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Review Article

Simple Sequence Repeat Marker as a Guided Genetic Approach to Increase Cardiovascular Health-Promoting Withanolides from *Withania somnifera* (L.) Dunal: A Review

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Abstract

Withania somnifera (L.) Dunal is a globally important medicinal plant of the Solanaceae family, primarily valued for its roots, which are rich in bioactive secondary metabolites, particularly withanolides. In Ayurveda, *W. somnifera* is widely recognized for its cardioprotective properties and additional pharmacological activities, including antioxidant and anti-inflammatory effects. However, wild populations are rapidly declining due to overharvesting, habitat loss, low seed germination, poor natural regeneration, and genetic erosion. Considerable genetic variability in withanolide content further increases the vulnerability of natural populations to environmental stress. Recent advances in molecular genetics, especially the use of simple sequence repeat (SSR) markers, enable high-resolution assessment of genetic diversity and identification of allelic variants associated with withanolide biosynthesis. To address poor germination and weak regeneration, emerging approaches such as nano-priming have demonstrated potential for enhancing seed germination and seedling vigor. Additionally, breeding superior genotypes offers opportunities to improve phytochemical productivity. This review integrates current genetic and biotechnological strategies, emphasizing SSR marker applications linked to withanolide biosynthetic pathways and their relevance to cardiovascular health. It also identifies existing knowledge gaps and highlights the translational potential of molecular breeding for sustainable phytopharmaceutical development of *W. somnifera*.

Key Words: Breeding; Cardio protection; Genotypes; Genetic Markers; Therapeutics

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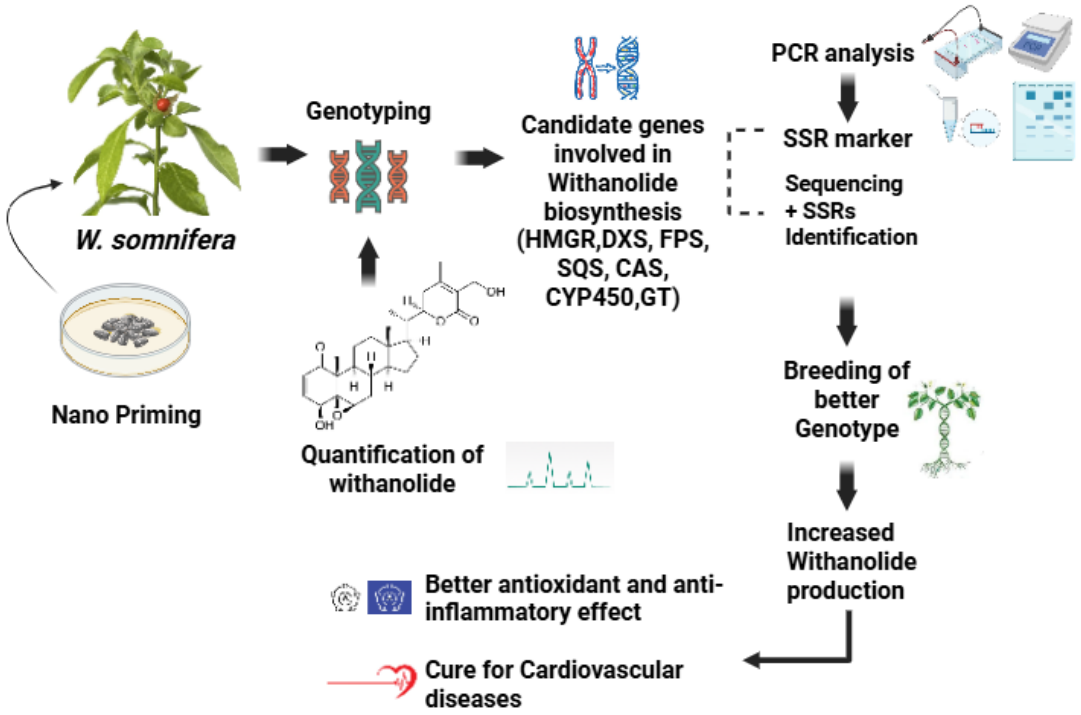
Graphical Abstract



1. Introduction

Plant biodiversity is declining worldwide at an alarming rate due to habitat loss, overexploitation, climate change, and unsustainable agricultural practices. This decline not only disrupts ecosystems but also threatens valuable plant species that provide essential medicines and bioactive compounds for human health. Among medicinal plants, natural populations of *Withania somnifera* are decreasing rapidly, reducing the availability of high-quality raw material and key bioactive compounds, particularly withanolides, which are responsible for its therapeutic properties, including anti-inflammatory, antioxidant, immunomodulatory, and cardioprotective effects (Sumaira *et al*., 2017).

W. somnifera, commonly known as Ashwagandha, Indian ginseng, or winter cherry, is a member of the Solanaceae family. It is widely distributed across Africa, Asia, Australia, and Europe. It has been used for over 3,000 years in Ayurvedic and Unani medicine to promote stress relief, fertility, vitality, and overall well-being attributed to its tonic, anti-inflammatory, vasodilatory, and nervous system-supporting properties (Poojari *et al*., 2019). The plant contains approximately 130 bioactive constituents (Afewerky *et al*., 2021), with withanolides being abundant in roots and leaves. Withanolides are steroidal lactones biosynthesized through the mevalonate pathway. Their steroidal backbone and characteristic unsaturated lactone ring are critical for bioactivity and are associated with diverse pharmacological activities, including antioxidant, anti-inflammatory, and cardioprotective effects (Saleem *et al*., 2020; Bashir *et al*., 2023).

Against this pharmacological backdrop, cardiovascular diseases (CVDs) remain the leading cause of mortality and morbidity worldwide, including coronary artery disease, hypertension, stroke, and heart failure. Their development is strongly linked to oxidative stress, endothelial dysfunction, chronic inflammation, and metabolic imbalances, which collectively drive disease progression (Donia and Khamis, 2021). Globally, over 523 million people are affected by CVDs, with nearly 18 million deaths annually, emphasizing the urgent need for effective preventive and therapeutic strategies (WHO, 2025). Although conventional pharmacological treatments exist, their long-term use is

often limited by side effects, high costs, and variable efficacy, prompting growing interest in plant-based alternatives. In this context, *W. somnifera*, rich in bioactive withanolides, has shown promising cardiovascular benefits by reducing oxidative stress, improving endothelial function, modulating lipid metabolism, and suppressing inflammatory pathways implicated in CVD progression (Singh *et al*., 2022; Nisar *et al*., 2018; Alaoui Mdarhri *et al*., 2022).

