin-interacting protein; Nucleotide-binding and oligomerization domain-like receptor protein 3

1. Introduction

Osteoarthritis (OA) is a debilitating articular cartilage disease that significantly impairs lower limb function, exacerbating the social and monetary strain.[1] The key pathological features of OA include articular cartilage degeneration, subchondral bone damage, synovial inflammation, capsular damage, and associated musculature.[2] Traditional OA treatments provide only limited symptomatic relief, with potential adverse effects and risk of recurrence.

Thioredoxin-interacting protein (TXNIP) plays a crucial role in OA by modulating oxidative stress and inflammation.[3] Elevated TXNIP levels in OA patients enhance oxidative stress and trigger activation of the nucleotide-binding and oligomerization domain-like receptor protein 3 (NLRP3) inflammasome.[4] Therefore, targeting TXNIP and its related pathways represents a promising strategy for developing therapies that mitigate inflammation and slow OA progression. Pyroptosis is a form of cell death initiated by inflammasomes and serves as a key innate immune mechanism.[5] The NLRP3 inflammasome is a primary driver of pyroptosis—upon activation, it triggers caspase-1 enzymatic activity, leading to pyroptotic cell death.[6] Activated caspase-1 cleaves gasdermin D (GSDMD) and promotes the release of pyroptotic cytokines, including interleukin (IL)-1β and IL-18.[7] Studies have demonstrated increased levels of NLRP3, IL-1β, and IL-18 in the synovial layer of OA animal knee models, indicating that the inflammasome signaling pathway contributes to OA pathogenesis.[8] Although pyroptosis has been implicated in OA, the specific role of the TXNIP/NLRP3 pathway in mediating this process remains incompletely understood.

Melatonin, a hormone known for regulating sleep and circadian rhythms, also has emerging roles in managing OA.[9,10] By reducing oxidative damage and suppressing inflammatory responses, melatonin can potentially slow the progression of OA and alleviate symptoms, offering a promising adjunctive therapy or alternative to traditional treatments for this debilitating joint disease.[11]

Exercise therapy has emerged as an effective, reliable, and accessible long-term strategy for the prevention and management of OA,[12,13] and is closely associated with the regulation of pyroptosis.[14] Previous studies have demonstrated that exercise can reduce the expression of the NLRP3 inflammasome and significantly inhibit the activation of its downstream components, such as caspase-1, IL-1β, and IL-18.[14,15]

Melatonin and exercise both exert anti-pyroptotic effects. Melatonin inhibits NLRP3 inflammasome activation, while exercise downregulates NLRP3 and its downstream effectors. The combined modulation of pyroptosis pathways by these two interventions may provide additive or synergistic therapeutic benefits for pyroptosis-related diseases such as OA.

The objective of this study is to investigate how treadmill exercise (TE) enhances the therapeutic effects of melatonin. Specifically, the study aims to examine the regulation of the TXNIP/NLRP3 axis by the combined intervention of TE and melatonin treatment, and how this leads to the inhibition of OA progression.

2. Materials and methods

2.1. Experimental animals

A total of 32 6-week-old Sprague-Dawley male rats weighing approximately 220 ± 20 g were used in this study. The training protocol was administered at the Experimental Animal Centre of Tongji Medical University, where the experimental animals were kept in a specific pathogen-free environment. Two rats were housed per plastic cage under controlled conditions (temperature: 22 ± 2°C; humidity: 60 ± 5%) on a 12 h light/dark cycle. Animals had ad libitum access to food and water and unrestricted mobility in their cages.

2.2. OA model and experimental groups

The study protocols are illustrated in Figure 1. A total of 32 Sprague-Dawley rats underwent a 1-week acclimation period of TE training. The exercise protocol was performed using the Zhenghua Biological Instrument Equipment (China) with the following parameters: 10 min/day, 5 days per week, at a speed of 10 m/min.

After the 1st week of TE training for acclimation, the rats were randomly divided into two experimental cohorts: Sham (n = 8) and destabilization of the medial meniscus (DMM; n = 24). Regarding anesthesia, isoflurane was administered throughout all surgical procedures. Briefly, the DMM group underwent surgical interventions on the right knee joint, which included anterior cruciate ligament transection and DMM surgery. The sham group served as the control group and underwent an incision of the skin and medial capsule only.

Four weeks post-surgery, eight animals from each group were randomly selected and euthanized to assess the success of the DMM model. Four weeks after surgery, the rats in the DMM group were randomly allocated into three groups: DMM (n = 8), DMM + melatonin (n = 8), and DMM + melatonin + TE groups, while the sham group (n = 8) included all sham animals in the study. The exercise training protocol was adapted from a previously published study.[16]

