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Investigation of Anti-oxidative Stress and Anti-inflammatory Constituents from *Sphaerocoryne affinis* Leaves

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Abstract: *Sphaerocoryne affinis* is prevalent in numerous forests throughout Vietnam and has traditionally been used in the production of alcoholic beverages, as well as in folk medicines for treating various diseases. This study provided the first comprehensive investigation into the anti-oxidative stress and anti-inflammatory properties of *S. affinis* leaves. These results revealed that the *S. affinis* leaf extract and its fractions demonstrated notable antioxidant activity and stress-mitigating effects, as indicated by the prolonged lifespan of *Drosophila melanogaster* subjected to paraquat (PQ)-induced oxidative stress. Furthermore, the anti-inflammatory potential of *S. affinis* leaf extract and its ethyl acetate and hexane fractions was validated through their ability to inhibit NO secretion in LPS-stimulated RAW264.7 macrophage cells. Besides, 12 known bioactive compounds were isolated from the ethyl acetate fraction of *S. affinis* leaf extract, among which 8 compounds were identified for the first time in the genus *Sphaerocoryne*. These newly discovered compounds included allantoin, apigenin, rutin, isatin, 3-hydroxy-3-(2-oxopropyl)indolin-2-one, ethylene glycol dibenzoate, nicotiflorin, and 3,3',4,4'-tetrahydroxybiphenyl. These findings emphasized the potential of *S. affinis* leaves as a valuable source of bioactive compounds, paving the way for the development of innovative healthcare products and natural therapeutic interventions for various diseases.

Keywords: Antioxidant; anti-oxidative stress; anti-inflammatory; chemical composition; *Sphaerocoryne affinis*

1. Introduction

Oxidative stress is a condition caused by an imbalance between free radicals and antioxidants, leading to cellular damage and potential health complications [1]. At low concentrations, reactive nitrogen and oxygen species (RNS/ROS) are beneficial in physiological processes such as redox regulation, mitogenic responses, cellular signaling, and immune function; however, at higher levels, they lead to nitrosative and oxidative stress [2]. Notably, the overproduction of ROS from endogenous or exogenous sources leads to oxidative stress, which is frequently observed in various cancer types and serious diseases [1]. Furthermore, accumulating evidence indicated that oxidative stress plays a significant role in initiating and sustaining chronic inflammatory diseases [3]. Therefore, the human

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body has evolved an antioxidant defense system to mitigate or prevent oxidative damage from free radicals, involving free radical scavenging, metal chelation, and enzymatic activities that neutralize reactive species. Besides, consuming dietary antioxidants supports the maintenance of sufficient antioxidant levels in the body [2]. To address these issues, natural products derived from plants and herbs, including foods and healthcare supplements, are gaining increasing attention and becoming a preferred choice. However, these natural products must undergo comprehensive studies to evaluate their properties, active ingredients, and safety for human use. Researchers have utilized a variety of screening models to examine and discover anti-oxidative stress or anti-inflammatory ingredients. *Drosophila melanogaster,* widely used as a model organism, has facilitated the study of how different environmental oxygen conditions impact lifespan and oxidative stress. *D. melanogaster* serves as a valuable model organism for researching human physiology and diseases due to its brief lifespan, simple reproduction, and genetic manipulability while preserving key cellular mechanisms present in mammals [4,5]. Remarkably, substantial evidence demonstrated that dietary paraquat-induced oxidative stress significantly affects the longevity of different *D. melanogaster* strains [5]. Besides, macrophages, known for their role in inflammation, are often used to evaluate anti-inflammatory agents. During Lipopolysaccharide (LPS)-activated macrophage cells, inducible nitric oxide synthase (iNOS) catalyzes the production of high levels of nitric oxide (NO). As a result, the measurement of NO secretion and proinflammatory cytokines can reflect the intensity of inflammation, offering a basis for evaluating the efficacy of pharmacological agents in treating inflammation-related conditions [6]. The models mentioned above are well-suited for assessing the potential of ingredients that exhibit anti-oxidative stress and anti-inflammatory properties.

Sphaerocoryne affinis is prevalent in numerous forests throughout Vietnam, including Phu Quoc National Park and Tay Ninh. While the fruit of this tree is widely used for commercial products like wine and non-alcoholic beverages, its leaves have been less studied and are mainly associated with traditional remedies. Previous studies have reported that *S. affinis* fruit exhibited antioxidant properties and showed potential as a candidate for treating Parkinson's disease, particularly anti-cervical cancer [7,8]. This study evaluates bioactive compounds from *S. affinis* leaves in *D. melanogaster* models of Paraquat (PQ)-induced oxidative stress, and further examines anti-inflammatory constituents in LPS-activated macrophage cells. These findings suggested that the active ingredients in *S. affinis* leaves, especially their antioxidant and anti-inflammatory properties, hold significant potential for developing innovative healthcare products to aid in disease treatment and boost immune function.