The demonstrated cardiovascular benefits of *W. somnifera* highlight the importance of sustainable cultivation and the reliable production of its bioactive withanolides, which can be achieved through advanced biotechnological approaches. One such promising strategy is nano-priming, an advanced seed treatment approach in which seeds are exposed to nanoparticles (NPs) prior to germination to enhance their physiological and biochemical performance. This technique has been shown to improve germination, stress tolerance, biomass production, and the accumulation of valuable compounds such as withanolides, making it a valuable tool for optimizing the cultivation and productivity of *W. somnifera* (Nile *et al*., 2022). In addition, genetic variability among *W. somnifera* genotypes significantly influences withanolide biosynthesis, making genotype-specific analysis essential (Singh *et al*., 2024). Several genetic and functional markers are available for assessing plant genetic diversity; however, simple sequence repeat (SSR) markers are considered highly reproducible and reliable. SSR markers consist of short, tandemly repeated DNA sequences which are widely used as molecular tools for evaluating genetic diversity to identify elite genotypes, and to associate genetic variation with metabolite production. Expressed sequence tag (EST)-derived SSRs, in particular, exhibit high polymorphism and cross-species transferability, enabling precise and robust genotype characterization (Ahmad *et al*., 2018; Karaca *et al*., 2013). The integration of nano-priming and SSR marker-assisted selection promotes robust, resilient plant growth while optimizing desirable genetic traits, thereby enabling the sustainable production of high-quality, consistent cardioprotective withanolides in *W. somnifera*.

Despite these advances, significant research gaps remain. Few studies integrate SSR marker analysis with functional gene validation (Khabiya *et al*., 2024; Chawla *et al*., 2025), cardiovascular bioassays (Suganya *et al*., 2020; Al-Eisa, 2025), broad germplasm screening, or multi-omics approaches, and the influence of environmental factors on marker trait associations is largely unexplored (Singh *et al*., 2024). Therefore, this review synthesizes current knowledge on the genetic improvement of *W. somnifera* using SSR markers, with a focus on enhancing withanolide content relevant to cardiovascular health, while highlighting research gaps and future directions to support the development of improved varieties with consistent therapeutic efficacy.

2. General Information of *W. somnifera*

2.1: Description and Morphology of *W. somnifera*

The origin of the genus *Withania* is uncertain, while the species epithet *somnifera* refers to the narcotic properties of its leaves. It is an erect, evergreen, densely branched, tomentose shrub reaching 30–150 cm in height. Leaves are simple, petiolate, elliptic-ovate to broadly ovate, 4–10 cm long and 2–7 cm wide, with entire margins, acute to obtuse apices, oblique bases, and both surfaces covered with persistent grayish tomentum. Vegetative shoots bear large, alternate leaves, whereas floral branches have opposite leaves arranged in lateral pairs, each axil supporting a cymose cluster of 4–25 small, pale green, monoecious flowers (Fig. 1). Flowering occurs year-round, peaking from March to July in the Northern hemisphere (Dutta *et al*., 2025).

2.2: Phytochemistry of *W. somnifera*: Terpenoids and Other Bioactive Compounds

W. somnifera is a rich source of over 80 bioactive phytochemicals, particularly steroidal lactones known as withanolides, being the most pharmacologically significant. Key

Figure 1: Morphological features of *W. somnifera*: (a) Twig, (b) Flower, (c) Ripped Fruit, and (d) Roots (Pandian *et al*., 2020)

withanolides, including withaferin A, withanolide D, withanone, and sitoindosides, contribute to the plant's adaptogenic, anti-inflammatory, antioxidant, neuroprotective, and anticancer properties. Mechanistically, withanolides modulate stress response pathways by regulating cortisol levels, inhibit pro-inflammatory mediators such as NF-κB and cytokines, scavenge reactive oxygen species, and induce apoptosis in cancer cells (Singirala *et al*., 2025) (Table 1).

Beyond terpenoids, *W. somnifera* contains a diverse array of other bioactive compounds, including alkaloids (somniferine, tropine, cuscohygrine), phenolic compounds, flavonoids, saponins, fatty acids, and sterols. These constituents enhance the plant's pharmacological potential by modulating neurotransmitter activity, strengthening antioxidant defenses, stabilizing cell membranes, and regulating genes involved in immune function and cellular repair. Collectively, the phytochemical richness of *W. somnifera* underpins its extensive therapeutic applications in both traditional and modern medicine (Khalid *et al*., 2025)

2.2.1 Withanolides: Structural Features and Diversity

Withanolides are a class of naturally occurring C28 steroidal compounds derived from an ergostane framework. In these molecules, oxidation typically occurs at C-26 and C-22 or C-26 and C-23, leading to the formation of δ- or γ-lactone rings. Additionally, oxidation at C-1 often produces a 1-one group, with over 90% of known withanolides classified as 1-oxosteroids. Structural diversity arises from modifications in both the steroidal nucleus and side chain, often accompanied by the formation of additional ring systems (White *et al*., 2016).

Withaferin A was the first withanolide isolated from *W. somnifera* by Lavie in 1965 and has since become a focus of extensive research. Although withanolide D has been less studied, it also exhibits a broad spectrum of biological activities. Withanone, another notable withanolide, is characterized by its selective cytotoxicity, targeting cancer cells while sparing normal cells (Singh *et al*., 2022). Over the past 45 years, 40 distinct withanolides have been isolated and characterized (Anjali *et al*., 2025). **Fig. 2** depicts the chemical structures of Withaferin A, Withanolide D, and Withanone.

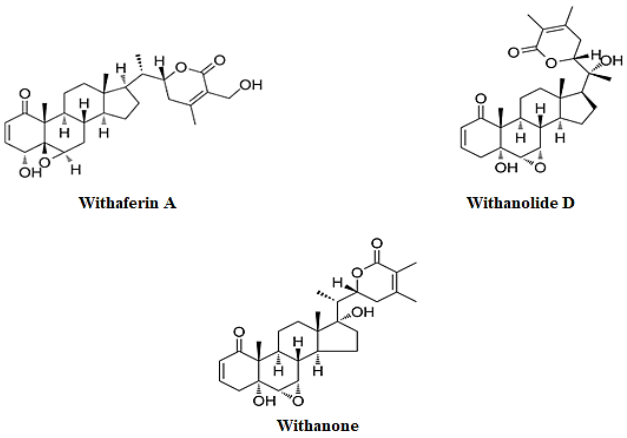


Fig. 2 Chemical structures of Withaferin A, Withanolide D, and Withanone

2.2.2 Biosynthesis pathway of Withanolides

Withanolides are steroidal lactones synthesized through complex secondary metabolic pathways, primarily originating from isoprenoid precursors via the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways **(Fig. 3)** (Ahmad *et al*., 2024).