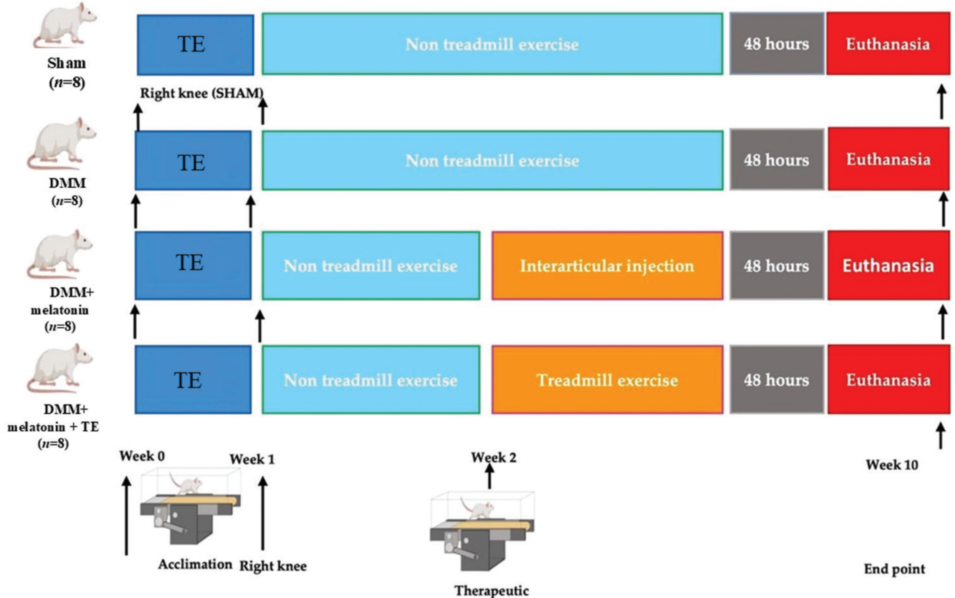

Figure 1. A total of 32 Sprague-Dawley rats underwent a 1-week acclimation training on a treadmill. Following this period, the rats were randomly assigned to four groups: sham, DMM, DMM + melatonin, and DMM + melatonin + TE. The DMM + melatonin group received an intra-articular injection of 10 μL melatonin solution. The DMM + melatonin + TE group received a combination of intra-articular melatonin injection (10 μL) and TE for 8 weeks. All rats were euthanized within 48 h after the final TE session.
Abbreviations: DMM: Destabilization of the medial meniscus; TE: Treadmill exercise.

2.3. Intervention treatment

In the experiment, melatonin was initially solubilized in 100% ethanol and subsequently diluted in saline (0.9% sodium chloride) to achieve a final concentration of 10 mg/mL.[17]

Following induction of the DMM model, rats in the DMM + melatonin and DMM + melatonin + TE groups received intra-articular injections of 10 μL of melatonin solution via the patellar tendon twice weekly for 6 weeks. The sham and DMM groups received an equal volume of normal saline. The DMM + melatonin + TE group underwent moderate TE for 8 weeks at a speed of 15 m/min for 30 min/day, 5 days per week. Within 48 h after the final TE session, all rats were euthanized. Blood plasma was collected via cardiac puncture, and tissue samples were harvested for subsequent analysis (Figure A1).

2.4. Microcomputed tomography with three-dimensional reconstruction

After euthanasia, samples of the rats' right knee joints were collected and scanned using a microcomputed tomography (micro-CT) (50 Scanco Medical, Switzerland) with a voxel size of 10.5 μm, 100 kV voltage, and 98 μA current. The integrated evaluation system generated three-dimensional (3D) images and quantitative data, including bone volume/tissue volume fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), and trabecular separation (Tb. Sp).

2.5. Histological staining

Samples of the rats' knee joints underwent decalcification in a 10% ethylenediaminetetraacetic acid solution. Following dehydration, the samples were embedded in paraffin. Sagittal sections (4 μm) were prepared for histological examination. Hematoxylin-eosin and safranin O/fast green staining were conducted to analyze the articular cartilage microscopically. Knee joint injury was evaluated using the OA Research Society International scoring system.[18]

2.6. Immunohistochemistry staining

The levels of tissue proteins were assessed using immunohistochemistry staining for caspase-1 (rabbit; 1:200; ER1905–47, Hua'an Biotechnology Co., LTD., China), GSDMD (GSDMD; rabbit; 1:200; ER1901–37, Hua'an Biotechnology Co., LTD., China), and NLRP3 (rabbit; 1:200; ET1610–93, Hua'an Biotechnology Co., LTD., China).

2.7. Immunofluorescence staining

Paraffin-embedded samples were processed following standard protocols and incubated with TXNIP (rabbit; 1:200, ET1705–72, Hua'an Biotechnology Co., LTD., China). Subsequently, slides were incubated with secondary

antibodies (Bosterbio, China) for 1 h. The nuclei were stained for 10 min with 4',6-diamidino-2-phenylindole (Bosterbio, China). Finally, images were captured using the EVOS FL Auto Imaging System (Hua'an Biotechnology Co., LTD., China).

