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ORIGINAL ARTICLE

Exploring the Phytochemical Diversity and Antioxidant Potential of the Vietnamese *Smilax glabra* Roxb: Insights from UPLC-QTOF-MS/MS and Zebrafish Model Studies

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Abstract
Research on natural products is growing due to their potential health benefits and medicinal properties. Despite regional variations in phytochemical composition and bioactivity, *Smilax glabra* Roxb (SGB) has attracted the interest of researchers. Scientists are particularly interested in the Vietnamese SGB variant, which is influenced by biological and environmental factors. Despite geographical differences in phytochemical makeup and bioactivities, SGB remains a fascinating subject in traditional herbal medicine. Using ultra-performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS/MS), the phytochemicals in Vietnamese SGB extracts were investigated. This study revealed a wide range of phytochemical compounds, including flavonoids, terpenoids, glycosides, alkaloids, organic acids, phenolics, and steroids. Furthermore, utilizing zebrafish as a model organism, we discovered that these extracts have the surprising ability to greatly improve the survival rate of zebrafish larvae exposed to oxidative stress caused by arsenite ($NaAsO_2$) and hydrogen peroxide (H_2O_2). Notably, our discoveries suggest the occurrence of new antioxidative pathways in addition to the kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, expanding the understanding of the antioxidant properties and potential therapeutic uses of these plants. To summarize, our research findings shed light on the phytochemical composition of Vietnamese SGB, revealing its potential as a natural antioxidant and encouraging further exploration of its underlying mechanisms for future innovative antioxidant therapies.

Keywords *Smilax glabra* Roxb · Phytochemical analysis · UPLC-QTOF-MS/MS · Zebrafish · Keap1/Nrf2-independent pathways · Natural antioxidants

Introduction

Research on natural products has experienced a surge in interest in recent years, driven by the compelling potential of these compounds to offer substantial health benefits and serve as valuable sources for medicinal applications. Among the myriad natural

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resources under scrutiny, SGB has emerged as a prominent focus of intensive research. Renowned for its rich history of traditional medicinal uses and promising pharmacological attributes, SGB has garnered increased amounts of attention from researchers seeking to unlock its therapeutic potential [1–3]. Given the extensive traditional medicinal legacy of SGB, concerted efforts have been made to decipher its phytochemical composition and explore its prospective therapeutic applications. However, the exceptional variability exhibited by SGB across different geographical regions has led to intriguing questions about its phytochemical profile and bioactive potential [4, 5].

The Vietnamese variant of SGB, influenced by biological and environmental factors, is unique in this botanical landscape [3, 6]. These geographical distinctions impart distinctive features to the phytochemical makeup of Vietnamese SGB, potentially shaping its medicinal properties [1]. Consequently, a comprehensive examination of these geographical variations is imperative to fully harness the health-promoting potential of botanical treasures.

The intricate phytochemical composition of SGB extracts necessitates the use of advanced analytical techniques. Among these methods, UPLC-QTOF-MS/MS is a potent tool for identifying and characterizing complex herbal extract mixtures [7–9]. Distinguished by its exceptional resolution and sensitivity, this analytical approach facilitates the precise determination of molecular weights and prediction of molecular compositions. The efficacy of this method has been demonstrated through the examination of secondary metabolites, including polyphenols, flavonoids, and alkaloids, in diverse botanical specimens [8, 10].

The Keap1/Nrf2 pathway, which coordinates antioxidant and cytoprotective activities, may not be the only mechanism of antioxidation [11, 12]. However, recent findings suggest the presence of alternate antioxidative pathways that function autonomously from Keap1/Nrf2 [13, 14]. These Keap1/Nrf2-independent processes hold tremendous promise, providing novel paths for exploring antioxidant activity and widening the boundaries of potential therapeutic applications.

The use of zebrafish as a model organism has received much attention in the scientific community because of the potential for researching the physiological effects of phytochemicals. The use of zebrafish as a model organism has received much attention in the scientific community because of the potential for researching the physiological effects of phytochemicals [15]. Boasting genetic similarities with humans and transparent embryos that facilitate real-time monitoring of physiological processes, zebrafish offer a unique advantage in scientific research [16]. Furthermore, the prolific reproductive capabilities of zebrafish enable high-throughput experimentation, rendering them an invaluable asset in the pursuit of scientific inquiry [17]. Therefore, the application of UPLC-QTOF-MS/MS technology to explore the phytochemical content of Vietnamese SGB extracts coupled with the assessment of their antioxidative activities in zebrafish represents a robust approach to unravel the therapeutic potential of this plant.

The primary goal of this research was to undertake a comprehensive investigation of the phytochemical composition of extracts of SGB, focusing in particular on the unique variety grown in Vietnam and affected by local environmental and geographical conditions. Additionally, we evaluated the antioxidative activities of SGB extracts using a zebrafish model, shedding light on potential therapeutic applications beyond the classical Keap1/Nrf2 pathway. Through this multifaceted approach, we aimed to elucidate the health-promoting potential of SGB and contribute to our understanding of natural product research for medicinal and therapeutic purposes.

Materials and Methods

Plant Material and Extract Preparation

SGB-dried rhizomes were obtained from Ea So commune, Ea Kar district, Daklak Province, Vietnam. Before being stored in a dry, shaded area, the material was subjected to stringent selection, cleaning, and fine grinding. The SGB roots were dried at 60 °C until the moisture content was reduced to less than 5%. The plants were subsequently crushed and sieved through a 0.25-mm sieve shaker (Retsch, Vietnam). At room temperature for 72 h, finely powdered samples were extracted with 96% ethanol (EtOH) at 1:20 (v/v) (3 times).