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Table 1. Major Withanolides, their biological activities, underlying mechanisms, and potential applications.

WITHANOLIDE	MAJOR BIOLOGICAL ACTIVITIES	MECHANISMS	POTENTIAL APPLICATIONS	REFERENCES
WITHAFERIN A	• **Anticancer:** Induces apoptosis in various cancer cell lines; inhibits angiogenesis and metastasis. • **Anti-inflammatory:** suppresses TNF-α, IL-6, IL-1β; inhibits NF-κB. • **Antioxidant:** enhances glutathione, catalase. • **Neuroprotective:** reduces oxidative stress, potential in Alzheimer's & Parkinson's. • **Antimicrobial:** antibacterial and antifungal activity.	• Modulates NF-κB, p53, Akt, HSP90. • Regulates oxidative stress. • Induces apoptosis	Cancer therapy, Inflammation, neurodegenerative disorders, infections	(Lee and Choi, 2016)
WITHANOLIDE D	• **Cytotoxic & anticancer:** induces apoptosis & cell cycle arrest in leukemia and breast cancer cells; modulates mitochondrial pathways and ROS. • **Anti-inflammatory:** inhibits COX-2 and iNOS. • **Cardioprotective & hepatoprotective:** protects liver and heart from oxidative damage. • **Immunomodulatory:** activates macrophages and NK cells.	• Mitochondrial modulation, ROS regulation. • Anti-inflammatory signaling.	Cancer therapy, immune support, cardioprotection, hepatoprotection	(Behl *et al*., 2020)
WITHANONE	• **Anticancer:** selectively induces oxidative stress and DNA damage in cancer cells; downregulates oncogenes, upregulates tumor suppressors (p53); inhibits metastasis. • **Antioxidant & anti-stress:** protects normal cells from oxidative/genotoxic stress. • **Neuroprotective:** maintains neuronal integrity under oxidative stress. • **Radioprotective:** protects normal cells from radiation-induced DNA damage.	• Selective cytotoxicity against cancer cells. • Modulates signaling pathways and stress responses.	Targeted cancer therapy, neuroprotection, radioprotection, stress management	(Alenazi *et al*., 2024)

TNF-α: Tumor Necrosis Factor-alpha; IL-6: Interleukin-6; IL-1β: Interleukin-1 beta; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; p53: Tumor Protein 53; Akt: Protein Kinase B (PKB); HSP90: Heat Shock Protein 90; **ROS: Reactive Oxygen Species; COX-2: Cyclooxygenase-2; iNOS: Inducible Nitric Oxide Synthase**

The biosynthetic process begins with the formation of key intermediates such as isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are subsequently converted into squalene by squalene synthase (SQS). Squalene is then

cyclized to cycloartenol by cycloartenol synthase, forming the steroidal backbone. This backbone undergoes multiple oxidations and hydroxylations mediated by cytochrome P450 monooxygenases (CYP450s), followed by glycosylation reactions catalyzed by UDP-glycosyltransferases (UGTs) to yield a diverse array of biologically active withanolides (Figure. 3) (Narayanan and Nagegowda, 2024).

Despite the characterization of several key enzymatic steps, many regulatory genes and intermediate reactions in the pathway remain poorly understood, which limits the precise manipulation of withanolide production (Poojari *et al*., 2019). Understanding the molecular regulation of this pathway, including the identification of transcription factors, signaling molecules, and environmental cues that influence enzyme activity can provide strategies to enhance or redirect metabolite flux toward desired withanolides (Sangwan, 2013).

Genes encoding critical enzymes in the steroidal pathway, such as Geranyl Pyrophosphate Synthase (GPPS), 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase (HMGR), 1-Deoxy-D-Xylulose-5-Phosphate Synthase (DXS), Farnesyl Pyrophosphate Synthase (FPPS), Squalene Synthase (SQS), Squalene Epoxidase (SQE), CYP450s, and UGTs, represent potential loci of genetic variation that influence both the yield and composition of withanolides (Shilpashree *et al*., 2022). Polymorphisms in these genes or their regulatory regions, detectable through SSR markers, facilitate genotype metabolite association studies. Genotypes with favorable SSR profiles are more likely to accumulate higher levels of cardioprotective withanolides, making them particularly valuable for targeted breeding programs and pharmaceutical applications (Ramesh *et al*., 2020). These goals can be achieved through targeted enhancement strategies. Molecular breeding approaches, including marker-assisted selection (MAS) or genome-editing techniques such as CRISPR/Cas9, can be used to develop plant varieties with higher withanolide content by focusing on genes that directly regulate the biosynthetic pathway. Additionally, metabolic engineering can modify enzymes or regulatory networks within the biosynthetic pathway, engineer microbial hosts such as yeast or bacteria for *In vitro* production, and enhance overall yield by overexpressing rate-limiting enzymes or silencing competing pathways (Ahmad, 2023)

2.3: Necessity to improve *W. somnifera* Cultivation

The cultivation of *W. somnifera* is often constrained by poor seed germination, low seedling vigor, uneven plant establishment, and susceptibility to abiotic stresses, including drought, salinity, and nutrient limitations dependent on soil capabilities. These challenges reduce biomass and secondary metabolite production, particularly the therapeutically important withanolides, limiting both the medicinal potential and commercial value of the crop. To overcome these constraints, advanced biotechnological approaches are required to improve both plant growth and phytochemical accumulation (Danish *et al*., 2024).

One such promising technique of seed conditioning is nano-priming, which can enhance germination, stimulate seedling vigor, and increase stress tolerance, thereby facilitating higher biomass and improved withanolide content. Coupling such techniques with molecular marker-assisted selection or breeding can further optimize cultivar performance. Nano-priming has emerged as a modern and efficient seed treatment approach because it not only improves early plant establishment but also helps plants tolerate environmental stresses that are becoming increasingly prevalent. Nanoparticles facilitate targeted delivery of nutrients and growth regulators at the nanoscale, enhancing physiological and biochemical responses during germination and

early seedling development (Kowsalya *et al*., 2024).