2. Materials and Methods

2.1. Plant Material

In April 2022, *Sphaerocoryne affinis* fresh leaf (SAL) was meticulously gathered from Phu Quoc Island (coordinates: VN 10°14′N 103°57′E), Viet Nam. The identification of the plant species was conducted using the Vietnamese plant classification system and subsequently verified by Associate Professor Dang Minh Quan and Dr. Nguyen Thi Kim Hue from the Department of Biology, College of Natural Sciences, Can Tho University. The specimen has been cataloged under a code number (SAL.2022-PQ002) at Biochemistry Laboratory of Can Tho University and recognized at Institute of Tropical Biology (ITB)-Vietnam under a herbarium barcode: VNM00030510.

2.2. Extraction and Isolation of Active Phytochemicals from S. affinis

SAL (12 kg) was collected, thoroughly cleaned, decontaminated, sliced into smaller pieces, and dried at 55°C. The dried samples were finely ground into powder using a waring two-speed blender (Cole-Parmer, USA) and stored at -4°C for future use. For supernatant extraction, the powdered sample was immersed in 96% ethanol for 24 hours at room temperature over five cycles and filtered through Whatman No. 1 filter paper. The crude extract was then concentrated using a rotary evaporator under reduced pressure, yielding 900 g.

The crude extract underwent liquid-liquid extraction to separate its components into distinct fractions based on solubility in various solvents. This sequential process yielded three primary fractions: n-hexane (217 g), ethyl acetate (275 g), and aqueous (106 g). Each fraction was meticulously collected

and labeled for clarity in further analysis. The extracts were designated as SALH, SALE, and SALW for the n-hexane, ethyl acetate, and aqueous fractions, respectively. This systematic partitioning effectively separated bioactive compounds into solvent-specific groups, facilitating targeted investigations in subsequent experiments.

Following initial analysis using thin-layer chromatography, the biological activities of SALH, SALE, and SALW extracts from *S. affinis* leaves were investigated, leading to the suggestion of the SALE extract for compound isolation. Thin-layer chromatography (TLC) was performed on pre-coated silica gel 60 plates with a fluorescent indicator F_{254} (Merck, Germany) to monitor the purification process. The TLC plates were visualized under UV light at 254 and 365 nm wavelengths. Subsequently, the spots were observed by spraying the plates with a mixture of ethanol and 5% vanillin/H_2SO_4 (v/v), followed by heating on a hot plate to enhance the visualization. The analysis of the structural composition was conducted utilizing a Bruker Avance III-600 MHz NMR spectrometer. For this examination, the solvents CD_3OD and DMSO-d_6 were employed, facilitating a comprehensive understanding of the samples' characteristics and configurations. All data were presented in Supporting Information.

The SALE extract was then employed in normal phase column chromatography with a solvent system of n-hexane:EtOAc:MeOH, which yielded five fractions, including SALE.I (30.6 g), SALE.II (31.1 g), SALE.III (36.8 g), SALE.IV (25.6 g), and SALE.V (42.5 g).

The SALE.I fraction was eluted using an n-hexane:EtOAc (100:0–0:100, *v/v*) solvent system via normal phase column chromatography. This fractionation resulted in 10 subfractions (SALE.I.1–SALE.I.10). Compound **1** (12 mg) was isolated from the SALE.I.2 fraction by normal phase column chromatography using the solvent system $CHCl_3$:MeOH (97:3, *v/v*). Similarly, compound **2** (13 mg) was extracted from the SALE.I.3 subfraction with a solvent system of n-hexane:EtOAc (75:25, *v/v*).

The SALE.II fraction (31.1 g) was processed similarly, eluted with an n-hexane:EtOAc gradient (100:0–0:100, *v/v*) via normal phase column chromatography. This resulted in the collection of 7 subfractions (SALE.II.1–SALE.II.7). Compound **3** (23 mg) was obtained from the SALE.II.1 subfraction using $CHCl_3$:MeOH (99:1, *v/v*). Compound **4** (14 mg) was isolated from SALE.II.2 using $CHCl_3$:MeOH (98:2, *v/v*), while compound **5** (30 mg) was extracted from SALE.II.3 by employing $CHCl_3$:MeOH (95:5, *v/v*). Compound **6** (17 mg) was obtained from SALE.II.5 using $CHCl_3$:MeOH (70:30, *v/v*).

For the SALE.III fraction (36.8 g), elution was performed using the n-hexane:EtOAc (100:0–0:100, *v/v*). This led to the isolation of 8 subfractions (SALE.III.1–SALE.III.8). Compound **7** (7 mg) was isolated from the SALE.III.1 subfraction with n-hexane:EtOAc (80:20, *v/v*), while compound **8** (20 mg) was extracted from SALE.III.2 using n-hexane: EtOAc (50:50, *v/v*).

