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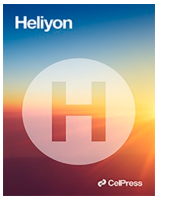

Review article

Advances in militarine: Pharmacology, synthesis, molecular regulation and regulatory mechanisms

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ABSTRACT

Militarine is the lead member of secondary metabolites found in multiple medicinal plants of the orchid family. It acts as not only an important inhibitor on plant growth, but also functions as the quality marker for medicinal materials. In addition, Militarine has been shown to possess remarkably medicinal value, with a definite potential for finding widespread adoption of treating various diseases, including lung injury, brain nerve injury, cognitive impairment, aging, tumors, inflammation, peptic ulcers, and more. Thus, it can serve as a material carrier for pharmacophore upon, so much so that it probes as natural source of lead compounds in the research and development of medication. The study reported herein makes an overview on the physicochemical properties and pharmacological mechanisms of Militarine compounds, summarizes the biogenic pathways of Militarine and organically integrates the biological characteristics of Militarine with multiple omics techniques. Besides, this review also constructs a regulatory system for the biological accumulation of Militarine around its precursor compounds, characteristic gene elements, key enzymes, important metabolic products, and critical steps and links. Exceptionally, emphasis on the biosynthesis of Militarine under both abiotic and biotic stress, as well as an elaboration of the signaling pathways and critical regulatory mechanisms that govern the metabolic flow of Militarine have been represented accordingly in this paper. These findings are expected to provide reference schemes and theoretical foundations for acquiring high-quality resources of Militarine and advancing its large-scale industrial production, drug development, and clinical applications to comprehensively elucidate the biosynthetic and metabolic pathways.

1. Introduction

Militarine is an active monomer molecule with abundant and distinctive biological characteristics, which presents in a variety of the Orchidaceae. Its hallmark feature lies in the 2-isobutylmatic acid of the chemical structures of O-glycosides and absorption maximum is shifted nearly 223 nm [1]. Not only hold its properties in plant growth bio-inhibition been defined [2], but it also possesses a rich pharmacological activities and serves as an invaluable source of pharmaceuticals that include the treatment of lung

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injuries [3], improvement in cognitive impairment [4], neuroprotection [5] and antioxidant capacity [6], among others. Militarine is proposed to be composed of saccharides and non-saccharide fractions (aglycone), materialized by condensation of hydroxyl groups in C2 epitopes of two molecules of glucose and phenolic hydroxyl in aglycone.

Militarine was originally identified in Orchidaceae in 1975 based on previous research outputs and experimental data, which initiated a new era of in the study of Militarine (Fig. 1 (A-C)). And the orchids exhibit the capacity for Militarine synthesis, as seen in species such as *Bletilla striata* (Thunb.) Reichb. f [7], *Pleione bulbocodioides* (Franch.) Rolfe [8], *Cremastra appendiculata* (D.Don) Makino, *Coeloglossum viride* var. *Bracteatum* [9] and *Gymnadenia conopsea* (L.) R. Br [10]. Despite this, Militarine has been not of adequate attention and remains in a stagnant stage of repetitive findings over lengthy periods because of low biomass and scarcity of pharmacological studies in the orchid species, while hindering research progression and industrial development of Militarine.

The foundation for the intensive study of Militarine was laid until Han discovered it in *B. striata*, which belongs to *Bletilla* genus of Orchidaceae in 2002 [11]. Since then, a growing report on the bioactivity of Militarine with substantial achievements that posed to its increasing appreciation in the past 20 years. There is a high and stable content of active Militarine in *B. striata*, which has been evidenced by scholars through UPLC fingerprints analysis, ranges between 18.98 mg/g and 44.87 mg/g, with up to 0.812–0.976 content-related similarity [7], thereby outperforming other species and genus of the Orchidaceae [1]. Militarine synthesized by *B. striata*, functions not only as an exclusive indicator, but also provides distinct advantages in terms of active fractions, which enables it a key medicinal ingredient of *B. striata*. It has determined that Militarine is the material basis for *B. striata* to exert multiple pharmacological effects, including nootropic activities and effects [4], hemostasis [12], anti-inflammatory properties [13], neuroprotection [5] and anti-ulcer effects [14], such that Militarine has gained significant attention.

However, there are no relevant reports on the systematic evaluation of Militarine, which limits its research and development. Therefore, it is important to comprehensively induce and summarize the research on the physicochemical properties, structure-activity relationships, pharmacological effects, synthetic metabolism, genetic regulation, and other aspects of Militarine. This paper focuses on detailed analysis and systematic summary of the progress made in the synthesis pathways of Militarine regarding its structure, physicochemical properties, structure-activity relationships, pharmacological actions and metabolic pathways, and genetic regulation mechanisms under adversity. And this review suggested that potential biological strategies to be explored in stimulating the accumulation of Militarine. Ultimately, comprehensive development, efficient utilization, and sustainable development, along with future biological manufacturing and industrial applications therefore will be implemented to in a thorough fashion as described above. In doing so, target for vigorous advancement of Militarine will be realized soon followed.

2. Structure-activity relationship of militarine

2.1. Chemical composition and structural features in militarine

Militarine is a bioactive product derived from various orchid plants, embodying in a unique and novel small molecule structure. It employs benzyl succinate as the mother nucleus (Fig. 2A), and thus, is also referred to as compound of glucosyloxybenzyl 2-

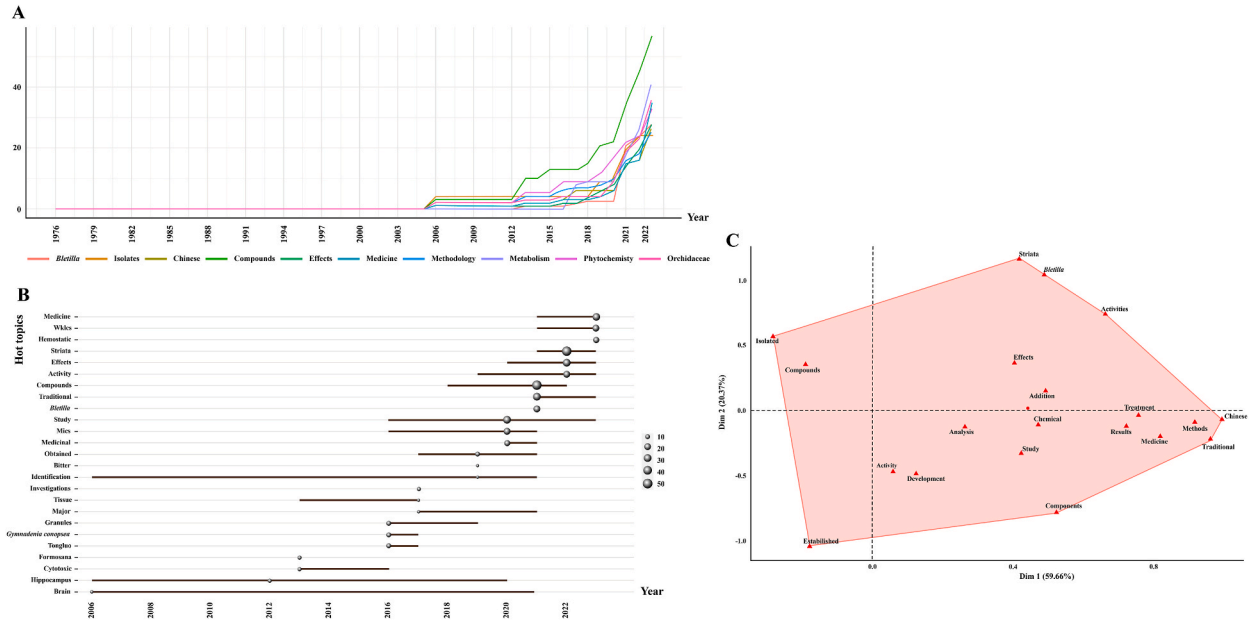

Fig. 1. The trends in the development of Militarine within 20 years. A. Frequency of evolution of Militarine research over the years. B. Trend analysis of hot topics in Militarine study over the years. C. Correlational study of hotpots in Militarine study.