To prepare the SGB extract, a Buchi R300 vacuum rotary evaporator from Buchi, Switzerland, was used. The process involved alcohol collection first at an initial evaporator pressure of 175 mBar, which was gradually decreased to 72 mBar for water collection. The evaporation operated at a rate of 100 rpm, with the heating tank temperature set at 60 °C and a condensation temperature of 10 °C. Mass consistency was monitored to confirm completion of the process. After weighing, the concentrated herbal extracts were transferred to Falcon tubes and dissolved in dimethyl sulfoxide (DMSO) obtained from Merck, Germany. The stock solution had a high concentration of 100 mg/mL. The solution was then aliquoted into 1.5-mL Eppendorf tubes for future use and preserved at − 20 °C.

To prepare for UPLC-QTOF-MS/MS analysis, 0.1 g of SGB extract was diluted in 1 mL of methanol (Merck, Germany) for 5 min and filtered through 0.22 μm PTFE (Millex, USA).

UPLC-QTOF-MS/MS Analysis

The phytochemical profile of the SGB extract was determined using a UPLC-QTOF-MS/MS system. Chromatographic separation was conducted at 40 °C with a Waters HSS T3 column (C18, 2.1 100 mm, 1.8 μm). The mobile phases comprised 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (Merck, Germany) (mobile phase B). The gradient elution program was as follows: 19–50% B (0–7 min), 50–96% B (7–12 min), 96–98% B (12–13 min), 98% B (13–25 min), and 98–19% B (25–29 min). The analyses were performed using an ESI source in positive mode, covering a mass range of 100 to 1200 Da.

The operating parameters for the ESI source were set as follows: capillary voltage, 3.0 kV; cone voltage, 30 V; and source temperature, 120 °C. Desolvation was carried out at 350 °C, and the MS/MS collision energy was set to 35 eV. The cone gas flow and desolvation gas flows were 110 and 1000 L/h, respectively [18].

LC−MS Dataset Analysis

The complete LC−MS dataset was subjected to meticulous processing, peak picking, and analysis through advanced UNIFI software from Water Corporation, USA. Employing a state-of-the-art 3D peak detection algorithm, this software intricately scrutinized the spatial shapes of ion responses, achieving precise identification of peak apexes. The results outperformed conventional 2D extracted ion chromatograms, which yielded significantly cleaner spectra and more accurate peak volumes. To gain a comprehensive understanding of the sample's composition, each ion's total intensity was normalized to the total ion

count, resulting in a data matrix that included crucial information such as *m/z* values, retention times, and normalized peak areas [19].

In refining the analysis, additional data processing with UNIFI's tools involved chromatogram smoothing using specified parameters (type, mean; half-width, 2 data points; iterations, 2), effectively reducing noise and enhancing peak resolution. Leveraging UNIFI's discovery tools, researchers identified variables of interest that seamlessly connected to esteemed natural product libraries, including the University of Ottawa Natural Product Library, the University of Mississippi Natural Product Library, and the Green Tea Library, serving as invaluable guides in navigating the molecular landscape. The stringent criterion for "good matches" was applied, considering compounds with a mass accuracy within 5 mDa, a response exceeding 2000, and adherence to the isotope distribution threshold. This rigorous approach ensured the precise identification of concealed molecular components within the sample.

With UNIFI software, the most abundant phytochemical was determined by analyzing the UPLC−QTOF–MS/MS data. The chromatogram was visually inspected, and the compound with the highest peak area, representing its abundance, was identified. The peak table, which lists compounds with relevant information, was sorted by area in descending order. The compound at the top of the list, which had the highest area, was considered the most abundant phytochemical in the sample.

Maintaining Zebrafish

Wild-type AB zebrafish were obtained from the Prof. Makoto Kobayashi Laboratory at the University of Tsukuba and were grown at the Biotechnology Center of Ho Chi Minh City Laboratory. Water temperatures were maintained between 26 and 28 °C, while pH values ranged from 6.8 to 7.5. To simulate the daily cycle of fish, each individual was exposed to 14 h of light and 10 h of darkness.

LC_{50} Determination and Survival Rescue Experiment

First, the acute toxicities of sodium arsenite ($NaAsO_2$) (Xiya Reagent, China) and hydrogen peroxide (H_2O_2) (Xilong Scientific, China) were established in 4-day postfertilization (dpf) zebrafish larvae. LC_{50} values were determined for $NaAsO_2$ (1–4 mM) and H_2O_2 (1–5 mM) concentrations.

To determine the safety of the SGB extract, 3.5 dpf larvae were exposed to a range of doses (50–1000 µg/mL) for 72 h in 6-well plates. This identified a safe concentration for subsequent experiments.

The protective effect of SGB against $NaAsO_2$- and H_2O_2-induced toxicity was then evaluated using a resecure assay modified from Fuse et al. [20]. Briefly, 3.5 dpf larvae were pretreated with the safe SGB dose for 12 h. The SGB solution was then replaced with $NaAsO_2$ or H_2O_2 solution, and the larvae were exposed for an additional 48 h. Fish mortality was monitored every 12 h throughout the exposure period, with no feeding.