The SALE.IV fraction (25.6 g) underwent normal phase column chromatography and was eluted with the solvent system n-hexane:EtOAc (100:0–0:100, *v/v*), resulting in 10 subfractions (SALE.IV.1–SALE.IV.10). Compound **9** (9 mg) was obtained from SALE.IV.2 using DC:MeOH (97:3, *v/v*), while compound **10** (5.8 mg) was isolated from SALE.IV.3 with $CHCl_3$:EtOAc (20:80, v/v).

Finally, the SALE.V fraction (42.5 g) was eluted using n-hexane:EtOAc (100:0–0:100, *v/v*), resulting in 7 subfractions (SALE.V.1–SALE.V.7). SALE.V.3 was further subjected with a solvent system of EtOAc:MeOH (100:0–70:30, *v/v*), yielding 6 segments (SALE.V.3.1–SALE.V.3.6). Compound **11** (16 mg) was isolated from SALE.V.3.1 using reverse-phase column chromatography with MeOH:W (30:70, *v/v*), while compound **12** (12 mg) was extracted from SALE.V.4 with DC:MeOH (80:20, *v/v*).

2.3. Total Polyphenolic, Flavonoid contents, and Antioxidant activity

Quantification of Total Flavonoids (TFC): Total Flavonoids (TFC) were quantified using the aluminum chloride ($AlCl_3$) coloration method, with a standard curve ($y = 0.0759x + 0.0345$; $R^2 = 0.999$) based on quercetin (QE). Results are expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g extract) [9].

Total polyphenol content (TPC) was determined utilizing the Folin-Ciocalteu reagent, following the method outlined in a previous report [10]. This measurement was based on a standard curve ($y = 0.0067x – 0.0025$; $R^2 = 0.998$) created using gallic acid, which serves as a reference point for

quantification. The results obtained from this analysis are expressed in terms of milligrams of gallic acid equivalent per gram of extract, abbreviated as mg GAE/g extract.

DPPH radical scavenging activity: DPPH (1,1-Diphenyl-2-picryl-hydrazyl) was sourced from Sigma–Aldrich (USA). Samples were diluted in methanol at various concentrations. The reaction mixture comprised an equal volume (100 µL) of DPPH and diluted samples, incubated in the dark at room temperature for 60 minutes, with measurements taken in dim light. The absorbance values were recorded at 517 nm using a Spectronic Genesys 5 spectrophotometer (Thermo Fisher Scientific, USA) [11].

$ABTS^{\bullet+}$ radical scavenging assay: The evaluation of the antioxidant activity of the test sample was conducted to determine its capability to scavenge the $ABTS^{\bullet+}$ radical (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), as described in a previous study [12]. In order to prepare the reaction mixture, 990 µL of the $ABTS^{\bullet+}$ solution was combined with 10 µL of the appropriately diluted samples. Following this preparation, the mixture was subjected to a 6-minutes incubation period at room temperature, allowing a proper reaction between the radical and the sample. Once the incubation was complete, the absorbance was measured spectrally at a wavelength of 734 nm to assess the extent of antioxidant activity demonstrated by samples.

The reducing power (RP), an essential indicator of its antioxidant activity, was assessed according to a previous method, with gallic acid as the positive control. The experiment was conducted in triplicate to ensure reliability of the results, and the average value was recorded for analysis. To establish the correlation between sample concentration and absorbance, the concentration corresponding to an absorbance of 0.5 ($Abs_{0.5}$) was determined from a plot of absorbance measured at 700 nm against sample concentration, illustrating the extract's reducing power [13].

Total antioxidant capacity (TAC): The antioxidant efficiency was measured following a previous study [14]. The procedure involved mixing 100 µL of extract at the tested concentrations with 1000 µL of phosphomolybdenum solution, followed by incubation at 95°C for 90 minutes. The absorbance was then measured at 695 nm, with higher absorbance indicating greater antioxidant activity.

In vivo anti-oxidative stress activities: The anti-oxidative stress potential of the samples was assessed using Canton-S wild type *Drosophila melanogaster* flies model (Kyoto Drosophila Stock Center, Kyoto, Japan), adhering to the methodologies outlined in previous reports [15,16]. The study evaluated oxidative stress resistance by exposing fruit flies to 20 mM paraquat (PQ), an oxidative stress inducer, to determine the samples' efficacy in reducing oxidative damage and enhancing survival.

2.4. Inhibition of Nitric oxide (NO) releasing

RAW264.7 cells (ATCC TIB-71™; USA) were cultured in a 25 cm^2 flask with Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Thermo Fisher Scientific, USA), supplemented with 10% (*v/v*) fetal bovine serum (FBS; Hyclone, USA), 1% (*v/v*) of L-glutamine and penicillin-streptomycin (Merck, Germany). Cells were maintained in an incubator that provided a properly humidified environment at a temperature of 37°C, along with a concentration of 5% CO_2 in the atmosphere.