isobutylmalates [15]. Militarine is composed of structure that linked by two units of *p*-hydroxybenzyl alcohol glucosides (glucosyloxybenzyl) and –COOH at both ends of the mother nucleus that react with each other further to form a unique glycosidic signature. The hemiacetal hydroxyl groups of the glucopyranosyl units (specifically point to the C2-sites hydroxyl group of glucose molecules, Fig. 2B) bond to *p*-phenolic hydroxyl group of *p*-hydroxybenzal alcohol (4-HBA) establishing polar hydrogen bonds connected by O-glycosidic bonds through a condensation (esterification) reaction. Therefore, Militarine is defined as compound of the oxindole series on the basis of its reaction features. Also, Militarine is, in turn, refined as a compound of the β-oxindole series, thanks to opposite configuration of the C2-sites and C3-sites hydroxyl group of glucose molecules in *p*-hydroxybenzyl alcoholglucoside that has S configuration and R configuration, respectively (Fig. 2A).

2.2. Characterization of physicochemical properties of militarine

Significant structure-activity relationship has been identified for the nature-based compound of Militarine. The relationship is directly captured in the physicochemical properties that are in intimate contact with both the basic molecular structure parameters and advanced conformation (e.g., spatial structure). Among them, basic molecular structure parameters include backbone chain, chemical composition, functional group species (such as the degree and substitutable manners of hydroxylation), corresponding molecular weights, branching degree and linkages (e.g., configuration and linked site of glycosidic bonds). The data from study suggested that Militarine belongs to the group of unsaturated glycosides include its taste quality of bitter, its molecular formula of $C_{34}H_{46}O_{17}$, and its molecular mass of 726.27. It is a compound of benzyl diglycosides with polyhydroxy, two benzene rings, multiple ester groups and two pyran rings, which has a maximum absorbance at 223 nm of ultraviolet spectrum [1].

Besides, Militarine is enriched with substantial branched chains and hydrophilic segments. Together with combined modes of how the hydroxy groups, the ester groups and pyrone rings and phenyl rings connect with each other that eventually to high diversity clades, stable chemistry, strong polarity, high solubility in water and alcohols and organic solvents with low polarity [16]. As a result, study staffs utilize methods such as aqueous or alcoholic extraction to isolate and obtain Militarine from plantarum metabolites, and chromatography is applied for the purification and identification of Militarine [17,18].

2.3. Structure-activity relationship between structures of militarine and its biological activity

Sophisticated and refined structures are not only the constituent basis for forming the skeleton of compounds but are also the prerequisite for various biological effects. The combined action of chemical groups, physiochemical performance, and structural features play a crucial role in the characterization of molecules and endow compounds pharmacological effects, pharmacokinetics, and potential medicinal purposes, which can be identified as a result of structural and functional integration. These factors, along with others, work in unison to create a range of biological activities in Militarine. This diversity in biological activities is essential for the effective research on Militarine of effective substances, mechanisms of effect, drug development, and Q-marker of medicinal plants.

2.3.1. The pharmacological mechanism of militarine on the host

Modern-day pharmacological studies confirmed that Militarine does not only act as an important inhibitor that suppresses the

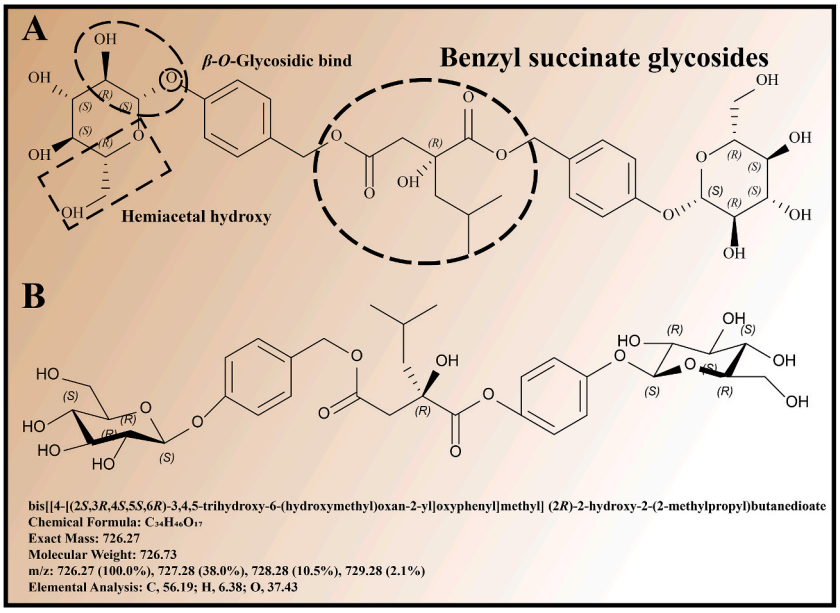

Fig. 2. Structural features of Militarine. A. Chemical structure and structural characteristics of Militarine. B. The Chair configuration of Militarine.

Table 1
Pharmacological mechanism of Militarine action in the body.

Pharmacological effects	Pathological models	Active substance	Material structure characteristics	Source of material plants	Study design	Regulatory mechanisms	Molecular targets/ mechanisms	Reference
Plant inhibitors	The growth of the host plant of Militarine is inhibited by surrounding weeds (environmental stress)	Militarine	The structural analogue of Militarine, Dactylorhin A (- O connected at the C2 position of Militarine)-β-D-Glu) does not exhibit plant inhibitory activity	Methanol extract of fresh *B. striata* and aboveground components	*In vivo*	Inhibiting the elongation of Lettuce embryonic roots and axes are achieved with EC50 values of 0.28 mmol L^{-1} and 1.03 mmol L^{-1}	As an allelochemical, Militarine inhibits the growth of weeds in its surroundings.	[2]
Protection of lung	SiO_2 thickens the alveolar septa and induces inflammatory infiltration, thereby inducing the Bax/ Bcl-2 pathways	Gymnoside II (the main active substance) and Militarine account for 1.31 ± 0.05 % and 12.12 ± 0.05 % of the ethanol extracts, respectively	Militarine stably binds to antioxidant HO-1 protein through its own hydrogen bonding	N-butanol extract from *B. striata* (40 mg/kg)	*In vivo* and *in vitro*	Inhibition of SiO_2 induced apoptosis and ROS production in alveolar epithelial cells through Nrf2 pathway dependent mechanisms	Nrf↑, HO-1↑, γ-GCSc↑, ROS↓, Bax↓, Bcl-2↑, cleaved-caspase 3↓, Bax/ Bcl-2↓	[24]
	SiO_2 has cytotoxicity and induces pneumonia, pulmonary fibrosis, and lung cancer by acting on alveolar epithelial cells	25 μmol/L Militarine processes 25 μg/cm^2 SiO_2	Gymnoside II is an ester hydrolysis product and synthetic raw material of Militarine	Alcohol extract from *B. striata*	*In vivo*	It significantly reduced SiO_2 induced lung inflammation and oxidative stress in mice	ROS↓, Nrf↑, Nrf downstream gene HO-1, and γ-GCSc↑	[19]
Anti-tumor	$PM_{2.5}$ induces cytotoxicity (activation of proinflammatory cytokines) of BV-2 microglia and lung morphological changes	0.31–1.25 μg/mL Militarine treated BV-2 that interfered by $PM_{2.5}$ (200 μ g/mL)	Militarine	–	*In vitro*	It significantly reduced $PM_{2.5}$ mediated ROS and cell apoptosis, driving anti-tumor immunity	TNF-α↓, IL-6↓, TLR4, TLR2, as well as COX-2 mRNA and its protein↓, NF-κB p65↓	[21]
Cognitive impairment	Permanent bilateral carotid artery ligation in rats poses chronic cerebral ischemia, which in turn induces brain protein damage	Militarine (purity ≥95 %)	Militarine	–	*In vivo*	Effect of brain proteins on improving exploration and navigation abilities in rats (morris water maze experiment)	Dense myelin sheath, basic protein cyclic nucleotide and phosphodiesterase positive cells in corpus callosum↑	[4]
Aortic ring relaxation	Norepinephrine induces contraction of the aortic ring	Militarine (Purity of 98 %)	Militarine	–	*In vivo*	Endothelial and non endothelial dependent vasodilation	It opens the K^+ channels and inhibits Ca^{2+} influx (possibly activating the NO/cGMP pathways)	[25]