2.8. Quantitative real-time polymerase chain reaction

Total RNA was extracted from articular cartilage using RNA Trizol (Powerful Biotechnology, China). Complementary DNA was synthesized using Hifair® III 1st Strand Synthesis Super Mix (Hua'an Biotechnology Co., LTD., China). The complementary DNA served as a template for PCR, with glyceraldehyde 3-phosphate dehydrogenase used as the internal reference. mRNA levels were quantified using SYBR Green Master Mix (Hua'an Biotechnology Co., LTD., China) and the $2^{-\Delta\Delta CT}$ method. Primer sequences, listed in Table 1, were obtained from GeneBank and synthesized by Bao Biomedical Technology Co., Ltd., China. All reactions were performed in triplicate, and fluorescence signals were detected using the ABI QuantStudio™ 1 System (Hua'an Biotechnology Co., LTD., China).

2.9. Western blot

Cartilage tissue samples were washed with phosphate-buffered saline and mechanically disrupted through grinding and sonication. Radioimmunoprecipitation buffer containing enzyme inhibitors was used for cell lysis. After centrifugation, the protein-containing supernatant was collected. Protein concentration was assessed using a BCA kit (BF0026, Bolff Biotechnology Co., LTD., China), and samples were denatured by heating at 100°C for 5 min in sample buffer.

Table 1. Forward and reverse primer sequences used for quantitative real-time polymerase chain reaction of articular cartilage samples (*n*=8)

Gene	RNA sequence (5' to 3')
R *GAPDH* F	ACTCTACCCACGGCAAGTTC
R *GAPDH* R	TGGGTTTCCCGTTGATGACC
R *CASP1* F	GACCGAGTGGTTCCCTCAAG
R *CASP1* R	GACGTGTACGAGTGGGTGTT
R *GSDMD* F	GATGCCTGCTTGTTGAGTTGG
R *GSDMD* R	AGAATTCCGAAGGCAGTCCA
R *TXNIP* F	TCTGCCTCACGGAGAGACTT
R *TXNIP* R	CATGATTGAGCCGAGTGGGT
R *NLRP3* F	CTGCATGCCGTATCTGGTTG
R *NLRP3* R	GGTACCCCATAGACTGGCAC

Abbreviations: F: Forward; R: Reverse.

The membranes were blocked with 5% milk solution to minimize non-specific binding. Primary antibodies—caspase-1 (1:1000), GSDMD (1:1000), NLRP3 (1:1000), TXNIP (1:2000), and glyceraldehyde 3-phosphate dehydrogenase (1:5000)—were incubated overnight at 4°C. After washing, membranes were incubated with secondary antibodies conjugated to fluorescent tags for 1 h at 4°C. Protein bands were detected using an infrared imaging system (software, Wuhan Guide Infrared Co., Ltd., China), and band intensities were quantified using ImageJ software (Laboratory for Optical and Computational Instrumentation, USA).

2.10. Enzyme-linked immunosorbent assay

Rats were anesthetized with isoflurane, and blood plasma was collected via cardiac puncture and stored at −80°C for subsequent assessment of inflammatory factors. The levels of IL-18 (FY-EH-6593, Feiyue Biotechnology, China) and IL-1β (FY-EH6675, Feiyue Biotechnology, China) were measured using enzyme-linked immunosorbent assay kits (A-A04, Qianbaidu Biotechnology Co., LTD., China) according to the manufacturer's instructions.

2.11. Statistical analyses

Data are presented as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism version 10 (Dotmatics, US). Comparisons among multiple groups were conducted using two-way analysis of variance, followed by Tukey's *post hoc* test for pairwise comparisons. Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Intervention treatment and body weight

As depicted in Figure 2, over the course of the intervention period, the body weights of rats in all groups showed an increasing trend, indicating normal growth and development.

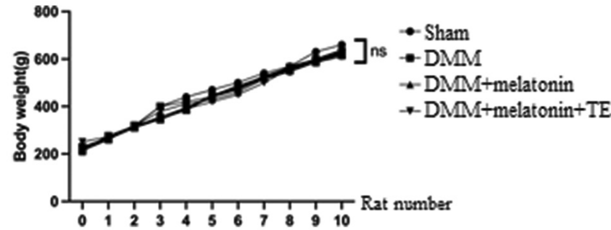

Figure 2. Intervention treatment and body weight of the rats at the study endpoint.

Notes: Data Data are presented as mean ± standard deviation. "ns" indicates no statistically significant difference.

Abbreviations: DMM: Destabilization of the medial meniscus; TE: Treadmill exercise.

3.2. Melatonin and TE treatment mitigate DMM-associated characteristics of the joint

To assess the impact of TE on enhancing melatonin's effects on the knee joint in the DMM model, microCT–3D analysis was performed to evaluate damage in the tibial trabecular bone (Figure 3A).

The combination of exercise and intra-articular melatonin injections significantly improved subchondral bone integrity and restored microstructural changes in the tibial bone following injury, compared to the DMM group that received only intra-articular melatonin injections.

The findings revealed that BV/TV, Tb. Th, and Tb. N values increased in the DMM + melatonin + TE group, whereas Tb. Sp decreased (Figure 3B).