For cell viability assessment, cells were seeded in 96-well plates at a density of 2×10^5 cells/mL and allowed to adhere for 24 hours. Each sample was diluted in DMEM to the desired concentration, and DMSO was added to a final concentration of 0.2 % (*v/v*). Cell cytotoxicity was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies Inc., USA). After removing the culture media, 10 µL of CCK-8 reagent was combined with 90 µL of DMEM and added to each well. The cells were pretreated for 24 hours with various sample concentrations, with or without 1 µg/mL lipopolysaccharide (LPS; Merck, Germany). Optical density was subsequently measured at 450 nm using a microplate spectrophotometer (Epoch, BioTek, USA).

NO secretion assay: Cells were similarly pretreated, as mentioned above. Then, an equal volume (100 µL) of culture supernatant and Griess reagent (FUJIFILM-Wako, Japan) was combined and incubated in the dark at room temperature for 10 minutes for color development. Absorbance was measured at 540 nm using a microplate spectrophotometer, referencing a sodium nitrite standard curve ($y = 0.0033x + 0.0087$, $R^2 = 0.9991$). Efficiency was assessed by IC_{50} value (half-maximal inhibitory concentration) [6].

2.5. Statistical Analysis

Values are presented as mean ± standard deviation (S.D.) from at least three independent replicates. Group differences were assessed using one-way ANOVA ($p < 0.05$) with GraphPad Prism software (v8.0.1; GraphPad Software Inc., CA, USA).

3. Results and Discussion

3.1 Anti-oxidative Stress of SAL and Its Fractions

Excessive production of oxidants can disrupt the balance within cells, resulting in oxidative stress, which is particularly associated with chronic infections caused by bacteria, viruses, and parasites [17]. Thus, antioxidants inhibit oxidative chain reactions by donating or accepting hydrogen from free radicals, leading to more stable radicals. By stabilizing the free radicals in this manner, antioxidants help to maintain cellular integrity and protect against potential oxidative damage that could otherwise disrupt normal biological processes [18]. Plant-derived compounds like polyphenols and flavonoids possess exceptional antioxidant properties that protect cells from oxidative stress [19]. Prior to antioxidant evaluation, the results indicated that SAL ethanol extract exhibited a relatively high total polyphenol and flavonoid content, highlighting the presence of numerous biologically active secondary metabolites. Specifically, the polyphenol content was measured at 671.83 ± 10.05 mg GAE/g extract, while the flavonoid content was determined to be 563.93 ± 12.87 mg QE/g extract, underscoring the significant phytochemical potential of the leaves. Moreover, the antioxidant efficiency of SAL extract and its respective fractions was assessed through their radical scavenging activity, reducing power, and total antioxidant capacity, as detailed in Table 1. Among them, TAC indicated resistance to oxidative stress and is closely linked to various diseases [20]. Antioxidant efficiency for radical scavenging activities was assessed using the EC_{50} value, which indicates the half-maximal effective concentration needed to inhibit initial free radicals. As presented in Table 1, SALE fraction displayed the highest radicals-scavenging activity among the fractions, with EC_{50} values of 11.12 µg/mL for DPPH and 9.74 µg/mL for ABTS, respectively. Similarly, SALE fraction also exerted the strongest activity through RP efficiency compared to other fractions, reaching $Abs_{0.5}$ of 15.06 µg/mL. Besides, the results also revealed that SALE fraction possessed the highest total antioxidant activity in comparison with SAL extract. Particularly, $Abs_{0.5}$ values for SALE, SAL, SALH , and SALW were 11.62 ± 0.28 µg/mL, 36.15 ± 1.03 µg/mL, 115.83 ± 3.12 µg/mL, and 166.42 ± 1.42 µg/mL, respectively. Together, the antioxidant efficiency of the extracts followed the descending order of SALE > SLA > SALH > SALW. This observation can be attributed to the abundant presence of active compounds, which became concentrated in the extract as a result of the fractionation process. Morever, the activity of bioactive compounds is often concealed by the presence of other inactive components, a phenomenon known as antagonism, which is commonly observed in mixtures derived from natural products [6]. Notably, antioxidants, as electron donors, regulate processes triggered by external stressors, effectively preventing oxidative stress [21]. Hence, these results indicated that moderately polar polyphenolic compounds from the SALE fraction may enhance antioxidant activity and anti-oxidative stress.

Table 1. Antioxidant efficiency of SAL extract and its fractions

No	Sample	Oxidant inhibitory activity			
		DPPH (EC_{50} µg/mL)	**ABTS$^{\bullet+}$** (EC_{50} µg/mL)	**RP** ($Abs_{0.5}$ µg/mL)	**TAC** ($Abs_{0.5}$ µg/mL)
1	SAL	19.20 ± 0.23^{c}	16.14 ± 0.11^{c}	26.36 ± 1.20^{c}	36.15 ± 1.03^{c}
2	SALH	87.18 ± 6.58^{d}	60.08 ± 0.83^{d}	61.23 ± 0.78^{d}	115.83 ± 3.12^{d}
3	SALE	11.12 ± 0.44^{b}	9.74 ± 0.13^{b}	15.06 ± 0.81^{b}	11.62 ± 0.28^{b}
4	SALW	171.10 ± 8.45^{e}	80.87 ± 1.66^{e}	101.13 ± 1.72^{e}	166.42 ± 1.42^{e}
5	Gallic acid	4.10 ± 0.20^{a}	1.80 ± 0.08^{a}	10.31 ± 0.10^{a}	25.31 ± 0.12^{a}

Mean values ± standard error with different superscript letters in the same column indicate significant differences at $p<0.05$.