2.4 Nano-priming as a Seed Treatment Strategy

Nano-priming involves pre-sowing treatment of seeds with engineered nanoparticles that can penetrate the seed coat and influence early metabolic pathways. This emerging technique enhances water uptake, activates key enzymes, regulates phytohormonal signaling, and strengthens antioxidant defense systems, thereby promoting faster and more uniform germination, improved seedling vigor, and potentially higher secondary metabolite production. When applied to *W. somnifera*, nano-priming improves seedling establishment and may enhance withanolide biosynthesis at early developmental stages (Mahakham *et al*., 2017; Abed *et al*., 2025). This approach is particularly valuable because withanolides, especially withaferin A and related compounds, exhibit anti-inflammatory, antioxidant, and endothelial-protective effects that contribute to cardioprotective benefits. Therefore, enhancing the yield of withanolide through nano-priming may directly support the development of more effective cardiometabolic phytopharmaceuticals from *W. somnifera*. Implementing this strategy requires careful selection and safety testing of nanoparticles, along with phytochemical profiling across developmental stages to ensure that improvements in germination and growth translate into reproducible increases in bioactive withanolides relevant to cardiovascular outcomes (Gupta *et al*., 2015; Wankhede *et al*., 2021). .

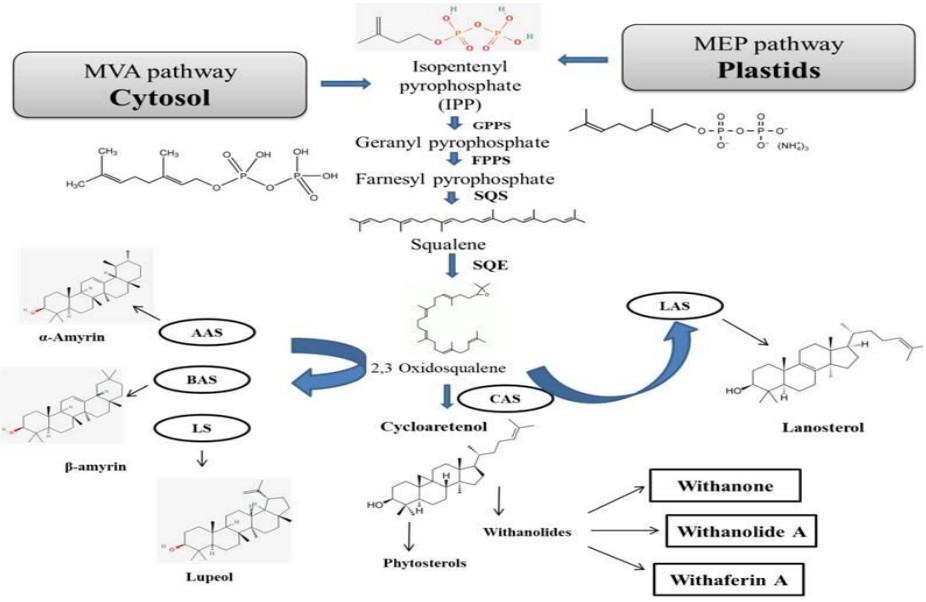

Fig. 3 Biosynthesis pathway of Withanolides: IPP-Isopentenyl Pyrophosphate; GPPS-Geranyl Pyrophosphate Synthase; FPPS-Farnesyl Pyrophosphate Synthase; SQS-Squalene Synthase; SQE-Squalene Epoxidase; CAS-Cycloartenol Synthase; LAS-Lanosterol Synthase; BAS-β-Amyrin Synthase; AAS-α-Amyrin Synthase; LS-Lupeol Synthase

Shami *et al.* (2024), reviewed the use of *W. somnifera* in the green synthesis of nanoparticles, emphasizing that plant-derived nanoparticles can enhance the bioavailability, solubility, and stability of bioactive compounds, thereby highlighting the potential of nanotechnology to optimize *W. somnifera* formulations. Chauriya *et al*. (2024) demonstrated that a nanosuspension prepared from *W. somnifera* root extract (particle size ≈ 133 nm) offers a promising strategy to enhance the ther-

apeutic efficacy of its hydrophobic constituents, supporting the integration of nanotechnology in herbal product development. Abed *et al.* (2025) showed that treatment of *Withania* callus cultures with silver nanoparticles (Ag-NPs) significantly increased the accumulation of several withanolide compounds (withastramonolide, withanolide-A), suggesting that nanoparticle-mediated elicitation can enhance secondary metabolite yield in *W. somnifera*

3:The Role of Genetic Markers in Enhancing Plant Diversity

Genetic markers are specific DNA sequences that serve as molecular tools to detect polymorphisms, study genetic diversity, and assist in breeding and conservation programs. They can be morphological, biochemical, or molecular, with molecular markers being the most precise and widely used due to their reproducibility and genome-wide coverage. Common molecular markers include RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), ISSR (Inter-Simple Sequence Repeat), SSR (Simple Sequence Repeat), and SNP (Single Nucleotide Polymorphism) (Ramesh *et al*., 2020).

Among these, SSRs are highly polymorphic, co-dominant, abundant across the genome, and easily detected using PCR, making them particularly effective for assessing genetic variation, constructing linkage maps, and performing marker-assisted selection (Sinha *et al*., 2025). In *W. somnifera,* SSR markers are widely used because of their high polymorphism, co-dominant inheritance, and genome-wide abundance. These features allow SSRs to reveal population structure, identify elite genotypes, and correlate genetic polymorphisms with the biosynthesis of bioactive compounds, such as withanolides. SSRs can also be identified directly from genome or transcriptome sequencing data, enabling species-specific marker development and accelerating studies on genetic diversity, functional genomics, and pharmacologically important traits (Rahayu *et al*., 2025).

3.1: Simple Sequence Repeats: Genetic and Functional Studies and Their Classification in Withanolide Biosynthesis

SSRs, also called microsatellites, are short tandem repeats of 1–6 nucleotides distributed throughout the genome. Their high mutation rate makes them highly polymorphic even among individuals of the same species, making them excellent markers for studying genetic diversity, population structure, and evolutionary relationships. SSRs exhibit co-dominant inheritance, allowing researchers to distinguish homozygous and heterozygous alleles, and they are highly reproducible and easily detected using PCR-based techniques (Kiritbhai, 2019).