Gene Expression Analysis

We extensively examined the gene expression of Nrf2 target genes, including glutathione S-transferase Pi 1 (*gstp1*) and peroxiredoxin 1 (*prdx1*), utilizing real-time PCR. The initial

steps involved the careful extraction of total RNA from 20 chemically treated larvae using TRIzol Reagent (Invitrogen, USA), followed by cDNA synthesis with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA). Subsequently, real-time quantitative PCR (qPCR) was performed with a Real-time LightCycler® 96 System (Roche Life Science, USA) and Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher, USA). The sequences of primers used for the targeted genes were *ef1α* (5′-CGTGGTAATGTGGCTGGAGA and 5′-CTGAGCGTTGAAGTTGGCAG), *gstp1* (5′-CAACGCCATGCTGAGACATC; and 5′-GAAGATCTTCAACGCCGCCGTCG), and *prdx1* (5′-GTCCCACTGAGATCATCGCCCCCC; and 5′-AACCACCTTTTTTTTTTTTGGGGT) [14].

Data analysis was performed using the $2^{-\Delta\Delta CT}$ relative quantification method [14, 21] to gain precise insight into gene expression dynamics. This method made it possible to accurately assess changes in gene expression levels in comparison to reference genes and control conditions, providing a rigorous and robust quantitative framework for the investigation.

Analytical Statistics

The log-rank test was used to analyze survival data, while two-tailed Student's *t* test was utilized to evaluate gene expression levels. To determine the statistical significance of the results, a significance threshold of $p < 0.05$ was used.

Results

Phytochemical Profile of the Ethanol Extract of SGB

To elucidate the complex phytochemical composition of SGB, a botanical species known for its versatile medicinal properties, an extensive chemical analysis was performed. This study revealed a diverse array of bioactive compounds from various chemical categories, each with distinct structural properties and bioactive potential. In this research, we formulated equations based on the excimer $[M+H]^+$ with a mass error tolerance of 10 ppm and partial isotopic abundance. Next, we conducted searches in chemical databases, including ChemSpider (www.chemspider.com) and MassBank (http://www.massbank.jp), to pinpoint the most plausible chemical formulas. Employing UPLC-QTOF-MS/MS analysis of the ethanol extract of SGB, we successfully identified a wide range of compounds from different chemical categories, and their distinctive attributes, which are consistent with those of natural products (mass accuracy within 5 mD, response > 2000, isotope distribution threshold), are comprehensively documented in Table 1.

The UPLC/TOF-MS^E base peak intensity (BPI) chromatogram of the SGB extracts is shown in Fig. 1, while UPLC-QTOF-MS/MS analysis methodically revealed the comprehensive phytochemical profile of the SGB ethanol extract. This investigation revealed a diverse range of molecules from several chemical groups. For thorough investigation, a sophisticated UPLC−QTOF−MS/MS mass spectrometry system was used, and accurate data analysis was performed using Waters UNIFI software. Flavonoids, alkaloids, terpenoids, glycosides, organic acids, phenolics, and steroids were among the 39 chemicals found. Table 1 provides a detailed summary of these compounds, including crucial information such as reported retention durations (RTs), component names, chemical formulas, observed *m*/*z* values, and mass errors (ppm).

Table 1 Compounds identified in the ethanolic extract of *Smilax glabra* Roxb (SGB) using UPLC-QTOF-MS/MS

Row No	Observed RT (min)	Component name	Formula	Observed m/z	Mass error (ppm)	Area	Area ratio (%)	Reference (component of *SGB*)
Flavonoids								
1	2.23	Astilbin	C21H22O11	451.1202	−3.2	12,840,689	6.69	(Zhao et al., 2020)
2	1.88	Cinchonain Ia	C24H20O9	453.1213	3.3	1,536,967	0.80	(Xu et al., 2013)
3	7.65	Skullcapflavone	C17H14O6	315.0833	−9.7	595,885	0.31	
4	2.38	3-O-β-D-Galacopyanosyl quercetin	C21H22O12	467.119	0.6	136,563	0.07	(Xu et al., 2013)
5	1.99	Neoeriocitrin	C27H32O15	597.1769	−7.5	2,064,145	1.08	
Alkaloids								
6	3.74	Catenarin	C15H10O6	287.0524	−8.9	6,262,388	3.26	
7	1.06	Aloenin	C19H22O10	411.1246	−9.7	6,512,643	3.39	
8	9.04	Pingpeimine A	C27H45NO5	464.3326	−9.5	3,360,005	1.75	
9	10.33	14-Formyldihydrorutaecarpine	C19H15N3O2	318.1259	7.1	453,702	0.24	
10	10.66	Corynoxeine	C22H26N2O4	383.2001	9.4	7,882,516	4.11	
11	11.08	Oxofangchirine	C37H34N2O7	619.2422	−2.7	2,239,429	1.17	
Terpenes/terpenoids								
12	12.69	Cimiside B	C40H64O13	753.438	−5.3	2,919,677	1.52	
13	21.39	Acanthopanax cerebroside B	C47H91NO10	830.6701	−1.8	627,378	0.33	
14	11.72	Noracanthopanin A	C19H26O4	319.1908	1.3	2,133,360	1.11	
15	10.31	Xiongterpene	C39H54O5	603.4064	3.2	6,402,062	3.33	
16	12.53	22-Hydroxy-25(R,S)-furostan-5-en-12-one-3β,22,26-triol-26-O-β-D-glucopyranoside	C33H52O10	609.3636	0.5	1,259,219	0.66	
Glycosides								
17	2	1,3-Dihydroxy-2-methoxyxanthone	C14H10O5	259.0576	−9.8	13,866,202	7.22	
18	3.39	Pelargonidin 3,5-diglucoside	C27H31ClO15	631.1389	−5.6	1,197,255	0.62	
19	11.5	Lappaol D	C31H36O10	569.2383	0.3	2,423,617	1.26	