The OA Research Society International score in the DMM group was significantly higher than that in the sham group. In contrast, the DMM group receiving intra-articular melatonin and TE exhibited reduced damage severity, as evidenced by less safranin O/fast green loss and improved surface regularity (Figure 4A and B). These findings indicate that combined TE and melatonin treatment effectively inhibits cartilage lesion progression and maintains structural integrity compared to the DMM + melatonin group.

In addition, immunohistochemistry analysis was performed to assess pyroptosis-related biomarkers and investigate the effects of TE combined with melatonin on the pyroptosis pathway. Rats in the DMM-only group showed elevated levels of NLRP3, caspase-1, and GSDMD compared to the DMM + melatonin group (Figure 4C and D). In contrast, TE combined with intra-articular melatonin significantly reduced the number of NLRP3-, caspase-1–, and GSDMD-positive cells in rat cartilage tissue (Figure 4C and D). These findings demonstrate that TE enhances the effects of melatonin in suppressing OA progression and reduces the expression of NLRP3, caspase-1, and GSDMD.

3.3. Effects of TE and melatonin on TXNIP expression levels

Immunofluorescence staining was used to assess TXNIP protein expression in cartilage tissue. The levels of TXNIP protein in the cartilage of the DMM group were significantly higher than those in the sham group ($p<0.0001$). In contrast, TXNIP protein expression in the DMM + melatonin group was significantly higher than that in the DMM + melatonin + TE group ($p<0.05$; Figure 5A and B).

3.4. Comparative impact of TE and melatonin on pyroptosis markers, TXNIP in cartilage tissue, and pyroptotic cytokine levels

The expression of pyroptosis biomarkers and TXNIP in cartilage tissues was compared between the DMM + melatonin group and the DMM + melatonin + TE groups (Figure 6). The mRNA (Figure 6A-D) and protein levels (Figure 6E and F) of caspase-1, GSDMD, NLRP3, and TXNIP in the DMM group were significantly higher than those in the DMM + melatonin and DMM + melatonin + TE groups ($p<0.01$). Additionally, the DMM + melatonin group exhibited elevated mRNA ($p<0.001$) and protein ($p<0.01$) levels compared to the DMM + melatonin + TE group.

Blood samples collected 48 h post-intervention were analyzed for serum inflammatory factor levels to assess the effects of exercise on inflammation during OA progression. Results indicated that serum inflammatory factor levels in the DMM group were significantly higher than those in the DMM + melatonin and DMM + melatonin + TE groups ($p<0.05$). Furthermore, the DMM + melatonin

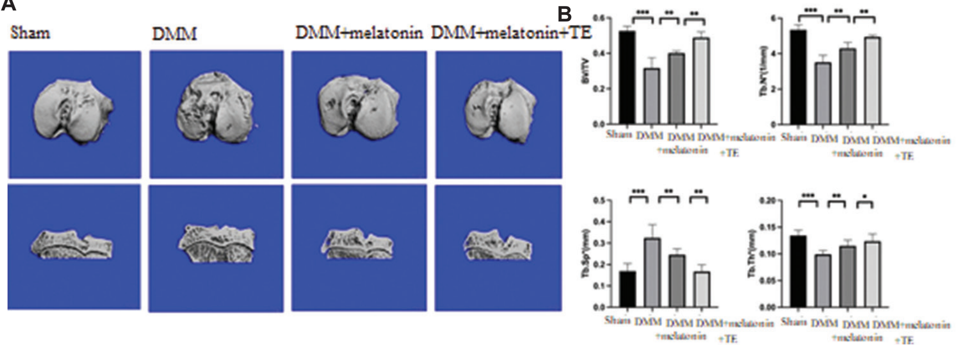

Figure 3. Microcomputed tomography and three-dimensional reconstruction images. (A) Representative microcomputed tomography and three-dimensional reconstruction images of the sham, DMM, DMM + melatonin, and DMM + melatonin + TE groups (scale bar: 1 mm). (B) Quantitative analysis of bone parameters. Note: Data are presented as mean ± standard deviation. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ ($n = 8$).

Abbreviations: BV/TV: Bone volume/tissue volume; DMM: Destabilization of the medial meniscus; Tb. N: Trabecular number; Tb. Sp: Trabecular separation; Tb. Th: Trabecular thickness; TE: Treadmill exercise.