These characteristics make SSRs particularly useful for constructing genetic linkage maps, identifying allelic variations at specific loci, and associating genomic regions with phenotypic traits such as metabolite production, yield, or disease resistance (Ahmed *et al*., 2022; Bhattarai *et al*., 2021). In *W. somnifera*, SSR markers have been extensively used to reveal genetic polymorphisms, identify elite genotypes, and link allelic variation with the biosynthesis of bioactive compounds, such as withanolides. Chawla *et al*. (2025) reported that SSR, EST-SSR, and ISSR markers can successfully differentiate up to 80 genotypes, thereby facilitating the identification of genetically diverse and superior lines for breeding programs. Functional SSR markers associated with sterol Δ-7 reductase genes (WsDWF5-1, WsDWF5-2) have aided the mapping of withanolide biosynthetic pathways, supporting metabolic enhancement and pathway-focused breeding strategies (Agarwal *et al*., 2017). SSR-based genotyping also allows correlation of genetic polymorphism with chemotype variation in metabolites such as withaferin A, facilitating the selection of high-yielding chemotypes (Mirjalili *et al*., 2009; Tetali, 2020). SSR markers can be identified from genome or transcriptome sequences without relying on pre-existing libraries, which accelerates genotyping, functional analysis, and pharmacogenomic research (Singh *et al*., 2017; Song *et al*., 2021).

Simple Sequence Repeats (SSRs) are classified based on the length of their repeat units mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide repeats (Table 2) (Parmar *et al*., 2025). Additional classification criteria include the number of repeat copies and their distribution in coding or non-coding regions. Understanding these classifications allows researchers to identify genetic variations associated with genes involved in withanolide biosynthesis, supporting the development of high-withanolide-producing genotypes with therapeutic potential. SSRs are classified based on repeat unit length (Parmar *et al*., 2025).

Table 2. Classification of SSRs and unit lengths

Repeat Type	***Unit Length (bp)***	***Example***	***Notes***
Di-nucleotide	2	(AT)n	Highly polymorphic
Tri-nucleotide	3	(GTA)n	Frequently found in coding regions; may affect protein function
Tetra-nucleotide	4	(AGCT)n	Less frequent, useful for molecular marker development

3.2: Development and Characterization of SSRs in *W. somnifera*

In *W. somnifera,* both genomic SSRs and EST-SSRs have been developed. EST-SSRs located in expressed or regulatory regions help link genetic variation with metabolic traits, including withanolide production (Parmar *et al*., 2025). Chawla *et al.* (2025) reported that EST-SSRs derived from transcriptome sequences of *W. somnifera* help identify functional allelic variations linked to metabolic pathway genes, particularly those associated with withanolide production. Bindu *et al.* (2014) demonstrated that genomic SSR markers can distinguish high versus low withanolide-yielding chemotypes, showing their usefulness in chemotype classification and selection of elite accessions. Singh *et al.* (2019) found that functional SSR markers located near triterpenoid biosynthesis genes offer greater predictive value for withanolide content than random SSRs, highlighting their application in functional genomics. Kulkarni *et al.* (2012) found that dinucleotide SSRs (especially AG/CT repeats) show higher polymorphism levels in *W. somnifera* populations, making them suitable for genetic diversity and trait linkage analysis. Fatima *et al.* (2018) suggested that EST-SSR markers targeting genes involved in the MVA and MEP pathways can support pathway mapping of withanolide biosynthesis under stress conditions like salinity and elicitor treatment. Patil *et al.* (2016) reported the successful use of tri-nucleotide SSRs in differentiating tissue-specific expression profiles, especially between leaf and root tissues, where withanolide biosynthesis differs. Girish *et al*. (2013) showed that SSR marker analysis combined with metabolic profiling helps correlate genetic

variability with differential accumulation of withaferin A and other withanolides.

3.3: Genetic Diversity, Population Structure, and SSR-Based Insights in *W. somnifera*

In the literature, SSR analyses have revealed substantial genetic diversity among *W. somnifera* accessions originating from different geographical regions. This genetic variation is closely associated with differences in withanolide content, supporting the presence of distinct chemotypes within the species (Venugopal *et al*., 2024). Such diversity plays a critical role in understanding population structure and provides valuable insights for conservation, breeding, and pharmaceutical utilization.

Several studies have demonstrated the effectiveness of molecular markers in linking genetic variability with metabolite profiles. Mirjalili *et al*. (2009) investigated Iranian natural populations of *W. somnifera* and *W. coagulans* using RAPD markers in combination with HPTLC (High-Performance Thin-Layer Chromatography) analysis, establishing a clear relationship between genetic diversity and withaferin A content. Similarly, Gopinath *et al*. (2024) evaluated 50 Indian *W. somnifera* genotypes and reported significant genetic variability in root- and seed-related traits, identifying WS-2 as a superior germplasm with high withaferin A content suitable for targeted breeding programs. Chawla *et al.* (2025) further analyzed genetic diversity and population structure using EST-SSR, ISSR, and genomic SSR markers, highlighting their importance in enhancing the agricultural and industrial value of *W. somnifera.*

Notably, several SSR markers have been found in close proximity to key biosynthetic genes involved in withanolide production, including 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR), 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), and Cycloartenol synthase (CAS). Associations between specific SSR genotypes and withanolide levels suggest that these markers may serve as valuable tools for regulating biosynthetic pathways and for marker-assisted selection of high-yielding genotypes (Parmar *et al*., 2025).

SSR identification in *W. somnifera* has been achieved using diverse sequence resources, including expressed sequence tags (ESTs), transcriptomes, and whole-genome sequences. EST-derived SSRs are particularly useful as they are located within expressed genes, including those involved in secondary metabolite biosynthesis. Transcriptome (RNA-Seq) data allows the discovery of SSRs in actively expressed metabolic pathways, facilitating the identification of functional markers linked to bioactive compound synthesis. Whole-genome sequencing provides a comprehensive overview of SSR distribution across the genome, enabling robust genetic analyses and marker development. Bioinformatics tools, such as MIcroSAtellite identification tool (MISA), Genome-wide Microsatellite Analyzing Tool Package (GMATA), and Simple Sequence Repeat Locator (SSR Locator), are widely employed for SSR detection and primer design, supporting experimental validation and downstream applications (Chen *et al*., 2015).