(*continued on next page*)

Table 1 (*continued*)

Pharmacological effects	Pathological models	Active substance	Material structure characteristics	Source of material plants	Study design	Regulatory mechanisms	Molecular targets/ mechanisms	Reference
Anti peptic ulcer	–	Militarine and dactylorhin A accounted for 1.47 % and 0.99 % respectively of Weikangling, the classic traditional Chinese patent medicines and simple preparations for gastric diseases	Consistent with the literature (Sakuno et al., 2010)	–	*In vitro*	Militarine and dactylorhin A are the major active components in the Weikangling	–	[26,27]
Anti-inflammatory	LPS induces RAW264.7 to produce key inflammatory factors	Militarine (1.9 g) and its structural analogue dactylorhin A (30.3 mg)	Consistent with the literature (Sakuno et al., 2010)	95 % ethanol extract of *B. striata* tubers (50 g)	*In vitro*	Both showed moderate inhibition of NO, with IC50 values of 48.5 and 51.7 μM, respectively	NO↓	[22]
Whitening and antioxidant	Tyrosinase is a key rate limiting enzyme in melanin synthesis	Militarine accounts for 52.74 % and 31.12 % of the total peak area of *B. striata* tuberous and roots	Militarine	95 % ethanol extract of *B. striata* tubers and roots	*In vitro*	It has more affinity for tyrosinase and adenylate cyclase, and has antioxidant and anti melanin growth activities	DPPH↓, ABTS↓, FRAP↓ and Tyrosinase↓	[6]
Antimicrobial activity	Gram-negative and gram-positive bacteria	Militarine	Militarine	Fibrous root of *B. striata*	*In vitro*	It has certain inhibitory activity against gram-negative and gram-positive bacteria	–	[23]
Hemostasis	Bleeding caused by inserting a capillary glass tube into the venous plexus behind the inner canthus bulb in mice	Gastrodin, Gymnoside I and Militarine	Both gastrodin and Gymnoside I are precursor compounds of Militarine	N-butanol extract from *B. striata* (0.04 g/mL)	*In vivo*	They significantly shorten the clotting time and bleeding time	–	[12]

Note: ↑: upwards adjustment; ↓: downwards adjustment.

growth of plant [2], but also occupies important position within therapeutic management for various diseases. Studies by researchers have highlighted the significance of Militarine in pharmacology. For example, it induces apoptosis in cancer cell lines, protection of lung [19], anti-tumor properties [20,21], whitens in skin [6], reduces inflammatory [3,22], and exhibits anti-bacterial [23] and hemostasis [12] properties (Table 1).

Compounds deprived from natural sources are highly effective and easily degradable plant inhibitors that utilize allelopathy to affect the growth and development of adjacent plants. They assist in competing for sufficient nutrients, water, light, and other resources, and help improve the physical, chemical, and biological properties of soil. These compounds have great potential as drugs that can reduce the risk of bioconcentration of pesticides in the environment [28]. Of these, Militarine, acts as an allelochemical and inhibits the elongation of Lettuce radula and hypocotyl with EC50 values of 0.28 mmol L^{-1} and 1.03 mmol L^{-1}, respectively, depending on the pathway of environmental release. It appears the potential to inhibit plant growth and can served as a green source to improve crop productivity. In this study, it also revealed that dactylorhin A of Militarine structural analogue (-*O*-β-D-Glu (dactylorhin A) but not –OH (Militarine), which is linked in the C2 position of Militarine chemical structure) did not display any effect in inhibiting plant growth [2] (Table 1). Further analyses have shed that Militarine regulates the growth of Italian ryegrass and timothy with inhibitory concentration almost equivalent to Lettuce [2].

At the same time, both clinical research and concurrent experimental data have proven that Militarine is an important medicinal active molecule by which exhibits excellent efficacy in the treatment of various diseases such as melanin, oxidative stress [6], hemorrhages [29], and aortic ring contraction [25]. Moreover, Militarine is also cytotoxic in the A549 cell line that shows the activities of

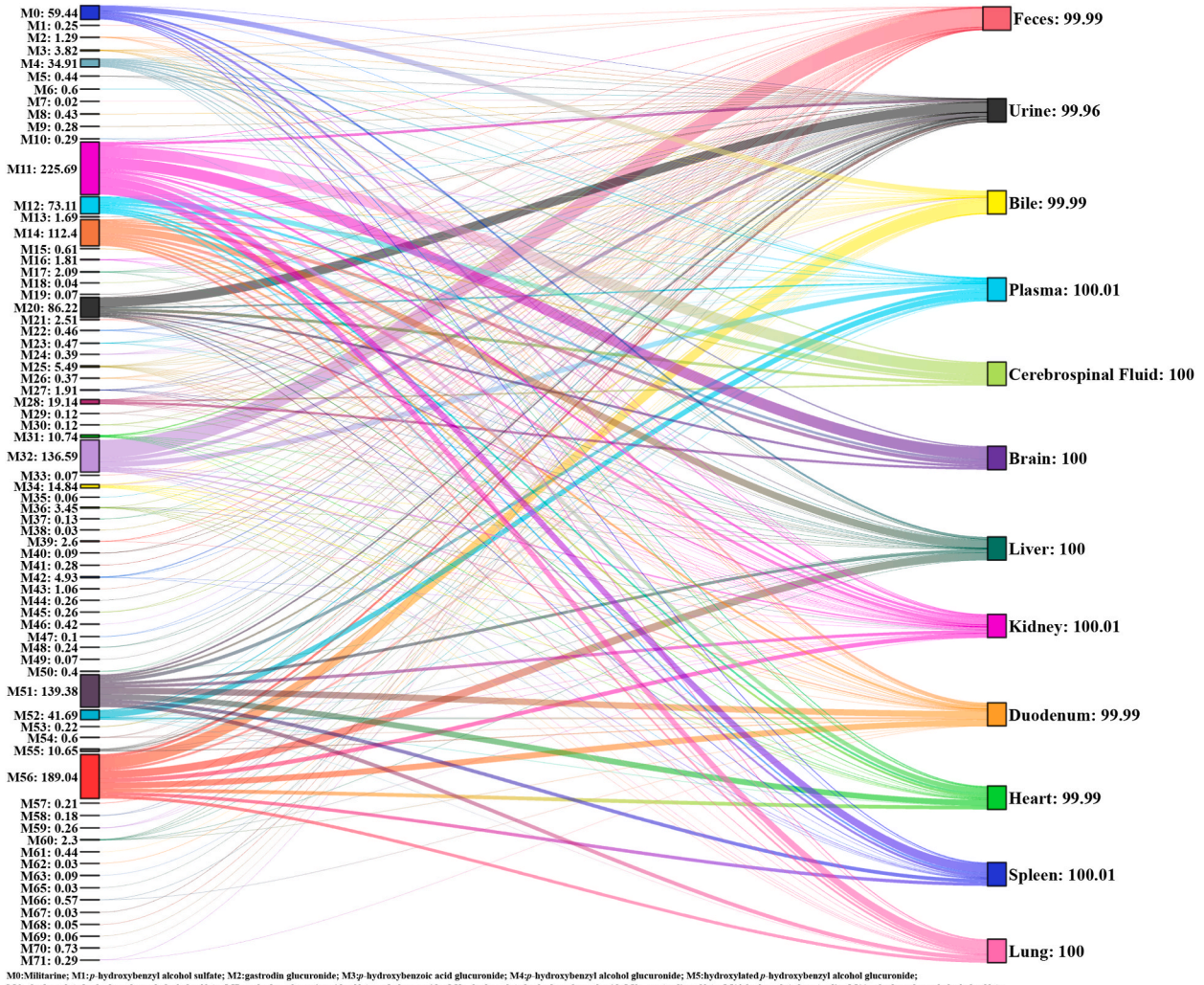

Fig. 3. Biotransformation pathways and metabolic distribution of Militarine.