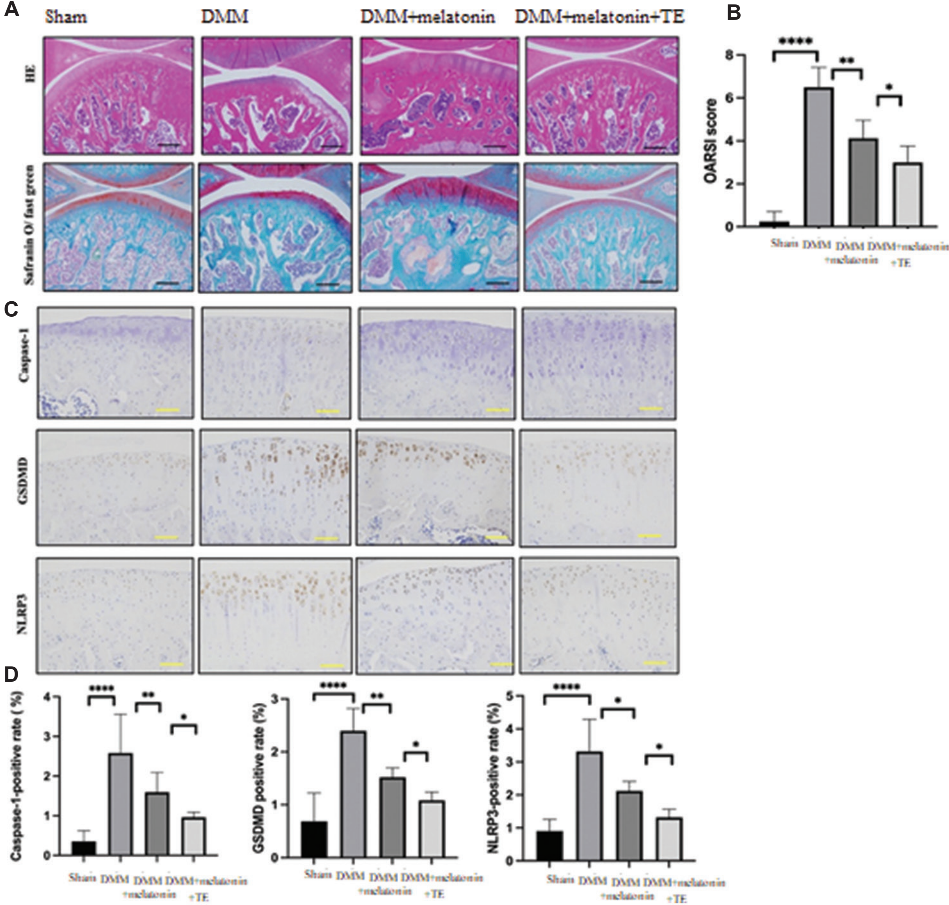

Figure 4. Histological and immunohistochemical analysis. (A) Representative HE and safranin O/fast green staining images (scale bar: 1,000 μm; magnification: ×100μm). (B) Osteoarthritis Research Society International score. (C) Representative immunohistochemical staining images of caspase-1, GSDMD, and NLRP3 (scale bar: 200 μm; magnification: ×200μm). (D) Quantification of the percentage of caspase-1–, GSDMD-, and NLRP3-positive cells. Note: Data are presented as mean ± standard deviation. *$p<0.05$, **$p<0.01$, and ****$p<0.0001$ ($n = 8$).

Abbreviations: DMM: Destabilization of the medial meniscus; GSDMD: Gasdermin D; HE: Hematoxylin–eosin; NLRP3: Nucleotide-binding and oligomerization domain-like receptor protein 3; TE: Treadmill exercise.

+ TE group showed lower serum levels compared to the DMM + melatonin group ($p<0.05$). These findings suggest that TE enhances the effects of melatonin by reducing inflammation in OA (Figure 7).

4. Discussion

The findings revealed that intra-articular injection of melatonin attenuates OA progression by modulating pyroptosis. TE further enhances melatonin's effects, leading to a greater reduction in OA progression through this pathway. In addition, TE amplifies melatonin's regulation of the TXNIP/NLRP3 signaling pathway, thereby inhibiting OA progression mediated by pyroptosis. These findings provide valuable insights into the molecular mechanisms through which TE contributes to the suppression of OA progression.

Exercise has been extensively employed in the management and treatment of numerous chronic conditions, helping patients alleviate symptoms, restore function, and improve overall well-being.[19-21] Previous studies have shown that moderate exercise can preserve joint integrity and mitigate disorder in DMM rats.[22,23] Exercise also exerts a notable anti-inflammatory effect associated with the regulation of pyroptosis.[24] It is well established that exercise can reduce chronic inflammation and effectively suppress inflammatory factor levels, thereby promoting the secretion of anti-inflammatory cytokines. Furthermore, studies have demonstrated that physical exercise can downregulate NLRP3 expression and significantly suppress the activation of apoptosis-associated speck-like protein containing a caspase recruitment domain, caspase-1, IL-1β, and IL-18.[10,24,25]