3.4: SSRs in Key Withanolide Biosynthesis Genes

SSRs associated with biosynthetic genes can affect withanolide levels and, indirectly, cardioprotective activity. Representative SSR markers associated with key withanolide biosynthetic pathway genes in *W. somnifera* are summarized in **Table 3**.

4: In Silico Functional Annotation and Therapeutic Potential of *W. somnifera* Genes and Metabolites

In silico approaches have become indispensable for elucidating the functional roles of genes, regulatory elements, and bioactive metabolites of *W. somnifera,* en-

abling the prediction of therapeutic potential and the prioritization of candidates for experimental validation. These computational strategies integrate sequence mining, functional annotation, structural biology, and molecular docking to provide a comprehensive understanding of genotype–phenotype relationships (Majumder and Bhattacharya, 2025).

Table 3. Classification of SSRs and their association with key withanolide biosynthetic genes in *W. somnifera*, highlighting potential implications for cardioprotective activity

Gene	Function	Cardioprotective Reference	Reference
WsSQS	Catalyzes squalene formation	Higher enzyme expression may increase Withanolides with antioxidant activity	(Patel *et al*., 2015)
WsSQE	Converts squalene to oxidosqualene	Influences downstream production of bioactive compounds that protect cardiac cells from oxidative stress	(Senbagalakshmi *et al*., 2019)
WsCYP85A	Hydroxylation/cyclization	Structural modifications of withanolides can enhance cardioprotective potency	(Shilpashree *et al*., 2022)
WsGT	Sugar addition to withanolides	Glycosylated withanolides show improved solubility and bioavailability, enhancing cardiac effects	(Anjali *et al*., 2025)

Key: WsSQS: Squalene Synthase; WsSQE: Squalene Epoxidase; WsCYP85A: Cytochrome P450; WsGT: Glycosyltransferase

In silico approaches have become indispensable for elucidating the functional roles of genes, regulatory elements, and bioactive metabolites of *W. somnifera*, enabling the prediction of therapeutic potential and the prioritization of candidates for experimental validation. These computational strategies integrate sequence mining, functional annotation, structural biology, and molecular docking to provide a comprehensive understanding of genotype–phenotype relationships (Majumder and Bhattacharya, 2025).

4.1: SSR Identification and Functional Annotation Workflow

The in silico workflow for SSR discovery in *W. somnifera* begins with the acquisition of genomic, transcriptomics, or expressed sequence tag (EST) datasets. SSR motifs are identified using bioinformatics tools such as MISA, Genome-wide Microsatellite Analyzing Tool Package (GMATA), and Simple Sequence Repeat Locator (SSR Locator) (Moosavi *et al*., 2025), which employ repeat-search algorithms to detect microsatellite regions across sequence datasets. Identified SSRs are subsequently mapped to genes involved in the withanolide biosynthetic pathway to establish gene–marker associations. Functional annotation is performed using Gene Ontology (GO) classification and pathway databases to link SSR-associated genes with metabolic and cardioprotective functions. Primer design for selected SSR loci enables downstream experimental validation and polymorphism assessment across diverse genotypes, facilitating the identification of accessions with enhanced cardioprotective potential (Parmar *et al*., 2015).

4.2 Transcription Factor and Gene Family Characterization

Several studies have focused on the in silico characterization of regulatory genes that govern secondary metabolite biosynthesis in *W. somnifera*. Sharma *et al*. (2020) performed structural and functional annotation of the WsMYB44 transcription factor, revealing its potential role in stress response and secondary metabolism. Similarly, Tripathi *et al*. (2024) conducted genome-wide mining and functional analysis of the AP2/ERF transcription factor family, highlighting their involvement in plant development, stress tolerance, and regulation of withanolide biosynthesis. These transcription factor family studies provide insight into regulatory networks that can be exploited for metabolic engineering and yield improvement.

4.3: Molecular Docking and Therapeutic Screening of Phytochemicals

Molecular docking is an in silico approach used to predict the binding affinity and interaction patterns between bioactive compounds and target proteins, providing rapid insights into their potential biological activity. It is an essential preliminary screening tool in drug discovery, as it enables the efficient prioritization of promising compounds for experimental validation while reducing time, cost, and resource requirements (Elbouzidi *et al*., 2024).

In silico molecular docking and molecular dynamics simulation studies have therefore been widely employed to assess the therapeutic potential of *W. somnifera* phytochemicals. Ali *et al.* (2023) investigated natural compounds from *W. somnifera* against bovine NLRP9, demonstrating stable ligand–protein interactions and suggesting anti-inflammatory potential. Jahagirdar *et al.* (2024) evaluated the anticancer activity of phytochemicals using molecular docking, molecular dynamics simulations, and ADME-T profiling, reporting that Withaferin A and Viscosalactone B interact effectively with the NQO1 protein and exhibit favorable oral bioavailability. Furthermore, Alam *et al.* (2024) identified potential GABA-A receptor agonists from *W. somnifera*, showing that hygrine, tropine, and withasomnine possess suitable drug-likeness, low predicted toxicity, stable receptor–ligand interactions, and promising pharmacokinetic profiles, indicating their potential as lead compounds for the treatment of insomnia.

5. Integrating SSR Markers with Metabolomic and Pharmacological Profiles in *W. somnifera*

Integrating SSR markers with metabolomic and pharmacological datasets provides a powerful framework for linking genetic variation with bioactive compound accumulation and therapeutic efficacy. This integrative strategy is particularly relevant for medicinal plants such as *W. somnifera*, in which secondary metabolites, especially withanolides, are responsible for the majority of pharmacological activities. By combining genotyping data with metabolite profiling and biological activity measurements, researchers can advance precision phytopharmacology and the identification of elite genotypes with superior therapeutic potential (Tripathi *et al.*, 2018).

SSR allelic variation serves as an indicator of genetic diversity, population structure, and genotype trait associations. In *W. somnifera*, withanolide content the primary determinant of medicinal value, can be quantitatively assessed using High-Performance Liquid Chromatography (HPLC) or Liquid Chromatography–Mass Spectrometry (LC-MS/MS). Correlating SSR allelic profiles with metabolite concentrations enables the identification of genetic loci associated with enhanced withanolide accumulation, thereby facilitating marker-assisted selection and quality improvement strategies (Kumar *et al.*, 2023). Complementing this genetic–metabolic linkage, metabolomic profiling has been emphasized as a critical tool for chemotype differentiation, quality control, and optimization of cultivation and harvesting practices in *W. somnifera* (Tetali *et al*., 2020).