Table 1 (continued)

Row No	Observed RT (min)	Component name	Formula	Observed m/z	Mass error (ppm)	Area	Area ratio (%)	Reference (component of *SGB*)
20	1.56	Asperuloside tetraacetate	C26H30O15	583.1636	−3.7	805,840	0.42	
Organic acids								
21	1.06	Diferulic acid	C20H18O8	387.1079	0.4	29,516	0.02	(Qin, 2007)
22	14.92	Vitamin K2	C41H56O2	581.4347	−1	2,275,534	1.19	
Phenolic								
23	10.68	(+)Syringaresinol-4-O-β-D-glucopyranoside	C28H36O13	581.2258	2.9	141,286	0.07	(Wu et al., 2022)
24	13.32	7-oxo-β-Sitosteryl tetra-O-acetyl-β-D-glycopyranoside	C43H66O11	759.469	1.2	64,911	0.03	(Wu et al., 2022)
25	15.67	Apigenin-7-O-β-D-glucuronopyranoside	C22H20O10	445.1165	3.7	933,606	0.49	(Wu et al., 2022)
26	1.45	Onjixanthone	C15H12O7	305.0626	−9.8	2,639,801	1.38	
27	7.85	Feralolide	C18H16O7	345.0939	−8.7	4,235,643	2.21	
28	2.58	Medicagol	C16H8O6	297.0367	−8.9	18,943,051	9.87	
29	3.81	Mururin A	C24H16O9	449.0831	−8.1	14,246,303	7.42	
30	11.4	Arbutin	C12H16O7	272.0896	−9.8	803,067	0.42	
31	11.07	23,27-Dihydroxypennogenin	C27H42O6	463.3029	−5.5	5,316,054	2.77	
32	11.5	Clinopodiside F	C49H82O20	991.5494	2.2	7,238,195	3.77	
Steroid								
33	14.79	7-oxo-β-Sitosterol	C29H48O2	429.3726	−0.2	247,728	0.13	(Wu et al., 2022)
34	10.27	(E,E)-9-Oxooctadeca-10,12-dienoic acid	C18H30O3	295.2265	−1	624,890	0.33	
35	12.9	Tetratriacontanamine	C34H71N	494.5611	−9.7	1,504,827	0.78	
36	12.52	11-O-p-Coumarylnepeticin	C39H56O4	589.4239	−2.1	20,456,652	10.66	
37	11.06	Decumbesterone A	C29H46O7	507.3292	−4.7	2,837,952	1.48	

Table 1 (continued)

Row No	Observed RT (min)	Component name	Formula	Observed m/z	Mass error (ppm)	Area	Area ratio (%)	Reference (component of *SGB*)
38	10.06	Cynanoside P5	C49H76O20	985.5036	3.4	1,504,827	0.78	
39	23.66	Phytolacca cerebroside	C48H93NO10	844.6846	−3.1	3,133,858	1.63	
40	8.51	Bufotalinin	C24H30O6	415.2079	−8.6	29,287,323	15.26	