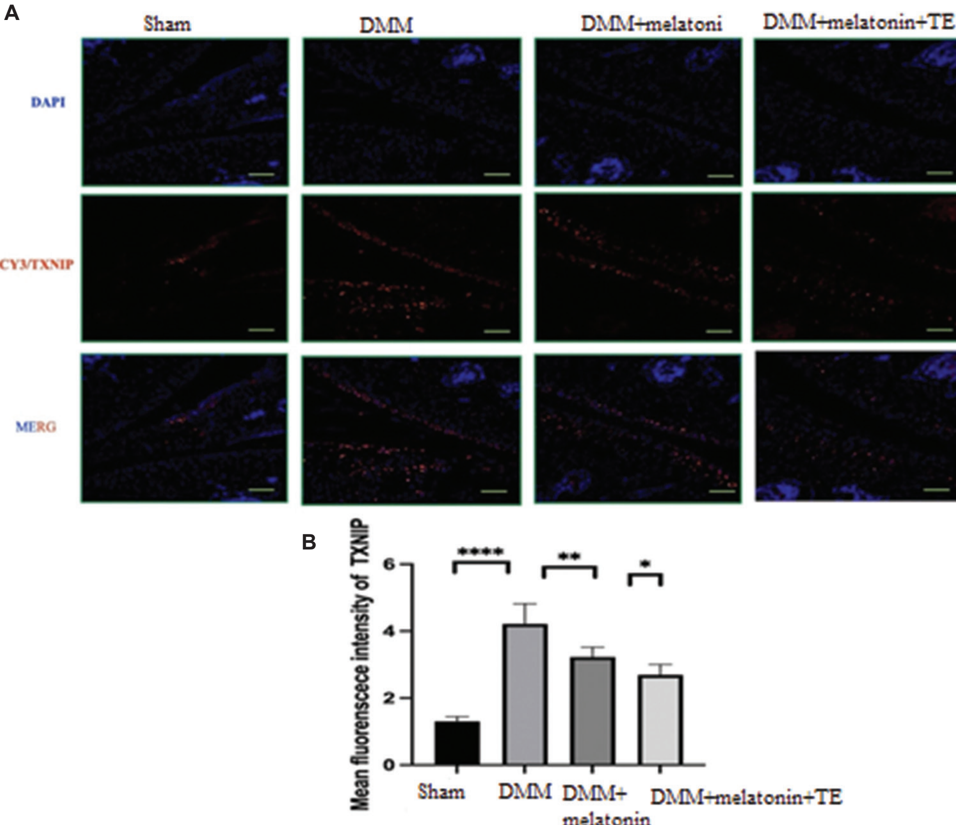

Figure 5. Effects of TE and melatonin on TXNIP expression. (A) Representative immunofluorescence staining images of TXNIP (scale bar: 200 μm; magnification: ×200μm). (B) Quantification of mean fluorescence intensity of TXNIP in cartilage.

Note: Data are presented as mean ± standard deviation. *p<0.05, **p<0.01, and ****p<0.0001 (n = 8).

Abbreviations: CV3: Caveolin-3; DAPI: 4',6-diamidino-2-phenylindole; DMM: Destabilization of the medial meniscus; MERG: DAPI with CV3/TXNIP; TE: Treadmill exercise; TXNIP: Thioredoxin-interacting protein

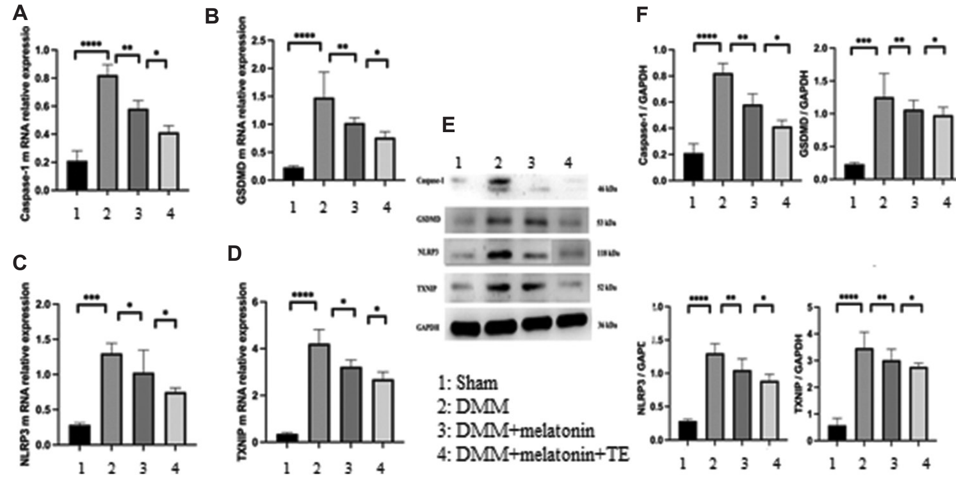

Figure 6. Comparative effects of TE and melatonin on pyroptosis markers and TXNIP. (A) Caspase-1 mRNA levels. (B) GSDMD mRNA levels. (C) NLRP3 mRNA levels. (D) TXNIP mRNA levels. (E) Protein expression of caspase-1, GSDMD, NLRP3, and TXNIP in cartilage detected by Western blot. (F) Quantification of protein expression levels.

Note: Data are expressed as mean ± standard deviation of two independent experiments. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 (n = 8).

Abbreviations: DMM: Destabilization of the medial meniscus; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GSDMD: Gasdermin D; NLRP3: Nucleotide-binding and oligomerization domain-like receptor protein 3; TE: Treadmill exercise; TXNIP: Thioredoxin-interacting protein.