Beyond metabolite quantification, SSR data can be integrated with pharmacodynamic endpoints that measure therapeutic effects such as anti-inflammatory, neuroprotective, and adaptogenic activities, typically evaluated through *In vitro* and *In vivo* assays

(Wróbel-Biedrawa and Podolak, 2024). Multivariate statistical approaches, including Principal Component Analysis (PCA), Partial Least Squares (PLS) regression, and Canonical Correlation Analysis (CCA), enable the integration of SSR genotypes with metabolomic and pharmacological datasets to identify genotypes associated with enhanced bioactivity or efficacy (Sallam *et al*., 2024). Expanding this approach, systems biology frameworks that combine genomics (SSR markers), metabolomics (withanolide profiling), transcriptomics, and pharmacological data allow the construction of network models that reveal regulatory pathways, gene–metabolite interactions, and molecular mechanisms underlying therapeutic effects (Pinu *et al*., 2019).

This integrated precision phytopharmacology framework supports the development of standardized, efficacious herbal formulations by enabling the selection of plant lines with optimized metabolite profiles and therapeutic outcomes. Furthermore, it facilitates the identification of biomarkers for efficacy, prediction of bioactivity across genotypes, and targeted breeding or cultivation strategies for high-value medicinal plants, including *W. somnifera* (Latif and Nawaz, 2025).

6. Cardiovascular Diseases: Global Burden, Mechanisms, and Phytopharmacological Interventions

Cardiovascular diseases (CVDs) remain the leading cause of morbidity and mortality worldwide, accounting for approximately one-third of all global deaths. Major CVDs include coronary artery disease, hypertension, stroke, heart failure, and atherosclerosis, all of which arise from complex interactions between genetic predisposition, environmental factors, and lifestyle choices. Key risk factors include hypertension, dyslipidemia, obesity, diabetes mellitus, smoking, physical inactivity, oxidative stress, and chronic inflammation. Rapid urbanization, dietary transitions, and sedentary lifestyles have further accelerated the global prevalence of cardiovascular disorders, particularly in low- and middle-income countries (Jagannathan *et al*., 2019).

Over the past two decades, the burden of CVDs in Pakistan has risen dramatically, mirroring global trends. These diseases are now a major contributor to premature mortality, affecting both urban and rural populations. Key risk factors include unhealthy dietary patterns, sedentary lifestyles, high prevalence of hypertension and diabetes, tobacco use, and limited access to preventive healthcare. Additionally, genetic predisposition combined with environmental stressors further amplifies susceptibility, underscoring the urgent need for affordable, effective, and culturally appropriate strategies for prevention and treatment (Siddiqi *et al*., 2026).

6.1: Molecular and Cellular Mechanisms Underlying Cardiovascular Disease

The pathogenesis of cardiovascular diseases involves multiple interconnected mechanisms (Frąk *et al*., 2022). Oxidative stress plays a central role by promoting lipid peroxidation, endothelial dysfunction, and mitochondrial damage. Excessive production of reactive oxygen species (ROS) reduces nitric oxide (NO) bioavailability, impairing vasodilation and promoting vascular stiffness (Higashi, 2025). Chronic inflammation further contributes to disease progression by activating of inflammatory pathways such as nuclear factor-kB (NF-κB), leading to the release of pro-inflammatory cytokines (TNF-α, IL-6) and adhesion molecules that promote atherosclerotic plaque formation (Valaitienė and Laučytė-Cibulskienė, 2024) (Table 4).

Additionally, dysregulation of lipid metabolism results in low-density lipoprotein (LDL) oxidation and foam cell formation, key events in atherogenesis. Apoptosis of cardiomyocytes and endothelial cells further weakens cardiac structure and function, while persistent metabolic imbalance aggravates vascular damage. Given the multifactorial nature of CVDs, therapeutic strategies that simultaneously target oxidative stress, inflammation, lipid peroxidation, and endothelial dysfunction are particularly desirable (Roy, 2025).

6.2 Pharmacological Relevance of Withanolide Biosynthesis in Cardio-protection

Natural products have gained increasing attention as complementary and alternative therapies for cardiovascular health. *W. somnifera* is a prominent medicinal plant known for its cardioprotective potential, primarily attributed to its bioactive withanolides. These compounds exert cardiovascular benefits by enhancing nitric oxide–mediated vasodilation, reducing oxidative stress, and limiting lipid peroxidation. Withanolides also suppress NF-κB signaling and prevent LDL oxidation, thereby reducing vascular inflammation and atherosclerotic progression. Importantly, SSR markers facilitate the identification of genetic polymorphisms associated with variations in cardioprotective withanolide levels, enabling genotype-based selection of superior plant lines **(Table 4)** (Wiciński *et al.*, 2024).

6.3: Antioxidant and Anti-Inflammatory Roles

Withanolides strengthen endogenous antioxidant defense systems by enhancing the activity of enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Logie and Vanden Berghe, 2020). They also downregulate inflammatory mediators, including TNF-α, IL-6, and cyclooxygenase-2 (COX-2) (Soh and Ong, 2022), thereby mitigating chronic vascular inflammation. Genetic variation in regulatory and biosynthetic genes, including cytochrome P450 (CYP450) enzymes, influences withanolide biosynthesis and consequently modulates their overall cardioprotective efficacy (White *et al*., 2016; Shilpashree *et al.*, 2022) **(Table 4)**.6.4 Applications in Cardioprotection and Marker-Assisted Selection

Marker-assisted selection represents a powerful strategy for identifying *W. somnifera* varieties enriched in cardioprotective metabolites. SSR-based genetic diversity studies enable the selection of genotypes exhibiting superior antioxidant and anti-inflammatory properties, which are critical determinants of cardiovascular health. Functional genomics approaches further elucidate the genetic regulation of secondary metabolite pathways involved in cardioprotection. In addition, metabolic engineering strategies targeting SSR-associated genes offer opportunities to enhance withanolide production, supporting the development of standardized formulations with improved cardioprotective potential (Niazian, 2019).