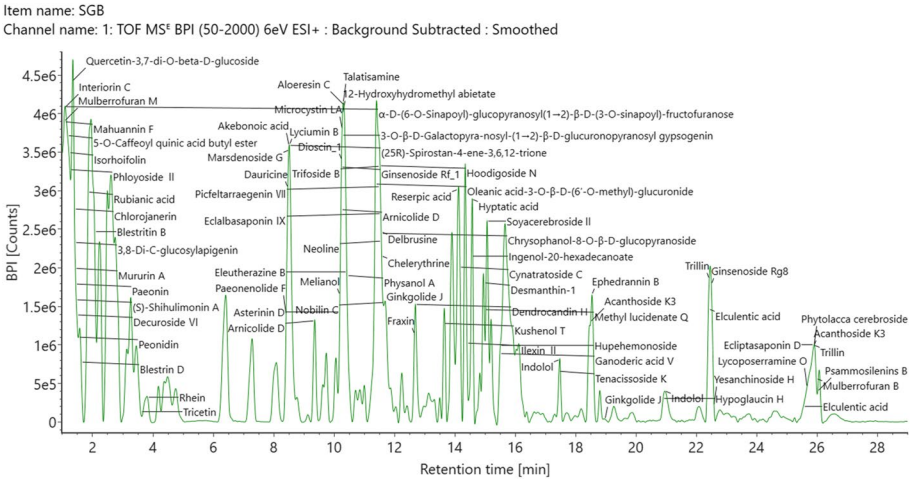

Fig. 1 Representative BPI chromatograms of *Smilax glabra* Roxb extract samples

SGB contains a variety of compounds, including **flavonoids** such as astilbin (RT: 2.23 min, m/z: 451.1202, − 3.2 ppm), cinchonain Ia (1.88 min, m/z: 453.1213, 3.3 ppm), Skullcapflavone (RT: 7.65 min, m/z: 315.0833, − 9.7 ppm), 3-O—D-Galacopyanosyl quercetin (RT: 2.38 min, m/z: 467.119, 0.6 ppm), and Neoaeriocitrin (RT: 1.99 min, m/z: 597.1769, − 7.5 ppm). Alkaloids, such as catenarin, aloenin, pingpeimine A, 14-formyl-dihydrorutaecarpine, and corynoxeine, were observed at specific m/z values. The terpene/terpenoid category included compounds such as Cimiside B, Acanthopanax cerebroside B, noracanthopanin A, and Xiongterpene. In the glycoside category, we identified 1,3-dihydroxy-2-methoxyxanthone, pelargonidin 3,5-diglucoside, lappaol D, and asperuloside tetraacetate. Organic acids, including diferulic acid and vitamin K2, were also present. The phenolic compounds included substances such as (+) syringaresinol-4-O-β-D-glucopyranoside, 7-oxo-β-sitosteryl tetra-O-acetyl-β-D-glycopyranoside, and apigenin-7-O-β-D-glucuronopyranoside. The steroids used included 7-oxo-β-sitosterol and (E,E)-9-oxooctadeca-10,12-dienoic acid. Finally, compounds from various chemical classes, such as tetratriacontanamine, 11-O-p-coumarylnepeticin, decumbesterone A, cynanoside P5, phytolacca cerebroside, and bufotalinin, were also found in SGB.

The predominant pollinins were bufotalinin (15.26%) and 11-O-p-coumarylnepeticin (10.66%), which together constituted more than a quarter (25.92%) of the SGB extract (Table 1). Interestingly, these two compounds belong to the steroid group and are known for their diverse biological properties, which aligns with the observed characteristics of SGB extract, including potential for growth stimulation, metabolic regulation, and anti-inflammatory and antiviral effects [22].

Other prominent contributors included medicagol (9.87%), 1,3-dihydroxy-2-methoxyxanthone (7.22%), mururin A (7.42%), astilbin (6.69%), catenarin (3.26%), alloenin (3.39%), corynoxeine (4.11%), Xiongterpenes (3.33%), and clinopodiside F (3.77%) (Table 1).

Intriguingly, a comparison with existing research revealed that 32 of the 40 compounds identified in the Vietnamese SGB extract are novel and not previously documented (Table 1). This highlights the remarkable diversity of natural products across regions, suggesting that geographical variations influence both chemical composition and biosynthetic pathways.

These findings offer a comprehensive overview of the intricate chemical composition of SGB, shedding light on the potential pharmacological applications and medicinal significance of this plant species in various domains of natural-product research.

LC_{50} Values of $NaAsO_2$, H_2O_2, and SGB Extracts

We tested the antioxidant properties of the SGB extract against hydrogen peroxide (H_2O_2) and arsenite ($NaAsO_2$), as shown in Table 1. Our assessment examined zebrafish larval survival at 3.5 dpf using SGB and 4 dpf using $NaAsO_2$ and H_2O_2. The main emphasis of this investigation was the LC_{50} values of the treatments. The effects of the $NaAsO_2$, H_2O_2, and SGB extracts on larvae are shown in Fig. 2A.

Zebrafish larvae died at 48- and 12-h intervals at 4 dpf when exposed to 2–4 mM arsenic. Figure 2B shows that the LC_{50} indices for 1.5 mM arsenic were calculated after 24 h