To enhance the hypothesis regarding anti-oxidative stress ingredients, *D. melanogaster* model was utilized to assess the efficiency of SAL extract and its fractions against Paraquat (PQ)-induced oxidative stress. Indeed, short-term exposure of *D. melanogaster* to paraquat induces oxidative stress, adversely affecting cellular processes and causing considerable mitochondrial dysfunction, thereby compromising the organism's overall physiological health [22]. Briefly, the radical byproduct of paraquat donates an electron to molecular oxygen, generating superoxide anions. This process heightens oxidative stress and leads to additional damage to dopaminergic neurons [23]. Moreover, a previous study showed that antioxidant supplements, such as polyphenols, prolonged the lifespan of Canton-S wild-type *D. melanogaster* and alleviated locomotor impairments caused by acute and chronic PQ exposure [24]. The results revealed that the survival time of fruit flies significantly increased when their diet was supplemented with gallic acid and SAL extract (a dosage of 1 mg/mL) compared to those maintained in the control (Table 2). The antioxidant effects of SAL extract and its fractions against PQ were assessed by measuring the mean lifespan, 50% survival time, and maximum lifespan of the fruit flies. Under PQ exposure, the extract-supplemented treatments outperformed the control group, indicating that the extracts effectively neutralize free radicals and extend the lifespan of fruit flies. Especially, the average lifespan of fruit flies supplemented with SAL extract and SALE fraction was 25.67 hours and 26.30 hours, respectively. Notably, both of these extracts demonstrated not only higher 50% survival times but also maximum lifespans that surpassed those observed with the other extracts that were tested. Therefore, our findings confirmed that SAL extract and its fractions exhibited antioxidant activity and stress-reducing capabilities, as evidenced by the extended lifespan of PQ-induced *D. melanogaster*.

Table 2. Anti-oxidative stress effect of SAL extract and its fractions under 20 mM PQ conditions

Sample	Mean lifespan (hour)	50% survival time (hour)	Maximum lifespan (hour)
Control	17.27 ± 0.32^{d}	15.33 ± 1.53^{d}	22.33 ± 1.53^{d}
Gallic acid	31.30 ± 1.51^{a}	26.33 ± 1.53^{a}	58.33 ± 2.08^{a}
SAL	25.67 ± 1.16^{b}	21.43 ± 1.75^{bc}	44.33 ± 1.73^{b}
SALH	19.53 ± 0.27^{c}	17.00 ± 1.49^{cd}	32.30 ± 1.16^{c}
SALE	26.30 ± 0.76^{b}	22.70 ± 2.03^{b}	45.50 ± 0.78^{b}
SALW	21.13 ± 2.00^{c}	19.40 ± 1.23^{cd}	34.80 ± 1.16^{c}

Mean values ± standard error with different superscript letters in the same column indicate significant differences at $p<0.05$.

3.2 Anti-Inflammatory Activities of SAL Extract and Its Fractions

To assess the anti-inflammatory properties of SAL and its fractions, an initial investigation of cell cytotoxicity was conducted to determine the safe concentration levels with minimal toxicity. LPS activates macrophages, leading to apoptosis and pyroptosis, which significantly reduce cell viability and affect overall cellular health. Additionally, LPS-induced macrophage activation causes notable changes in cellular morphology, highlighting its impact on the structural characteristics of the cells [6,25]. As illustrated in Figure 2, cell viability remained similar to the non-pretreated group up to 100 µg/mL, with the exception of the SALH fraction at 200 µg/mL (Fig. 2A). Furthermore, this phenomenon was comparable to the treatment with SAL extract and its fractions following LPS stimulation, whereas incubation with LPS alone resulted in cell death (Fig. 2B). Likewise, SALW did not affect cell survival (data not shown). It could be suggested that SAL extract and its fraction were chosen at concentrations below 100 µg/mL to ensure minimal toxicity, effectively safeguarding cells against LPS-induced cell damage.