anti-inflammatory and anticancer [3]. And Militarine also displays antibacterial activity and has a certain inhibitory effect on gram-positive and gram-negative bacteria [23]. In conclusion, Militarine is an essential functional ingredient that equips with broad-spectrum pharmacological properties with tremendous application potential in the pharmaceutical field, thus offering possibilities and innovative ideas for treating human diseases.

2.3.2. The biotransformation and drug-metabolizing route of militarine

Militarine is all in addition to significant associations with the brain, lungs, liver, intestine, stomach, and other organs. Through direct and/or indirect ways of biotransformation, Militarine exerts its pharmacological effects (such as neuroprotection [5], improvement of learning and memory [30], ameliorative effects on cognitive impairment, anti-shock effects [4], preservation of lung function [24] and treatment in peptic ulcer [26,27]) on the host [5] (Fig. 3 and Table 1). The diverse pharmacological activities of Militarine render it an important therapeutic option for different conditions.

Study confirmed that Militarine and its intermediate metabolite coelonin significantly inhibit the induction of multiple inflammatory transcription factors (TFs, such as IL-1 and TNF-α) by $PM_{2.5}$. Furthermore, they slow down the activation of the proinflammatory factors and $PM_{2.5}$-induced NF-κB pathway and cell migration. Therefore, these substances have potential to be developed into drugs for the prevention and/or treatment of various lung diseases caused by air pollution [3]. As reported in the literature, the key hydrolysis pathway linked to Militarine is of metabolism and differentiation mediated by esterase β-glucosidase to active materials of 4-HBA and gastrodin, which proved that positive effects on neuroprotection, enhancement of learning and memory and improvements in intellectual development engaged by central nervous system. These metabolites closely resemble those found in rats following oral administration of gastrodin [5]. Moreover, both *in vivo* and *in vitro* experiments have proved that Militarine, in combination with its metabolites (such as 4-HBA) are mostly enriched in the liver. The primary routes of Militarine metabolism in liver microsomes is through the hydrolysis of the ester bond containing C1 and/or C4. It is worth noting that its metabolism is not reliant on the cytochrome P450 enzyme metabolic pathway initiated by NADPH [31].

These findings indicated the pathways of mlitarine drug metabolism in the liver. Simultaneously, they are of great significance in clarifying the active ingredients, pharmacological effects, toxicity studies and structural modifications of drugs (such as lead compound) and conducting basic research on the metabolic process of candidate compounds. Further research has revealed that Militarine follows specific metabolic pathways, including ester hydrolysis, deglycosylation, and sulfation in the liver, resulting in the formation of metabolites such as methylquinone. The unique chemical structure is attributed to its specific metabolic characteristic. Intensive investigations have proven that there is a specific affinity between aromatic hydroxylamines, benzyl alcohols, and allyl alcohols in phase II metabolic enzyme sulfonyltransferases (SULTs) and Militarine chemical groups. This affinity dramatically activates the water solubility and metabolic activity of Militarine [32]. As expected, given the above relationship between pharmacological effects and chemical structure, drug metabolism and chemical changes, it can conclude that the pharmacophore is the basis and guarantee for the specific efficacy of Militarine, in turn, the establishment of the pharmacophore is strongly correlated with the chemical structure.

Taken together, Militarine exists prominent structure-activity relationship, promoting it versatile as a chemical index of pharmacodynamics and carrier for substances, that is notably promising in the arena of drug development. Additionally, Militarine is one of the most important active ingredients in orchid plants and serves as a key indicator of authentic medicinal herbs. As a Q-marker for medicinal plants, it is used to screen and evaluate the quality of medicinal products, such as the traditional Chinese medicine *B. striata*. The 2020 edition of the Chinese Pharmacopoeia has listed the content of Militarine in *B. striata* dried products and its decoction pieces as the primary indicators for quality control of *B. striata* finished products, with its corresponding data should not be less than 2.0 % and 1.5 %, respectively [1,33].

Still, insufficient biological resources, difficulty in obtaining it, low extraction efficiency, and uncertain biological content of Militarine severely limit its pharmacological applications and industrial development, rendering it unable to meet the growing demand of people. Clearer insights into and tighter grasps to these synthetic pathways and key regulatory mechanisms of Militarine is extremely crucial and necessary for solving the aforementioned issues. And the elucidation of metabolic pathways is the driving force in the biosynthesis of Militarine, facilitating exploration into pathways and regulatory mechanisms that regulate Militarine synthesis.

3. Synthetic pathways of the militarine

At present, the methods for obtaining the effectiveness of Militarine can be roughly owing to chemical synthesis and biosynthesis. However, there is currently no accessible literature reporting on the chemical synthesis pathways of Militarine. In the context of these issues, this paragraph aims to reveal the biogenic pathways of Militarine, highlighting the key technologies and main means of regulating its biosynthesis. It also analyzes and evaluates the quality formation laws of Militarine. This study strategy is directly related to the acquisition, genetic information analysis, innovative drug development, as well as the protection of medicinal plant and biological research of Militarine.

3.1. Militarine accumulation biological pathways

Since complexity of "the medicinal properties" of Militarine bears close relationship to the diversity in groups of "pharmacophores" [34], obtaining Militarine from medicinal plants remains the principal method of production. And the combination of stable yield, high content, and high activity of Militarine is a crucial value indicator of medicinal materials, and good germplasm resources are an important prerequisite to ensuring the quality of medicinal materials [35]. The biosynthesis of the secondary metabolite Militarine is believed to be converted by the host plant using the primary metabolites glucose, protein, lipid and nucleic acid. Elaborate conversion

processes are realized through various chemical reactions, including oxidation, glycosylation, esterification, sulfation, glucuronization and glycine conjugation [5] in the main metabolic pathways. Complementary to this, metabolic pathways contain glycolysis, gluconeogenesis, malonic acid, shikimic acid, phenylalanine, amino acids and other major metabolic pathways (Fig. 4) [36,37]. The efficient acquisition of mlitarine through the utilization of synthetic biology and metabolic engineering has many advantages, such as green environmental protection, strong maneuverability and sustainable development, among which the key steps in the biosynthetic pathway, biosynthetic intermediates, characteristic gene elements and key enzymes are more targeted links that regulate the synthesis of Militarine.