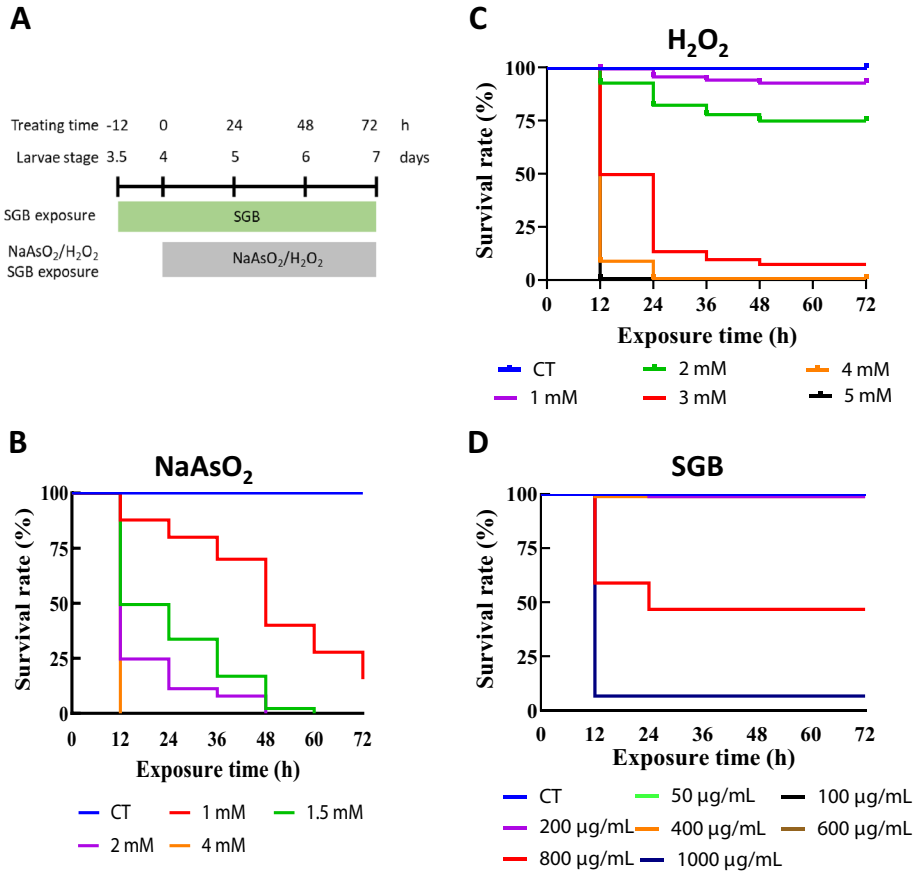

Fig. 2 LC_{50} values for the $NaAsO_2$, H_2O_2, and SGB extracts are presented. **A** The experimental setup is described, where AB larvae were exposed to varying concentrations of $NaAsO_2$ and H_2O_2 at 4 dpf and treated with *Smilax glabra* Roxb (SGB) extract at 3.5 dpf for 12 h, with survival recorded every 12 h until 7 dpf. Survival curves for $NaAsO_2$-treated (**B**), H_2O_2-treated (**C**), and SGB-treated (**D**) larvae are shown, and data from three experiments were pooled for analysis

and for 1 mM after more than 60 h. Thus, acute toxicity experiments were performed with a concentration of 1 mM.

Similarly, 4 dpf larvae exposed to 5 mM H_2O_2 died after 12 h, while 4 mM H_2O_2-treated larvae died after 24 h. The survival rate of the zebrafish larvae ranged between 1 and 3 mM H_2O_2, with a concentration of 3 mM considered adequate for measuring the LC_{50} within 24 h (Fig. 3C), allowing for additional studies of acute toxicity.

Establishment of the LC_{50} for SGB extract-treated zebrafish larvae is at 3.5 dpf. Zebrafish larvae were exposed to various concentrations of SGB for 72 h, spanning a concentration range of 50–1000 µg/mL (Fig. 2A). The resultant data, as depicted in Fig. 2D, indicated that the LC_{50} value of the SGB extract was 800 µg/mL after 24 h of exposure. Remarkably, SGB concentrations less than 600 µg/mL had minimal impact on the overall well-being of zebrafish larvae at this developmental stage. Consequently, a concentration range of 100 − 400 µg/mL was identified as safe for subsequent experiments focused on assessing the protective efficacy of SGB on zebrafish larvae.

In Vivo Antioxidant Effect of the SGB Extract

We used 3.5-dpf larvae as our experimental model to investigate the potential of SGB extract to mitigate oxidative stress caused by 3 mM H_2O_2 and 1 mM $NaAsO_2$. SGB concentrations ranging from 100 to 400 µg/mL were applied to the specimens (Fig. 3A and B).

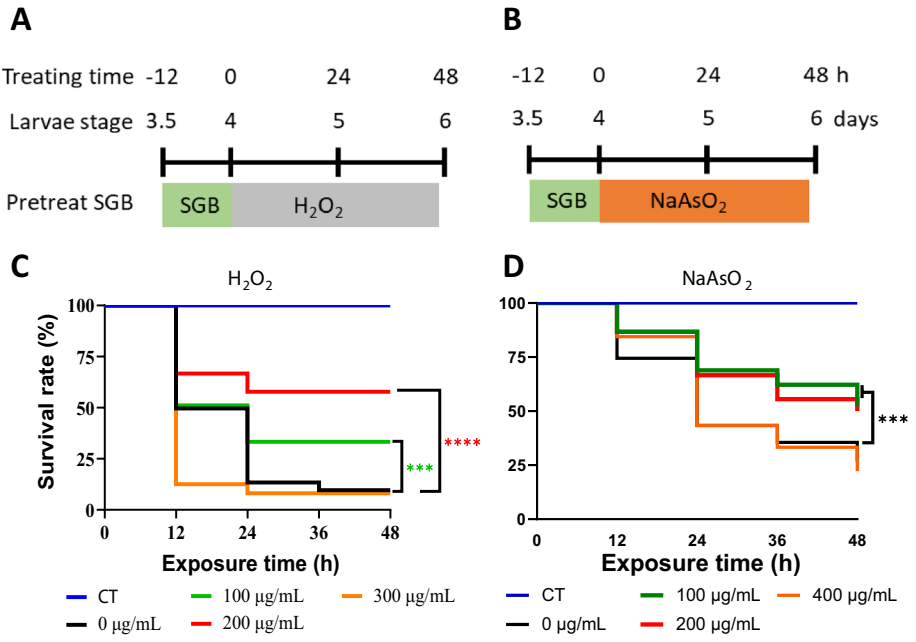

Fig. 3 Antioxidant activity of *Smilax glabra* Roxb (SGB) extract in zebrafish larvae. **A, B** The experimental setup for survival testing with $NaAsO_2$ and H_2O_2, respectively. **C** Protective effects of SGB at concentrations ranging from 100 to 400 µg/mL against 1 mM H_2O_2 toxicity. **D** The protective effects of SGB within the same concentration range against 3 mM H_2O_2 toxicity. The experiment was conducted three times, with a sample size of $n = 135$. Statistical significance is denoted by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$), and "ns" signifies no statistical significance

Treatment with SGB at dosages ranging from 100–200 µg/mL significantly improved the survival rate in cells exposed to 3 mM H_2O_2 (Fig. 3C). After 48 h of H_2O_2 exposure, the 200 µg/mL SGB extract solution increased survival by 57.8% (Fig. 3C). Additionally, SGB concentrations ranging from 100 to 200 g/mL dramatically enhanced larval survival in the presence of 1 mM arsenic (Fig. 3D). Survival rates within this concentration range reached astonishingly high levels of 33.3% and 57.8%, respectively, in stark contrast to the 7.4% survival rate of the non-SGB control group.