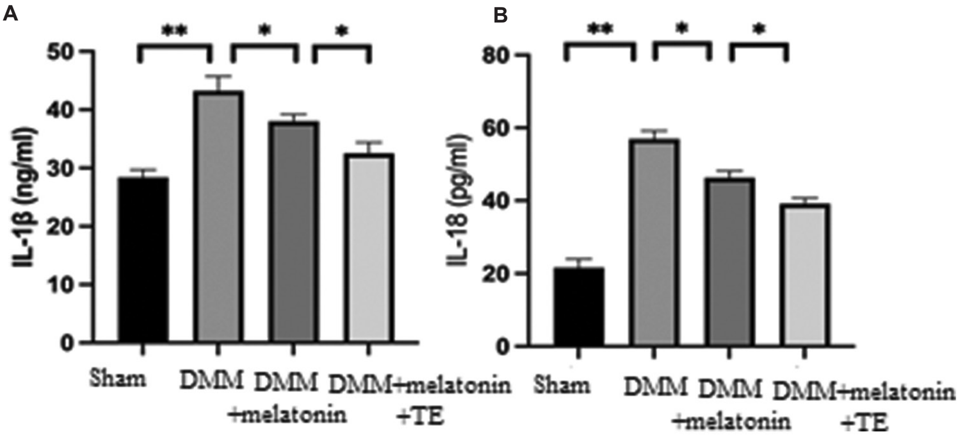

Figure 7. Quantification of serum inflammatory cytokines: (A) IL-1β and (B) IL-18.
Note: Data are expressed as mean ± standard deviation. $p<0.05$ and $^{**}p<0.01$ ($n = 8$).
Abbreviations: DMM: Destabilization of the medial meniscus; IL: Interleukin; TE: Treadmill exercise.

The present study investigated how TE enhances the effects of melatonin treatment in mitigating cartilage degradation in OA through the pyroptosis pathway. Using the DMM model, we found that both melatonin treatment and its combination with exercise significantly reduced inflammation and cartilage damage in the knee joints of DMM rats. Multiple analytical methods, including micro-CT and histological staining, were used to assess OA progression. The DMM + melatonin + TE group showed significantly greater protective effects on cartilage and reduced subchondral bone damage compared to the DMM + melatonin group. In addition, moderate TE enhanced melatonin's inhibitory effects on the expression of pyrotosis-related proteins, which are associated with OA. Furthermore, serum analysis showed a significant reduction in the levels of IL-1β and IL-18. These findings are consistent with previous studies.[24,26-28]

This study further demonstrated that TE enhances the effects of melatonin treatment, leading to a more pronounced attenuation of OA progression through the pyroptosis pathway. TXNIP plays a key role in this process, as it regulates cellular redox homeostasis and activates NLRP3, a crucial component of the pyroptosis-mediated inflammatory response.[29] Melatonin, with its potent antioxidant and anti-inflammatory properties, is known to inhibit the TXNIP/NLRP3 signaling axis, thereby suppressing pyroptosis and the associated inflammation.[30] The findings suggest that the combination of TE and melatonin treatment enhances this regulatory mechanism, resulting in a more significant reduction in OA progression compared to melatonin treatment alone. By elucidating the interplay between TXNIP, melatonin, and NLRP3 within the pyroptosis pathway, this study highlights the potential therapeutic benefits of this combined approach in mitigating OA progression.

Immunofluorescence staining showed that treatment with melatonin significantly reduced TXNIP protein expression in the DMM + melatonin group compared to the untreated DMM group. This finding is consistent with previous studies that investigated the effects of melatonin on TXNIP expression.[27,31-35] In addition, exercise notably enhanced the effect of melatonin treatment by further reducing TXNIP protein expression. This finding is consistent with previous studies conducted on different diseases.[36-38]

In addition, quantitative real-time polymerase chain reaction and Western blot analyses demonstrated that rats in the DMM + melatonin group showed significantly reduced mRNA and protein levels of pyroptosis-related proteins and TXNIP. Furthermore, combining melatonin with TE led to an even greater decrease in these markers compared to the DMM + melatonin group. These findings provide strong evidence that melatonin treatment, especially in combination with TE, effectively reduces the expression of key inflammatory and apoptotic markers in the DMM model. Overall, these findings suggest that melatonin, particularly when paired with exercise, could alleviate inflammation and pyroptosis, highlighting the potential of combined therapeutic strategies for managing inflammatory conditions.

This study has several limitations that should be considered when interpreting the findings. First, the short experimental duration limits the ability to assess the long-term effects of OA progression. Second, the small sample size may reduce the statistical power and generalizability of the results. Third, the study focused primarily on advanced OA stages, which may not fully reflect the potential benefits during earlier phases of the

disease. Fourth, functional assessments (e.g., gait analysis and weight-bearing tests) were not performed, thereby limiting our understanding of the clinical relevance of the observed molecular and structural changes. Future research should evaluate the effects of moderate TE in larger cohorts, including early OA stages, and assess its impact on cartilage-subchondral bone interactions and the pyroptosis pathway. Furthermore, investigating the long-term effects of different training durations and varied doses or delivery methods of melatonin would enhance our understanding of their therapeutic potential in OA management.