6.5:Synergistic Effects and Pharmacogenomics Perspectives

SSR markers reveal substantial genetic variation linked to differences in withanolide accumulation and pharmacological activity, supporting the development of consistent, genotype-specific herbal formulations. Tripathi *et al.* (2021) demonstrated that SSR-based genotyping uncovered allelic variation associated with differential expression of key biosynthetic genes such as squalene synthase and DWF5, leading to genotype-specific enhancement of withanolide production. Kulkarni and Patwardhan (2020) reported significant variation in anti-stress and neuroprotective effects among SSR-classified genotypes, underscoring the relationship between genetic markers and pharmacological efficacy. Further studies have shown that SSR diversity correlates with metabolomic signatures of withanolides, enabling the screening of pharmacologically superior ecotypes for targeted herbal formulations (Mishra *et al.*, 2018). Rashid *et al.* (2022) demonstrated that genotype-specific responses to elicitors such as methyl jasmonate (MeJA) and salicylic acid (SA) were associated with SSR polymorphisms near key biosynthetic genes, highlighting the predictive value of markers for metabolite induction strategies. Bharti *et al.* (2015) and Pandey *et al*. (2023) emphasized the utility of SSR-based diversity profiling for identifying chemotypes with high root yield and elevated withanolide content, which are essential for standardized Phytomedicine manufacturing. Moreover, Hussain *et al.* (2017) linked SSR genotypes with anti-inflammatory and Immunomodulatory profiles, supporting pharmacogenomic tailoring of *W. somnifera* based cardiovascular interventions.

Table 4. Major Withanolides and their cardioprotective mechanisms

Compound	Primary molecular targets	Experimental model / system	Observed cardioprotective effects	Mechanistic pathways involved	References
WITHAFERIN A	NF-κB, Nrf2, iNOS, caspase-3	Rat myocardial infarction, H9c2 cells	↓ Myocardial infarct size, ↑ antioxidant enzymes, ↓ apoptosis	Activation of Nrf2/HO^{-1}, inhibition of NF-κB-mediated inflammation	(Ali *et al*., 2023; Wang *et al*., 2024)
WITHANOLIDE A	PI3K/Akt, eNOS	Isoproterenol-induced cardiotoxicity in rats	Improved cardiac output, restored lipid profile, ↓ oxidative damage	PI3K/Akt-mediated survival signaling, NO production	(Wiciński, *et al*., 2024)
WITHANONE	MAPK, ERK1/2	H9c2 cardiomyocytes under oxidative stress	Protection against ROS-induced apoptosis	Regulation of mitochondrial membrane potential, MAPK signaling	(Yan *et al*., 2018)
12-DEOXYWITHASTRAMONOLIDE	TNF-α, COX-2	LPS-induced inflammation model	Reduced inflammatory markers and lipid peroxidation	Downregulation of pro-inflammatory cytokines	(Arulselvan *et al*., 2016)
WITHANOLIDE D	Bcl-2/Bax ratio, cytochrome c	Ischemia–reperfusion injury in rat heart	Maintains mitochondrial integrity and reduces apoptosis	Modulation of intrinsic apoptotic pathway	(Heidari *et al*., 2021)
WITHANOSIDE IV AND V	β-adrenergic signaling, Ca^{2+} homeostasis	Rat cardiomyocytes	Improved calcium handling, reduced arrhythmogenic potential	Stabilization of β-adrenergic receptors and antioxidant defense	(Ahmad, 2025)

NF-κB**:** Nuclear Factor kappa-light-chain-enhancer of activated B cells; Nrf2: Nuclear Factor Erythroid 2–Related Factor 2; iNOS: Inducible Nitric Oxide Synthase; Caspase-3: Cysteine-aspartic protease 3; PI3K/Akt: Phosphoinositide 3-Kinase / Protein Kinase B (Akt); eNOS: Endothelial Nitric Oxide Synthase; MAPK: Mitogen-Activated Protein Kinase; ERK1/2: Extracellular Signal–Regulated Kinases 1 and 2; TNF-α: Tumor Necrosis Factor-alpha; COX-2: Cyclooxygenase-2; Bcl-2/Bax ratio: B-cell lymphoma 2 / Bcl-2-associated protein ratio; LPS: Lipopolysaccharide; H9c2: Cardiomyoblast cell line; HO^{-1}: Heme Oxygenase-

7. Conclusion and Future Perspectives

This review suggests that *W. somnifera* exerts cardioprotective effects primarily through its bioactive withanolides, which act via multiple interconnected pathways. These include the enhancement of NO-mediated vasodilation, reduction of oxidative stress, inhibition of lipid peroxidation, suppression of NF-κB–mediated inflammatory signaling, and modulation of apoptotic pathways in cardiomyocytes. SSR-based genetic studies reveal significant allelic variation in genes regulating withanolide biosynthesis, indicating that polymorphisms in these loci influence both the amount and composition of withanolides produced in different genotypes. Such genetic variation directly affects metabolite accumulation patterns and, consequently, cardioprotective efficacy. Integration of SSR genotyping with metabolomic and pharmacological profiling has enabled the identification of elite genotypes characterized by higher concentrations of bioactive withanolides and favorable withanolide profiles, exhibiting superior antioxidant, anti-inflammatory, and vascular-protective properties. This integrative approach provides a clear mechanistic link between genetic variation, withanolide biosynthesis, and therapeutic potential.Although preclinical *In vitro* and *In vivo* studies have consistently demonstrated the cardioprotective properties of *W. somnifera*, clinical evaluation remains limited. Some preliminary human studies suggest benefits in reducing blood pressure, improving lipid profiles, and mitigating stress-related cardiovascular risk, but larger, well-controlled trials are needed to establish efficacy, optimal dosage, and safety in diverse populations. Therefore, while mechanistic and genetic evidence are strong, translation to clinical practice requires systematic evaluation.

Future research should focus on integrating SSR-based functional markers with advanced genome editing tools, such as CRISPR/Cas9, to enhance withanolide biosynthesis and standardize elite genotypes for therapeutic use. Combining genomics with transcriptomics, metabolomics, and proteomics can further unravel molecular networks governing cardioprotective pathways and identify novel withanolides with potential clinical relevance. Establishing genotype-specific pharmacological profiles and incorporating these into standardized herbal formulations will improve reproducibility, safety, and efficacy. Ultimately, *W. somnifera* has the potential to serve as a model system for genetically guided, natural-product–based cardioprotective drug discovery, bridging preclinical findings with evidence-based clinical applications.

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Conflicts of Interest

The authors declare no conflicts of interest.

Author Contribution

Z. S. Conceptualized the review and drafted the manuscript. U. S. and G. A. Supervised, provided guidance and reviewed the manuscript. E. A. A., S. G. C. S. and W. Z. A. critically reviewed the manuscript and helped with the structure. M. A. D. and S. M. contributed to manuscript review and editing.

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