These findings show that SGB has strong protective potential as a pretreatment method, as it significantly increases the survival capacity of zebrafish larvae exposed to arsenic and hydroperoxide-induced stresses. These findings show that SGB has strong protective potential as a pretreatment method, as it significantly increases the survival capacity of zebrafish larvae exposed to arsenic and hydroperoxide-induced stresses. Surprisingly, an SGB concentration of 200 µg/mL was found to be the most effective, necessitating additional research, especially in the context of gene expression analysis.

Keap1/Nrf2-Independent Antioxidant Effects of SGB Extract

To determine the protective effect of the Keap1/Nrf2 pathway against oxidative stress in zebrafish larvae, we evaluated the expression of Nrf2 target genes (gstp1 and prdx1). This was accomplished by exposing 3.5 dpf larvae for 12 h to 200 µg/mL SGB and 40 µM sulforaphane (SF) (Sigma, USA), a recognized Nrf2 activator derived from broccoli [20, 23–25]. The results, depicted in Fig. 4A and B, revealed robust induction of *gstp1* and *prdx1* expression in SF-treated larvae. However, larvae treated with SGB did not demonstrate such induction. These findings suggest that while the SGB extract positively influenced the survival rate of zebrafish larvae, it did not exert a discernible impact on the expression levels of the *gstp1* and *prdx1* genes compared to those in

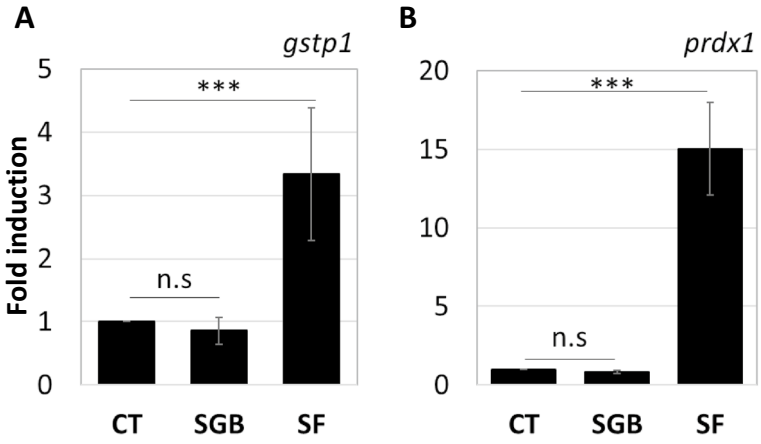

Fig. 4 Nrf2 target gene expression in *Smilax glabra* Roxb (SGB) extract-treated larvae. Larvae at 3.5 dpf were exposed to 400 µg/mL SGB and 40 µM sulforaphane (SF) for 12 h, and qRT−PCR analysis was performed to assess the expression levels of gstp1 (**A**) and prdx1 (**B**). The expression of untreated larvae (CT) was set to 1 for normalization. Each experiment was independently replicated at least three times with duplicate samples

control larvae. This finding suggested that the modulatory effect of SGB on antioxidant activity may occur through mechanisms other than the Keap1/Nrf2 pathway.

Discussion

A comprehensive analysis of ethanol extracts from Vietnamese SGB using UPLC-QTOF-MS/MS mass spectrometry revealed a vast array of phytochemical compounds. As detailed in Table 1 and visually depicted in Fig. 1, these compounds belong to a variety of chemical classes, including flavonoids, alkaloids, terpenoids, glycosides, organic acids, phenolics, and steroids. This paper explores in detail the possible pharmacological relevance of Vietnamese SGB and its prospective applications in numerous areas, including medicine and health.

The extract contains pharmacologically significant flavonoids. Previous studies have shown these compounds to be antioxidant, anti-inflammatory, antiviral, anticancer, and hepatoprotective agents [26–28]. The flavonoids in the ethanol extract included astillbin, cinchonain Ia, skullcapflavone, 3-O-D-galactopyranosyl quercetin, and neoeriocitrin. Astelbin activates Nrf2 and upregulates antioxidant genes in HEK-293 cells to reduce ROS accumulation [29]. Additionally, astuline has anti-inflammatory, antioxidant, and anticancer effects [27]. Like skullcapflavone, 3-O-D-galactopyranosyl quercetin activates Nrf2 and has anti-inflammatory and antibacterial effects, making it a viable medicinal candidate [26, 28, 30].

The anti-inflammatory, antioxidant, and anticancer alkaloids in the ethanol extract included catenarin, aloenin, pingpeimine A, 14-formyldihydrorutaecarpine, corynoxeine, and oxofangchirine. Inhibiting the CXCR4 and CCR5 pathways and decreasing p38 and JNK phosphorylation effectively ameliorate type 1 diabetes in NOD mice [31]. Pingpeimine A (Pingpeimine A) exhibits anti-inflammatory, anticancer, and antioxidant properties, making it a promising candidate for neuroprotection, cognitive enhancement, and cancer, Alzheimer's disease, and Parkinson's disease treatment [32, 33].