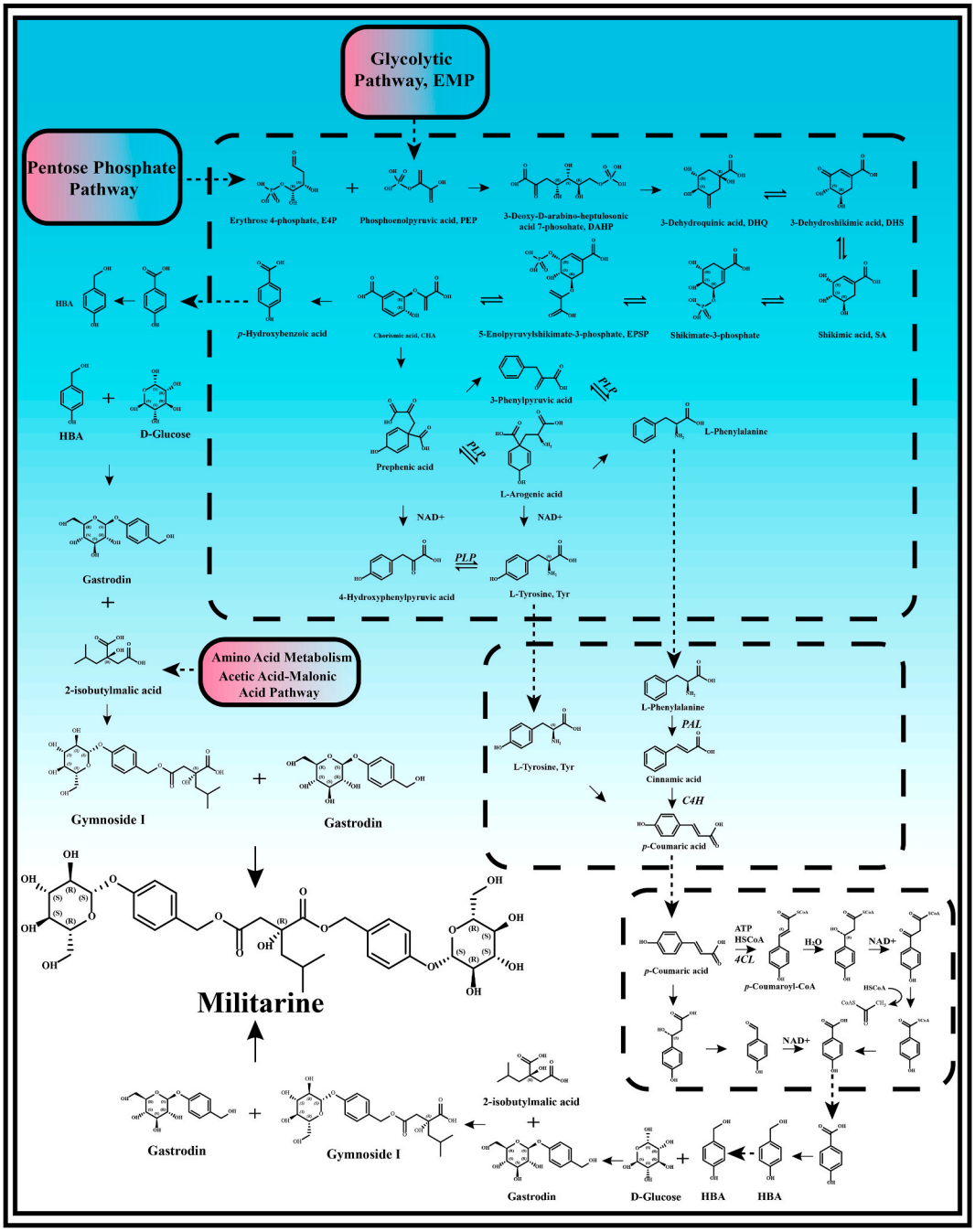

Fig. 4. The biosynthetic pathways of Militarine.

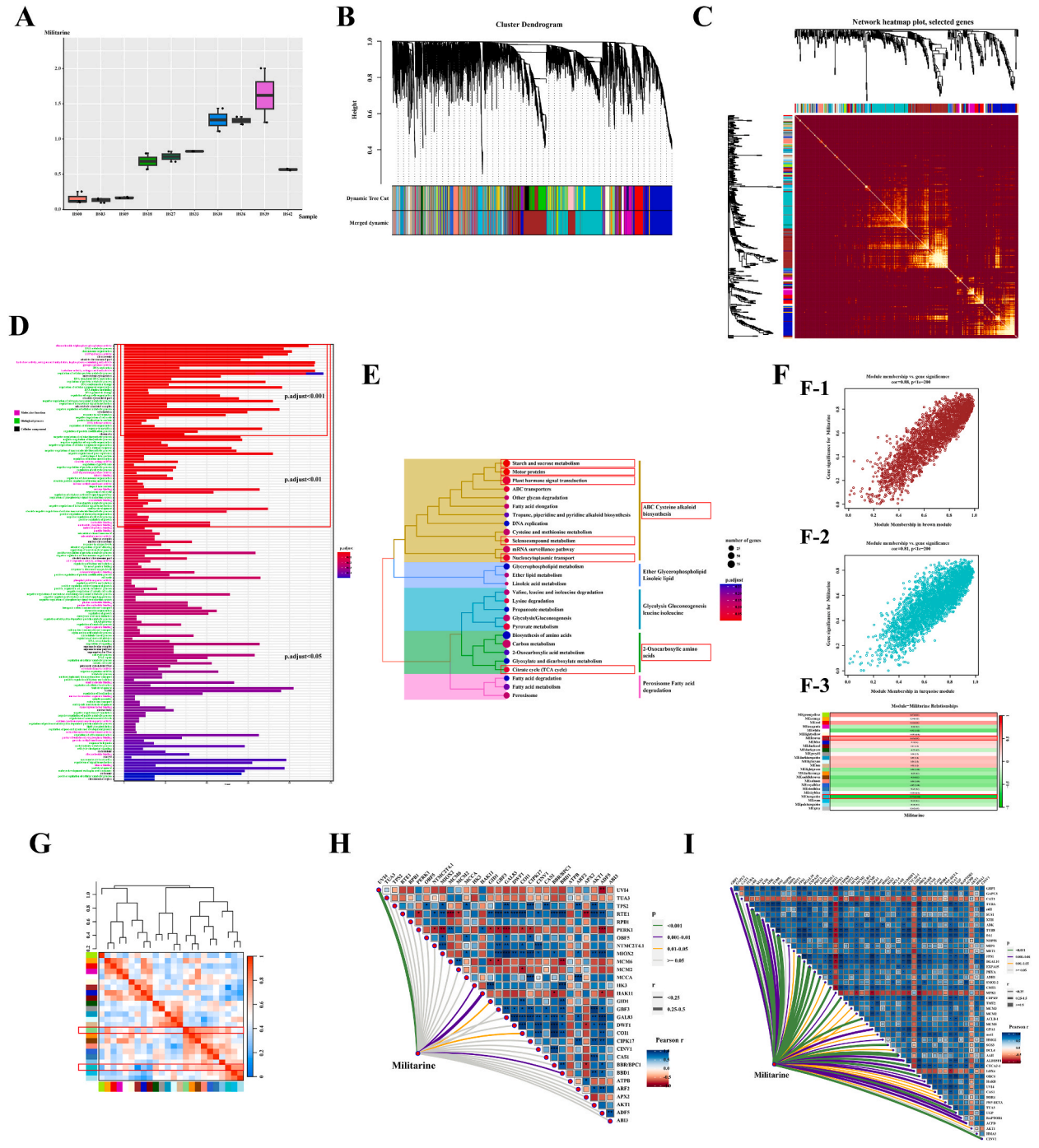

Fig. 5. Transcriptome analysis of genes, key pathways, and transcription factors related to the expression of Militarine. A. The expression level of Militarine at different time nodes. B. Weighted co expression analysis of differentially expressed genes related to Militarine. C. Selected Militarine differential gene heatmap and weighted co expression network map. D. 158 GO enrichment pathways related to Militarine expression. E. 5KEGG enrichment pathways associated with Militarine expression. F. F-1. Brown module with strong correlation with gene significance. F-2. Turquoise module with strong correlation with gene significance. F-3. Correlation diagram between different modules and the expression level of Militarine. G. Hierarchical clustering and correlation analysis between different expression modules of Militarine. H. A heatmap of the correlation between the expression levels of module member brown and Militarine. I. Heat map drawing of the correlation between the expression levels of turquoise and Militarine among module members.