5. Conclusion

TE significantly enhances melatonin-mediated regulation of the TXNIP/NLRP3 pathway and inhibits pyroptosis-driven OA in the DMM rat model. These findings suggest that combining melatonin treatment with moderate TE may offer a promising therapeutic strategy for managing OA by effectively reducing pyroptotic cell death.

Acknowledgments

None.

Funding

None.

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Yongping Wang, Li Chaoyi
Formal analysis: Jamal Alshorman
Investigation: Yongping Wang
Methodology: Ruba Altahla
Writing – original draft: Ruba Altahla, Jamal Alshorman
Writing – review & editing: Ruba Altahla, Jamal Alshorman

Ethics approval and consent to participate

All subjects involved in this study were treated in accordance with the ethical principles outlined in the Declaration of Helsinki, and the study was approved by the ethics committee (No. 2025-M20-21).

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author on reasonable request.

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Appendix

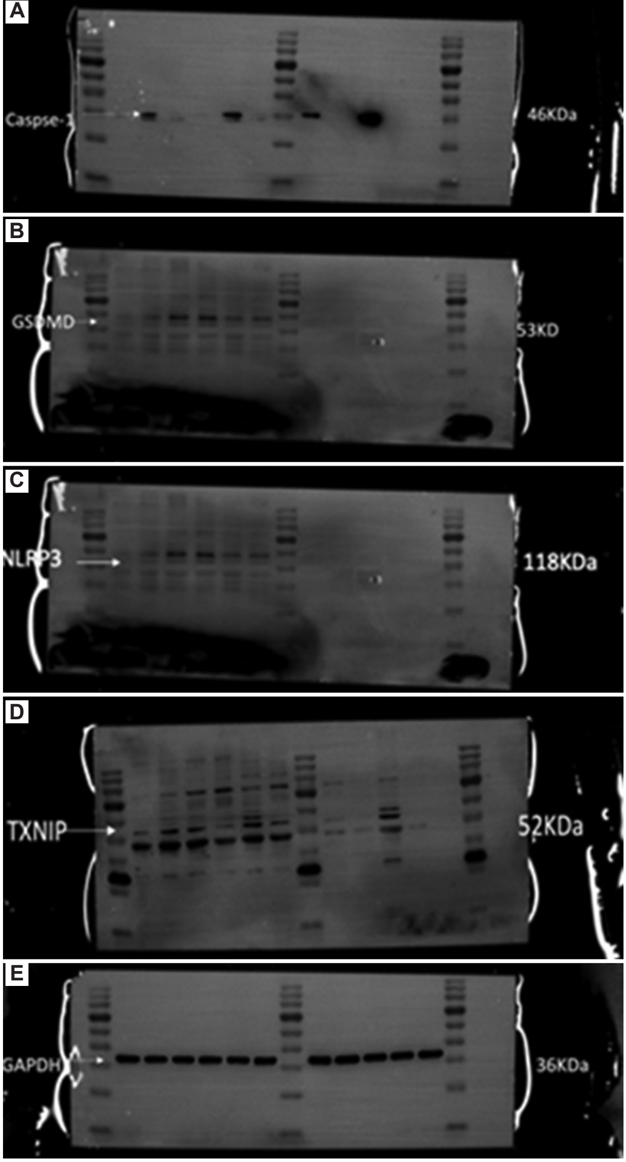

Figure A1. Original Western blot images of (A) caspase-1, (B) GSDMD, (C) NLRP3, (D) TXNIP, and (E) GAPDH in rat cartilage
Abbreviations: GSDMD: Gasdermin D; NLRP3: Nucleotide-binding and oligomerization domain-like receptor protein 3; TXNIP: Thioredoxin-interacting protein; GAPDH: Glyeceraldehyde-3-phosphate dehydrogenase.

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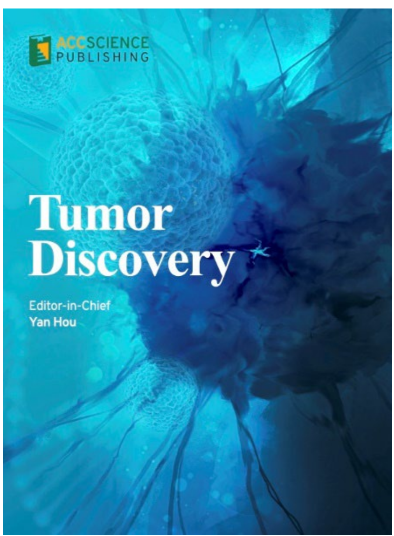

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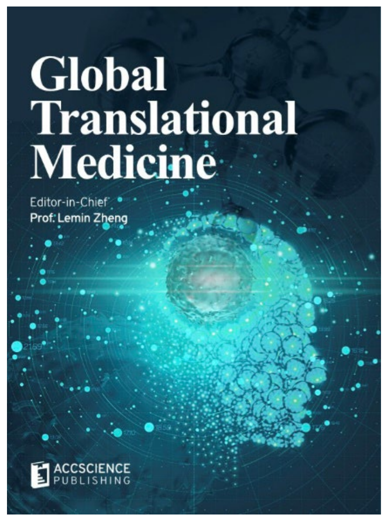

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