Phenolic compounds, which have hydroxyl (-OH) groups and phenol rings, offer antioxidant, antibacterial, antifungal, and anticancer benefits [34, 35]. The extract contains various phenolic compounds, including (+)syringaresinol-4-O-β-D-glucopyranoside, 7-oxo-β-sitosteryl tetra-O-acetyl-β-D-glycopyranoside, apigenin-7-O-β-D-glucuronopyranoside, onjixanthone, fulvicolide, medicagol, mururin A, arbutin, 23,27-dihydroxypennogenin, and (+)syringaresinol-4-O-D-glucopyranoside, which activate Nrf2 to reduce H_2O_2-induced oxidative stress in HepG2 cells [35]. Arbutin has antioxidant and anti-inflammatory effects, and SIRT1 activation promotes Nrf2 and downstream genes such as HO-1, contributing to anti-inflammatory and antioxidant effects, including mitigating lung injury [36, 37].

The ethanol extract contains numerous steroid compounds, such as 7-oxo-sitosterol, (E,E)-9-oxooctadeca-10,12-dienoic acid, tetratriacontanamine, 11-O-p-coumarylnepeticin, decumbesterone A, cynananside P5, phytolacca cerebroside, and bufotalinin. The enhanced interaction between the antibiotic amoxicillin and 7-oxo-sitosterol, which results in a more potent antibacterial effect [38], is an intriguing observation. Furthermore, Mishra et al. (2005) reported that phytolacca cerebroside plays a role in immune regulation and infection prevention [39]. In contrast, bufotalinin possesses antiviral and anticancer properties [32].

The ethanol extract of Vietnamese SGB was analyzed and compared to that of SGB from other nations. This analysis revealed that the extract contains a number of compounds that

are likely the consequence of Vietnam's tropical monsoon climate and soil conditions [40]. Approximately 31 of the 39 identified compounds are either novel or have only been studied recently in relation to SGB. These findings suggest that SGB has a promising future in medical research. Six of these compounds, astilbin, skullcapflavone, pingpeimine A, diferulic-gykpl acid, (+) syringaresinol-4-O—D-glucopyranoside, and arbutin, are of particular interest because they activate Nrf2 and induce the expression of genes involved in cellular antioxidant defense [26, 27, 32, 36, 41]. Contrary to the findings presented in Fig. 4, the SGB extract did not trigger the Nrf2 target gene signal. This could be because the concentration of Nrf2 activators in SGB is insufficient to amplify this gene's signal in zebrafish [42]. Interestingly, our research suggested that the antioxidative effects of SGB may not rely on the Nrf2 pathway. This finding suggested that SGB could be an effective strategy for mitigating arsenite-induced oxidative stress, without causing potential adverse effects from Nrf2 activation [43, 44].

Additional investigations into the pharmacological effects of SGB compounds are necessary, especially to clarify their biological mechanisms, to advance clinical therapeutic applications and therapies in the future. Furthermore, although SGB has been the focus of most domestic and international research efforts, thorough analyses of the leaves, flowers, and fruits of this plant are crucial for obtaining a comprehensive understanding of the properties of its chemical components. Additionally, differences in chemical composition, as observed in the pharmacological actions of substances such as astilbin and neoastilbin, can occur [1]. Despite the extensive isolation and identification of chemical compounds from SGB, fundamental research into their pharmacological effects, particularly their underlying biological mechanisms, is currently essential for future advancements in therapeutic applications, such as antioxidant effects.

Our findings showed that the SGB extract strongly increased the survival of zebrafish larvae under oxidative stress caused by both $NaAsO_2$ and H_2O_2. Increased doses of the extract resulted in increased survival rates, indicating that the protective effects of the extract were dose dependent. Notably, SGB concentrations less than a particular threshold had little effect on the general health of the zebrafish larvae at this stage of development, indicating that the SGB extract can effectively reduce the damaging effects of oxidative stressors without harming the organisms. Our findings showed that when subjected to oxidative stress caused by both $NaAsO_2$ and H_2O_2, the SGB extract dramatically boosted the survival of zebrafish larvae. Higher doses of the extract increased survival rates, demonstrating dose-dependent protective effects of the substance. Notably, SGB extract at concentrations less than a specific threshold had little effect on the general health of the zebrafish larvae at this developmental stage, indicating that SGB extract can successfully reduce the damaging effects of oxidative stressors without harming the creatures. Arsenite is infamous for impairing liver and renal function, causing serious health concerns [45]. However, additional molecular biology studies are needed to fully understand how SGB improves the removal of arsenic and increases cell resistance to oxidative stress. By examining this process, we will be able to obtain most of the SGB extracts as antioxidants and detoxifiers.

Conclusion

The potential of the SGB extract as a powerful antioxidant for preventing oxidative stress caused by arsenite was highlighted by our study. Its Keap1/Nrf2-independent process opens up promising new options for future molecular biology studies and safer medical procedures. Additionally, we found that the extract has a rich phytochemical makeup with a

variety of pharmacological activities that are interesting for use in medicine. Fungal larvae exposed to the SGB extract exhibited strong protection against oxidative stress, demonstrating its potential as a natural antioxidant. Its mechanics, clinical applications, and chances for novel compound discovery in natural product research all call for further research.

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Data Availability Upon reasonable request, the corresponding author will provide access to the datasets created during and/or used in the current work.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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