3.1.1. Biosynthesis of militarine occurs via the shikimate pathway

Structurally, Militarine is a highly polymerized compound composed of two aromatic rings, nine hydroxyl groups, and two ester groups. As far as the shikimate pathway is concerned, it recruits two initial substrates: D-erythrose-4-phosphate (E4P), which is an intermediate product of the pentose phosphate pathway, and phosphoenolpyruvate (PEP), an intermediate product of glycolysis. These substrates is subjected to nine steps of hydroxyl condensation, acid hydrolysis, hydroxylation, phosphorylation, synthesis, dephosphorylation, and addition reactions to generate aromatic A and B rings of Militarine derived from 4-HB [38]. Next, 4-HB synthesized during the mono-glycosylation can efficiently proceed with reduce into important precursor of 4-HBA that then sequentially binds glucose and 2-isobutylmalate compound. Immediately after the response in a reciprocal fashion of mono-glycosylated products of gastrodin and gymnoside I leading to generation of Militarine as end product, in which 2-isobutylmalate plays a key role as derivative in the metabolic pathways for glucose, malonic acid, and the tricarboxylic acid cycle.

It is worthwhile noting that the shikimate pathway is an essential metabolic pathway presented in plants, microorganisms and fungi. This pathway encompasses multiple synthetic manners of Militarine and are also central to the Militarine metabolic pathways, such as acting as a reaction substrate source for the Militarine phenylpropane pathway.

3.1.2. Phenylpropanoid metabolism pathway as part of biosynthesis of militarine

Starting with the chorismic acid, which is an important pivot substance in the shikimate pathway, L-phenylalanine and L-tyrosine are synthesized/derived in the phenylpropanoid direction. These substances then undergo a deamination reaction to form cinnamic acid. Subsequently, through use of intermediate products like phenolic acids (such as coumarin), they can be further converted into lactones (such as coumarin), phenolic acid compounds (including hydroxybenzoic acid (such as 4-HB, gallic acid, protocatechuic acid)) and hydroxycinnamic acid (such as chlorogenic acid, caffeic acid). After that, these intermediates are finally transformed into flavonoids, lignin and other polyphenolic compounds.

Multiple pieces of evidence indicated that the biosynthesis of Militarine is strongly associated with the phenylpropanoid metabolic pathway, and that its precursor compound 4-HBA is synthesized through the phenolic acid branching pathway [37,38]. Procedures can be broadly classified as follows: (1) L-phenylalanine undergoes deamination and hydroxylation to produce 4-coumaric acid; (2) And L-tyrosine is able to generate 4-coumaric acid through deamination. Common intermediate 4-coumaric acid also generates phenolic compounds along two directions, *p*-hydroxybenzoic acid: (1) Firstly, 4-coumaric acid undergoes a reverse hydroxylation reaction to produce 1′,4′- dihydroxycoumaric acid that undergoes subsequently a reverse aldol reaction to produce *p*-hydroxybenzaldehyde, which is ultimately oxidized to 4-HBA. However, the second reaction mechanism for 4-coumaric acid which is different from the first reaction direction is similar to the β-oxidation pathway of fatty acid metabolism. (2) 4-coumarin acid passed successively acetyl-coenzyme A (acetyl-CoA) and 4-HB are generated via reactions such as para-hydroxylation (PHO), β-oxidation reaction with CoA, inverse Claisen reaction, and oxidative dehydrogenation. The three-step reactions of reduction, glycosylation and esterification are equally experienced by 4-HB, resulting in the production of Militarine.

3.2. The militarine analyses of association to genetic information

At the same time, Militarine occurs as a metabolic product during the plant growth cycle, which facilitates the transmission, expression, and regulation of genetic information in host plants through a series of metabolic processes. This capability to better reflect the current state of individuals and serve as a potential biomarker makes it highly valuable [39]. Identification and optimization of characteristic gene elements, TFs and key enzymes in the biogenic pathways of Militarine are the core and source of the application of synthetic biology technology to innovate the mode of Militarine production.

Due to the fact that Milltarine is a biologically active ingredient present in orchid plants, this study used suspension cells of *B. striata* at different time points during the growth cycle as materials, and conducted full-length transcriptome sequencing to obtain 50.02 Gb of raw data. These raw data were stored in the SRA database under the login number SRR18045794. Then, this article used transcriptome analysis methods of *B. striata* to map differential genes onto the *Phalaenopsis equestris,* the first reported orchid genome [40], from which a co-expression network of Militarine related genes is constructed. Of which, this review focuses on differentially expressed genes closely related to the expression of Milltarine during its growth process. More specifically, by exploring the relationship between gene networks and Militarine expression, and identifying core genes in the key pathway Militarine expression network, this study sought to identify the Militarine biological pathway and key genes and to target such pathways for the high expression of Militarine and providing a promising strategy to develop more efficient Militarine production (Fig. 5 (A-I)). Firstly, under the screening conditions of FDR$<$0.01 and $|$ log2FC $|>$1, combining with the expression levels of Militarine at different time nodes (Fig. 5A), a total of 11,221 DEGs (5253 upregulated and 6068 upregulated) were selected for weighted correlation network analysis (Fig. 5B). Then, low expression genes were filtered, high expression DEGs were screened and a heat map and dendrogram were then visualized to highlight the similarity within DEGs (Fig. 5C). After that, these coding genes were compared and mapped onto the orchid model organism *Phalaenopsis equestris* using alignment method of kobas, and combined with high expression DEGs heat map analysis, 25 modules with high co-expression were ultimately screened, totaling 2719 DEGs genes (1330 unigenes upregulated and 1389 unigenes downregulated). These module genes were analyzed through GO and KEGG functional enrichment (Fig. 5D and. E, respectively) to identify specific biological pathways or functional genes of Militarine, discover interdependent network of gene regulation and metabolism during Militarine biological processes, and predict the gene composition of specific biofunctions. Furthermore, this article screened two modules that are most relevant to gene significance (Fig. 5F1 & F2), and simultaneously combining with the correlation analysis between the modules and the expression level of Militarine (Fig. 5F3), while constructing a hierarchical clustering tree and heat map (Fig. 5G). Ultimately, characteristic gene clusters (brown and turqoise modules) with significant positive correlation in Militarine were

identified and characterized in the review. Afterwards, we employed p-weighted<0.001-based screening criteria in the brown and turquoise modules to screen for 30 and 49 genes that are tightly bound to high level of Militarine, respectively. Then, correlation heatmaps were plotted between the high expression genes in the brown and turquoise modules and the expression level of Militarine (Fig. 5H and I). Finally, this study investigated 59 hub genes and predicted their associations with the transcription factor database of *Phalaenopsis equestris*. It was found that the expression of Militarine was correlated with bZIP family protein, ARF family protein, B3 family protein, ARR-B family protein, and CAMTA family protein.

So far, researchers have undertaken detailed research on the biogenic pathways of Militarine, which confirmed that Militarine is an effective ingredient that can be achieved by people for development and utilization. However, Militarine is mostly stemmed from precious, endangered, and slow-growing orchid species. Concurrently coupled with the low content of Militarine biosynthesis in higher plants make obtaining Militarine organisms difficult. Furthermore, the complex chemical structure of Militarine renders it challenging to produce it on a large scale, which creates significant obstacles for its clinical use and quality control. While the biosynthesis of Militarine often occurs in response to environmental factors, thus, the modulation of secondary metabolites under environmental stress has become a crucial way to promote the biosynthesis of Militarine. And this provides an effective means for the deep-level resolution and regulation of Militarine synthesis.