During the inflammatory response, nitric oxide (NO) secretion as regarded as a key signaling molecule involved in the defense mechanism against infectious pathogens [26]. Indeed, controlling high nitric oxide (NO) levels is vital for correcting abnormal secretion in conditions like hepatic failure and sepsis. Targeting increased NO production can address these critical issues [27]. Here, NO levels increased after LPS incubation compared to the control group (data not shown). However, based on the inhibition of NO secretion, as indicated by the IC_{50} value, the results indicated that SAL extract had the highest NO inhibitory activity among the fractions, with an IC_{50} value of 15.7 µg/mL. Similarly, SALE and SLAH fractions also demonstrated NO inhibition, with IC_{50} values of 47.3 µg/mL and 51.4 µg/mL,

respectively. In contrast, SAW extract exhibited minimal NO inhibitory activity, with an IC_{50} value exceeding 100 µg/mL (Table 3). Thus, these findings further support the anti-inflammatory potential of SAL extract, along with the SALE and SLAH fractions, due to their ability to suppress NO production in LPS-stimulated cells. Considering all factors, SALE was chosen as the subject for more in-depth exploration and analysis of its various active components.

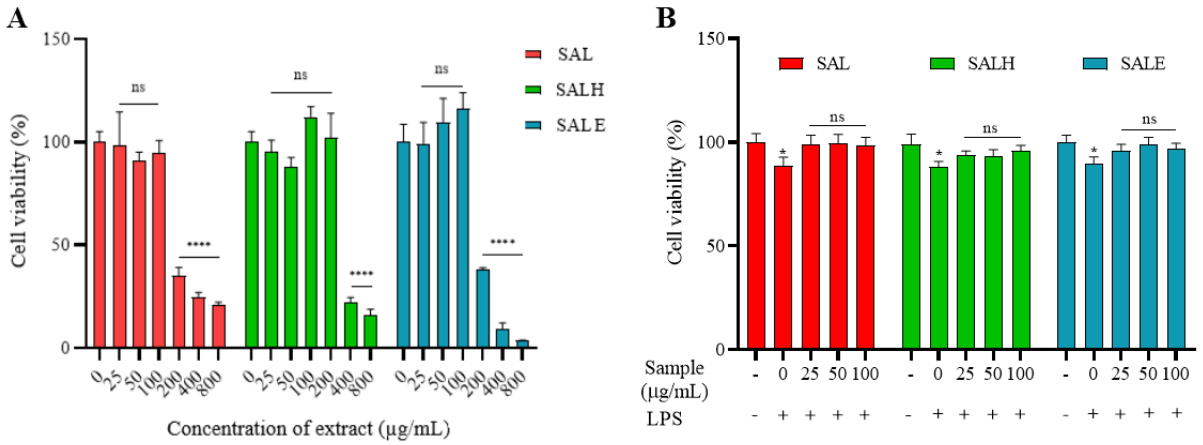

Figure 2. The cytotoxicity of SAL extract and its fraction in LPS-induced cell damage. (A) Cell viability was assessed following treatment with SAL extract and its fractions; (B) Cell viability was evaluated after incubation of SAL extract and its fraction with LPS; Data was presented as mean ± S.D. (n = 6); n.s., not significant; *, *p < 0.05;****, *p < 0.0001*: compared to non-treatment group

Table 3. Nitric oxide inhibitory activity from SAL extract and its fractions

Sample	Nitric oxide inhibitory activity			
	SAL	**SALH**	**SALE**	**SALW**
IC_{50} (µg/mL)	15.7 ± 1.08[a]	51.4 ± 1.67[b]	47.3 ± 0.59[c]	>100

Different superscript letters in each column depict a significant difference value ($p < 0.05$)

3.2 Evaluation and Structure Elucidation of Active Components from SALE

Based on the initial bioactive investigation above, twelve compounds were isolated using column chromatography. Then, for the confirmation of anti-inflammatory effectiveness, compounds **3**, **5**, **6**, and **7** were randomly selected to evaluate the NO inhibition. This aimed to re-evaluate the biological activity of isolated compounds based on the minimal amount obtained, which is sufficient to conduct biological assays. Firstly, the cytotoxicity of these compounds was accessed up to 50 µg/mL. As expected, the cell viability of compounds **5**, **6**, and **7** was similar to the control group, indicating non-toxicity at those concentrations. In contrast, compound **3** at 25 µg/mL caused cell death, while concentrations up to 12.5 µg/mL did not significantly differ from the control group (data not shown). As depicted in Figure 3, pretreatment with compounds **3**, **5**, **6**, and **7** markedly suppressed NO production. This observation highlighted the anti-inflammatory properties of these compounds, though their toxicity at high concentrations may limit their therapeutic application.

The molecular structures of all isolated compounds were thoroughly analyzed using both one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy techniques. These analyses were then compared in detail with the information and data available in the published literature, which can be found in the Supporting Information. Figure 4 illustrated the initial elucidation and numbering of the structures of isolated compounds, including chrysin **(1),** daucosterol **(2)**, allantoin **(3)**, (+)-catechin **(4)**, apigenin **(5)**, rutin **(6)**, isatin **(7)**, 3-hydroxy-3-(2-oxopropyl)indolin-2-one **(8)**, benzoic acid **(9)**, ethylene glycol dibenzoate **(10),** nicotiflorin **(11)**, 3,3',4,4'-tetrahydroxybiphenyl **(12)** [28–40]. Eight compounds were identified for the first time in both this plant and the genus *Sphaerocoryne*: allantoin **(3)**, apigenin (**5**), rutin (**6**), isatin (**7**), 3-hydroxy-3-(2-

oxopropyl)indolin-2-one (**8**), ethylene glycol dibenzoate **(10),** nicotiflorin (**11**), 3,3',4,4'-tetrahydroxybiphenyl **(12)**.

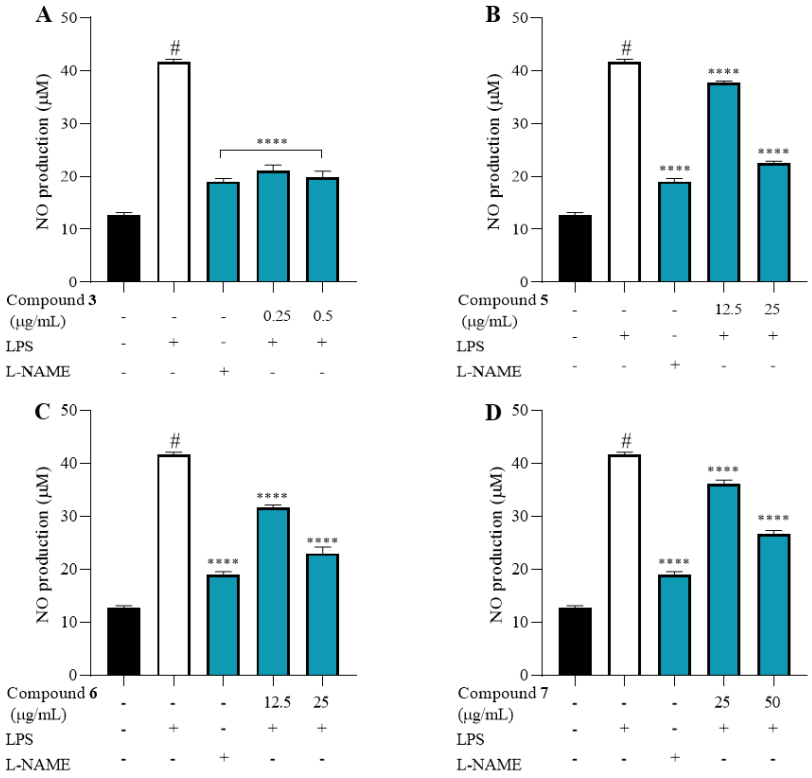

Figure 3. The effect of compounds **3**, **5**, **6**, and **7** on NO secretion. NO production was assessed following treatment with compound **3** (A), compound **5** (B), compound **6** (C) and compound **7** (D), respectively. Data were presented as mean ± S.D. (n = 6); #, $p < 0.05$: compared to non-treatment group*;* ****, $p < 0.0001$: compared to LPS group

In accordance with above results, allantoin exhibits several properties that facilitate a wound's transition from the inflammatory to the proliferative phase, including antioxidant and anti-inflammatory effects, direct antimicrobial action, and keratolytic properties that enhance healing [41]. Moreover, the results showed that allantoin (compound **3**) significantly inhibited NO secretion, reducing it by 53.66% compared to the LPS-stimulated group at a concentration of 0.25 µg/mL, equivalent to 1.58 µM (Fig. 3A). This finding was consistent with a previous report, in which IC_{50} for NO inhibition was achieved at 1.55 µM [42]. Besides, part of the flavonoid group, chrysin (compound **1**), apigenin (compound **5)**, rutin (compound **6**), and nicotiflorin (compound **11**), demonstrate strong antioxidant activity, reducing tissue damage and benefiting inflammatory diseases [28,43–45]. Similarly, apigenin and rutin also exerted their role in NO inhibition by reducing approximately 53% at 25 µg/mL (equivalent to 92,6 µM and 41 µM, respectively), which is consistent with the previous reports (Fig. 3B,C) [46,47]. Notably, nicotiflorin was demonstrated as a potent hepatoprotective agent that safeguards the liver from acute immunological and chemical damage, likely due to its antioxidant and immunoregulatory effects [48]. Additionally, (+)-catechin is a natural antioxidant recognized for its wide range of biological effects, such as anti-cancer, anti-obesity, anti-infective, anti-cardiovascular, hepatoprotective, and neuroprotective activities [49]. Daucosterol is an essential antioxidant that neutralizes free radicals and supports the immune system's inflammatory response [50]. Moreover, the presence of isatin (compound **7**) contributed to reducing NO secretion by approximately 36% at 50 µg/mL in comparison with the LPS-treated group (Fig. 3D). Indeed, the non-brominated compound of isatin has been reported to exhibit weak NO inhibitory activity, resulting in a 34% reduction at a concentration of 50 µg/mL. However, isatin indole derivatives have been patented for potential anti-inflammatory properties [51].