4. Environmental and biological stresses affecting the synthesis of militarine

Environmental factors change plant morphology at the histiocyte and molecular levels by influencing many aspects of plant physiology and biochemistry, hormone levels, membrane protective substances, active oxygen balance, DNA degradation, and other processes through stress. In order to adapt, defend against adversity, and improve their survival competitiveness, plants must interact with their environment to acquire the essential ecological functions (such as drought resistance, frost resistance, insect resistance, etc.). One of the most crucial pathways for plant development and evolution, endogenous Militarine is a byproduct of environmental adaptation in plant. It serves as the basis for interactions between plants and their environment and is a key driver of plant evolution, thereby is strictly controlled by biotic and abiotic stimuli.

4.1. Bioaccumulation of militarine under physical factors

Physical stresses demonstrated that of significance in plant physiological metabolism, such that light quality (including light quality, light cycle, and light intensity) has dramatical effects on the photosynthetic rate, leaf area, and leaf lifespan of plants. These factors, in turn, affect the metabolic network and material accumulation of Militarine [41,42]. Excessive light intensity can hinder plant growth (including plant height, leaf length, and leaf width), but can promote the accumulation of Militarine. In response to this phenomenon, some studies have suggested that high and medium frequency light quality treatments such as red light (600–700 nm, peak 625 nm) and yellow light (500–600 nm, peak 518 nm) significantly increase the expression of Militarine in white and middle. Concomitantly, the red and yellow light remarkably improve the ratio of chlorophyll *a*/b, indicating that the role of plant resistance acquisition to light inhibition may be in accordance with Militarine [42].

Beyond that, the red and yellow light can also increase the activity of the main antioxidant enzymes SOD, POD, and CAT in plants, thus accelerating the degradation of superoxide anion factor O^{2-} and preventing chlorophyll decomposition to enhance plant resistance. This aligns with the regulatory trend of increasing the accumulation of Militarine biomass [42]. In addition, water uptake efficiency (such as drought and waterlogging) [41], temperature (such as heat and freezing damage), wind, ionizing radiation, and other critical factors play a vital role in regulating the content of Militarine in plants. These factors affect plant physiological metabolism, drive the formation of stress avoidance, tolerance and resistance, along with promote Militarine bioaccumulation by intervening in pattern of response to plant. The exploration of these studies is of great significance in revealing the expression of Militarine under adversity.

4.2. Accumulation of biomass in militarine excited by chemical exposures

Under normal circumstances, plants tend to adapt to their environment over the long-term, selecting a more suitable natural habitat. As a result, their ability to compete for limited resources through physical means is greatly reduced when compared to other plants. Meanwhile, chemical and biotic stresses have become the main competitive considerations for the accumulation of biomass in Militarine [43]. Based on environmental stress and their own needs, plants utilize roots, root exudates, soil enzymes, and microorganisms to absorb mineral elements such as Na, K, C, N, P, Si, Ca, as well as their compounds, toxins, heavy metals, etc. from their surrounding environment as chemical stress.

The chemical exposures act among histiocyte to carry out information transmission, exchange and conversion. And they not only play crucial roles in nutrient absorption, the cell membrane system (including cell membrane potential, membrane ATPase activity and membrane permeability), osmotic pressure balance, salt tolerance mechanisms and signal transduction, but also are pivotal sources of organic molecules, including DNA, RNA, ATP and phenylpropane compounds. Some studies demonstrated that administering *B. striata* with carbon-based fertilizers (including pure carbon based fertilizers, N, P, K) rich in nutrients can increase Militarine content in the plant (up to 5.32 g·100 g^{-1}) [44]. And recent studies have shown that biochar can increase the content of available phosphorus and potassium (A-P and A-K), soil pH, soil microbial diversity, and organic matter 4-HBA in soil when treated with 6 t/ha, thus, in turn, improvements of soil properties [45]. Amongst others, 4-HBA is an important precursor compound in the biogenic pathways of Militarine. Therefore, we speculate that biochar may have a certain regulatory effect on the biosynthesis of Militarine.

Additionally, fertilizers or pesticides with the same or similar effects as ethylene, abscisic acid (ABA), auxin (IAA), cytokinin (CTK), gibberellin (GA), etc. are imposed by the outside and synthesized by plants themselves. They are also important members of chemical stress, and almost participate in and improve the whole process of biological accumulation and metabolism of Militarine in a small amount. Studies have shown that under the cultivation conditions of plant growth regulator MS+1.0 mg/L 6-BA+3.0 mg/L 2,4-D+30 g/L sucrose+20 mg/L phenylalanine, the content of Militarine in suspension cells of *B. striata* is relatively high, reaching 15.869 ± 0.245 mg/g [46]. Collectively, to enhance the mechanisms analysis of the bioaccumulation and molecular mechanisms of Militarine under stress, it is crucial to elucidate the existing regulatory mechanisms of Militarine and comprehend the promotion and inhibition of significant precursor compounds, intermediates, and metabolites in the Militarine biogenic pathways when exposed to chemical stress.

4.3. Mechanisms of biomass accumulation of militarine in biotic stimulations

A growing body of evidence supports the inducible role of biotic exposures that stimulate the synthesis of Militarine, induce systemic resistance or enhance stress tolerance, and protect host plants from pests, pathogens, competition, and other harmful factors. As previously demonstrated, Militarine generates a competitive ability to inhibit weeds growth in the surrounding environment of the host plant for function as an allelochemical, then enhancing crop competitiveness [2]. Recently, optimizing the accumulation of secondary metabolites such as Militarine by focusing on microbial release of signaling molecules to inducible expression and combined metabolism has always been a prominent area of research and garnered significant attention among scholars.

In the growth cycle of Militarine active ingredient, microorganisms are expected to play multiple roles. One of these roles is regulating the synthesis of Militarine through their direct actions on plant growth and development (such as germination rate, biomass, chlorophyll content, and length of roots and buds), thereby enabling the plant to resist biotic/abiotic stresses [47]; the second is to participate in the production of Militarine identical or similar compounds to Militarine; and thirdly, the bacteria' s role is to respond indirectly to biotic/abiotic stresses or produce innovative drugs through biological nitrogen fixation, production of plant hormones, phosphorus dissolution, inhibition of ethylene production, and other mechanisms [48]. According to reports, the fungal genera *Ilyolectria*, *Epicocus*, *Lexera*, and *Colletotrichum* are reported to benefit for the growth of *B. striata* and induce a reduction in the synthesis of Militarine; On the other hand, the bacterial genera *Streptomyces*, *Sphingomonas*, *Hailangium*, *Dongia*, and *Nocardioides* facilitate the accumulation of Militarine and inhibit the growth of *B. striata* [49].