The other mentioned compounds are well-known secondary metabolites produced by various plants, playing important roles in their biological processes and depending on environmental interactions. Remarkably, 3,3',4,4'-tetrahydroxybiphenyl showed notable activity against *Trypanosoma brucei trypomastigotes*, which is a species of parasitic, achieving an IC_{50} of 6.66 µg/mL [52]. Hence, these results indicated that these particular plant secondary metabolites play a significant role in enhancing the diversity of bioactive activities associated with oxidative stress and various inflammatory diseases. This underscores the importance of these compounds in potentially providing a wide range of beneficial effects that may help in combating conditions related to oxidative damage and inflammatory responses.

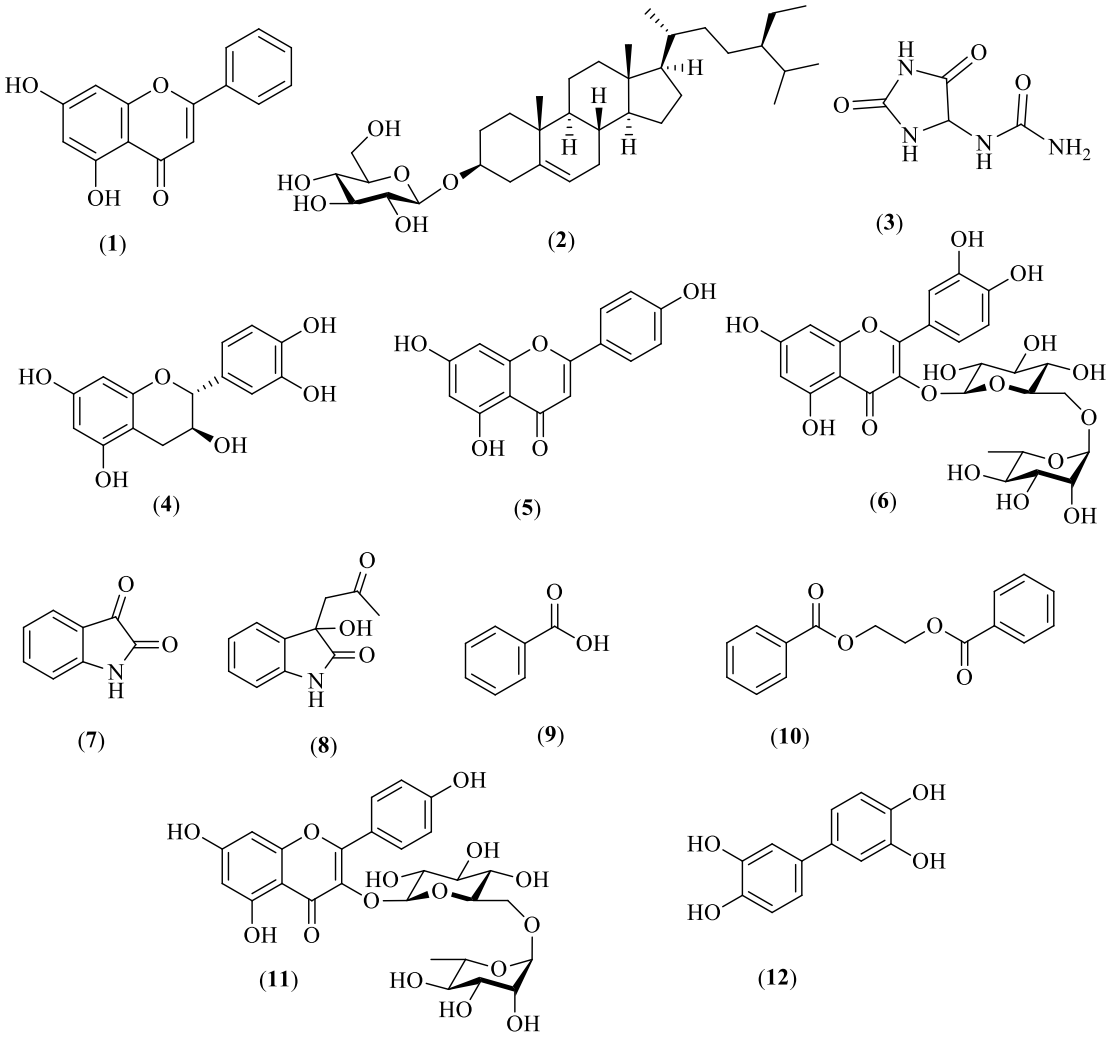

Figure 4. Structures of active compounds isolated from SALE fraction

In onclusion, our findings provide preliminary evidence of the antioxidant and anti-inflammatory properties of *Sphaerocoryne affinis* leaves. This research highlighted their potential as a source of bioactive compounds, which could lead to innovative healthcare products and natural therapies for various diseases. Our results open an encouraging path for further exploration of *Sphaerocoryne affinis* leaves in health applications, potentially offering new disease management and prevention solutions.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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