And researchers found that Militarine acts as a critical role in regulating plant growth cycle by altering the physical and chemical

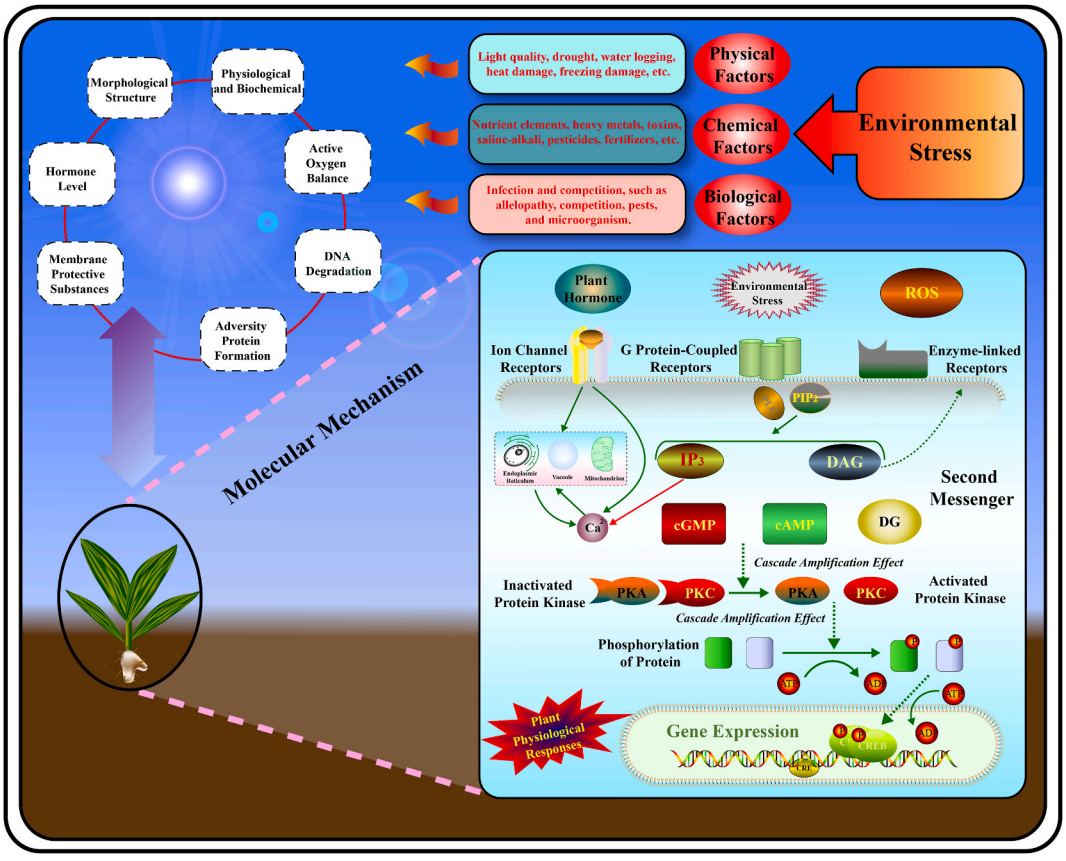

Fig. 6. The molecular mechanism of Militarine biosynthesis under environmental stress.

properties of the surrounding environment in a way that resembles biotic stress [49]. The soil treatment with the aforementioned fungal genera exhibited significantly higher contents of total calcium (T-Ca), T-N, T-Mg, T-Mn, T-Zn, A-Ca, A-Mn, and A-Cu, as compared to that treated with bacteria, but the T-P, T-K, T-Fe, and A-P contents showed significant decrease. This phenomenon is consistent with the potential chemical elements A-P and A-K promoting effects of Militarine as mentioned earlier. Besides, endophytic bacteria located between or within various histiocyte of the host plants, as a treasure-trove of natural product sources, play an important role in regulating Militarine, overcome the obstacles in traditional chemical synthesis and plant metabolic engineering, constantly excavate Militarine with significant biological activity, and explore important alternative resources for Militarine [50,51]. Relevant studies have confirmed that the endophytic fungus *Gibellulopsis nigrescens* exhibits a significant promoting effect on the increase of Militarine biomass [52,53].

Taken together these observations imply that environmental factors can be an important choice for studying the synthesis and regulatory mechanisms of Militarine, which are not just significant in terms of serving as an important resource to address the shortage of Militarine, but they can also act as an important breakthrough for optimizing Militarine synthesis.

5. Perspectives

5.1. Multiplicity optimization of militarine synthesis strategies

Quality control is the core in the acquisition of safe, efficient and stable medicinal plants, as well as the standard throughout genuine medicinal materials. The active substance Militarine, stands out as the Q-marker in the Orchidaceae, making it an important manifestation of high-quality resources in authentic medicinal herbs. Modern biology holds the belief that the true essence of medicinal herbs lies in their phenotypic variation, which is determined by both genetic variation and environmental modification [43]. It is evident that the quality control and shaping of Militarine is intricately linked to its own genetic background and natural habitat. Especially, the synergistic relationship between the two and elucidating the molecular mechanisms underlying the formation and accumulation of Militarine under environmental stress (Fig. 6) are crucial for optimizing the acquisition of Militarine.

The synthesis strategy of applying multi potency approaches to catalyze the formation of Militarine is essential in effectively and precisely regulating its activity. Multiple research strategies have been demonstrated to be involved in Militarine induction, primarily through targeted and non-targeted processes. (1) Non-targeted regulation: 1) Change nutritional conditions and cultivation status; 2) Change physical parameters; 3) Cocultures. (2) Targeted regulation: 1) Adding chemical molecules (self-induced molecules, signal molecules, and so on); 2) Heterologous expression and reconstruction; 3) Molecular modification of genes (such as promoter engineering, the activation and inhibition of positive and negative regulatory genes, etc.).

5.2. Molecular strategies for optimizing militarine synthesis

In recent years, cell tissue cultures of Militarine *in vitro*, including plant histiocytes (such as adventitious root, hairy roots, seeds, callus, suspension cells). And specifically, it takes wild Chinese herbal medicine entities or artificially cultivated medicinal plants as biomaterials, utilizes characteristics of Militarine, adopts appropriate biological, chemical and physical methods to target products, thus serving as the main methods to obtain Militarine. Examples include the callus culture system and/or suspension cell culture system of *B. striata* [46,53].

Regardless of the cultivation methods or extraction systems used, the ultimate goal of the experiment is to obtain a high Militarine content with high-quality. Understanding the molecular mechanism and its role in the biological processes of Militarine is a key strategy for optimizing Militarine synthesis. The general mechanisms regarding this process are detailed below (Fig. 6). After sensing environmental stress, the plasma membrane, reactive oxygen species (ROS), and cell wall of host plant cells produce second signaling molecules such as ROS, ABA, and phosphatidylinositol. The second signaling molecules can stimulate the inner membrane of cells, regulate intracellular Ca^{2+} levels, initiate protein phosphorylation cascade amplification reactions through signal transduction, and produce target phosphorylated protein molecules. Indeed, the vital roles for these functional proteins (also known as TFs) have been suggested in cell protection (transmitting signals or regulating gene expression) via activating or inhibiting the expression of downstream functional genes. They achieve this by specifically binding to the target gene promoter sequence and thereby manipulating the expression level of Militarine. In essence, they function as a buffer against adverse conditions [54].

Genome, transcriptome, metabolome and other emerging biological technologies are utilized to detect and identify changes in response genes, enzymes, TFs, and so on under environmental stress. Bioinformatics tools are employed to analyze, summarize and pinpoint key regulatory factors. Afterwards, potentially global regulatory factors are transformed at the molecular level to shape the phenotype of host plants, aiming to access Militarine with “high-quality” and fully stimulate the growth potential of crops. Meanwhile, the entire process of forming and producing of authentic medicinal materials is connected to substance efficacy as the core. Based on the premise of clarifying the structure-activity relationship, synthetic pathways and regulatory mechanism of Militarine. Therefore, utilization of Militarine as a lead compound to carry out structural transformation, modification and optimization to enhance its functional activity, so as to target direct or indirect development of innovative drugs that are efficient for the treatment of diseases.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Center for Biotechnology Information (NCBI) BioProject database under accession numbers

PRJNA807395 and PRJNA807396. The raw data for full-length transcriptome sequencing of *B. striata* were stored in the SRA database under the login number SRR18045794.

CRediT authorship contribution statement

Xueyan Jia: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Formal analysis, Data curation. **Qingqing Li:** Writing – original draft, Validation, Resources, Investigation, Data curation. **Mengwei Xu:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation. **Jie Zhang:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition. **Delin Xu:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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