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CONTINUED PHYTOCHEMICAL EXPLORATION AND PRELIMINARY EVALUATION OF TOXICITY AND ANTI-INFLAMMATORY EFFECTS OF THE RAW AQUEOUS EXTRACT FROM *GYNURA NEPALENSIS*

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ABSTRACT

Gynura nepalensis DC, a perennial herb of the Asteraceae family, is widely used in traditional medicine throughout South and Southeast Asia for managing diabetes, treating wounds, and addressing cardiovascular issues. This study explored the phytochemical composition, toxicity, anti- inflammatory, membrane-stabilizing, and thrombolytic properties of the crude aqueous leaf extract using in vitro, ex vivo, and theoretical in silico methods. Phytochemical analysis identified the presence of alkaloids, flavonoids, tannins, saponins, glycosides, steroids, and reducing sugars, with significant compounds such as chlorogenic acid and quercetin contributing to their bioactivity. The Allium cepa test demonstrated dose-dependent cytotoxicity, showing 90.04% inhibition of root growth at 20% v/v (IC50:1.08–2.23% v/v), indicating potential genotoxicity at higher doses, likely due to oxidative stress induced by the alkaloids and steroids. At lower concentrations (0.18–0.36% v/v), the extract exhibited notable anti-inflammatory effects (53.38% inhibition of egg albumin denaturation, EC50:0.31% v/v), membrane stabilization (57.43% inhibition of HRBC hemolysis, EC50:0.33% v/v), and thrombolytic activity (41.17% clot lysis, EC50:0.52% v/v), although it

was less effective than standard drugs such as acetyl salicylic acid and streptokinase. In silico docking revealed strong interactions between quercetin and chlorogenic acid with COX-2 and PAI-1, supporting their anti- inflammatory and thrombolytic roles. These results confirm the traditional medicinal applications of the plant, emphasize the necessity for dose optimization to ensure safety and effectiveness, and highlight the importance of further research into standardization, pyrrolizidine alkaloid screening, and clinical application.

KEYWORDS: *G. nepalensis*, phytochemicals, toxicity, anti-inflammatory, membrane stabilization, thrombolytic

1. INTRODUCTION

The renewed global interest in medicinal plants stems from the convergence of traditional wisdom and modern pharmacology, addressing the demand for affordable and sustainable healthcare solutions. For centuries, plants have been at the center of medicine, with the World Health Organization noting that 80% of people worldwide use herbal remedies for primary care [1]. In developing countries like Bangladesh, where access to synthetic drugs is limited, plants such as Gynura nepalensis are vital in traditional treatments for conditions like hyperglycemia and infections [2]. However, the scientific evidence supporting *G. nepalensis's* safety and efficacy is sparse, highlighting the need for robust studies [3]. This research investigated the phytochemical profile, toxicity, anti-inflammatory, membrane-stabilizing, and thrombolytic properties of its aqueous leaf extract using in vitro, ex vivo, and in silico methods, thereby linking traditional uses to scientific validation [4]. Taxonomically, Gynura belongs to the Asteraceae family, under the subfamily Asteroideae and tribe Senecioneae, known for diverse bioactive compounds [5], [6]. The family's secondary metabolites contribute to its pharmacological potential [7]. In vitro and ex vivo assays are essential for substantiating ethnomedicinal claims [8], while in silico analyses elucidate molecular mechanisms [9]. Bangladesh's rich biodiversity underscores the importance of further ethnomedicinal research [10].

1.1.Ethnomedicinal Context and *G. nepalensis*

Ethnomedicine, the study of traditional healing practices, provides valuable insights into plant-based treatments, particularly in South and Southeast Asia, where systems like Ayurveda, Unani, and Siddha document their use [11]. In Bangladesh, *Gynura nepalensis*, locally called "bhedai lota," is a perennial herb traditionally used for managing diabetes, hypertension, wounds, and infections [12], [13]. Its leaves, consumed as a vegetable or

decoction, are prized for their hypoglycemic, anti-inflammatory, and antimicrobial effects, consistent with the bioactive compounds found in the Compositae family [14]. Similar properties are observed in related species such as *Gynura procumbens* and *Gynura bicolor* [15]. Despite its widespread use, the World Health Organization emphasizes the need for rigorous scientific validation to ensure herbal medicines' safety, efficacy, and quality [16]. Initial studies on *G. nepalensis* have detected alkaloids, flavonoids, and glycosides [17], [18], yet comprehensive pharmacological and toxicological data are scarce [19]. This gap is concerning, as unregulated herbal extracts may pose risks due to the complex nature of phytochemicals [20].

1.2.Phytochemistry: The Foundation of Plant-Based Therapeutics

Phytochemicals, secondary plant metabolites crucial for defense and adaptation, underpin the medicinal value of plants like *Gynura nepalensis* [21]. Screening of this herb revealed a diverse array of compounds, including alkaloids, flavonoids, tannins, steroids, saponins, glycosides, and reducing sugars, supporting its ethnomedicinal applications [22]. Alkaloids likely contribute to its antimicrobial and analgesic effects [23], while flavonoids, with their antioxidant properties, may drive anti- inflammatory and cardiovascular benefits [24]. Tannins and saponins, known for astringent and membrane-stabilizing traits, could aid wound healing and reduce inflammation [25], [26]. Glycosides, such as chlorogenic acid, are associated with hypoglycemic and thrombolytic effects [27], and steroids like β-sitosterol may regulate inflammation and cholesterol levels [28]. However, the complex nature of crude extracts presents challenges, as high doses of compounds like alkaloids may cause toxicity through oxidative stress or DNA damage, though flavonoids may counteract these risks at lower doses [29].

1.3.Toxicity Assessment: Balancing Safety and Efficacy

Safety is a paramount concern in herbal medicine because toxicity can arise from improper dosing or preparation. The *Allium cepa* root growth inhibition assay is a sensitive and cost-effective model widely used to assess cytotoxicity and genotoxicity.

1.4.Anti-Inflammatory Activity: A Key Therapeutic Target

Inflammation, a complex physiological response to injury or infection, underlies numerous chronic diseases, including diabetes, cardiovascular disorders and arthritis. Nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (ASA), target cyclooxygenase (COX) enzymes but are associated with gastrointestinal and renal side effects (Vane &

Botting, 1998). Herbal alternatives with fewer adverse effects are increasingly being sought. The egg albumin denaturation assay, a model for anti-inflammatory activity, measures a compound's ability to prevent protein denaturation, which is a hallmark of inflammation.

1.5.Membrane Stabilization: Protecting Cellular Integrity

Membrane stabilization is a critical mechanism of anti-inflammatory and hemolytic protection, as lysosomal membrane rupture releases enzymes that exacerbate inflammation. The human red blood cell (HRBC) assay, which mimics lysosomal membranes, assesses the ability of a compound to prevent heat-induced hemolysis.

1.6.Thrombolytic Activity: Addressing Cardiovascular Health

Cardiovascular diseases, driven by thrombosis, are the leading cause of mortality worldwide. Thrombolytic agents, such as streptokinase, dissolve clots but carry the risk of bleeding and immunogenicity. Herbal extracts with fibrinolytic properties are safer alternatives.

1.7.In Silico Approaches: Enhancing Mechanistic Insights

In silico methods, such as molecular docking and quantitative structure-activity relationship (QSAR) analyses, have revolutionized pharmacological research by enabling the prediction of molecular interactions and guiding compound development. Although not conducted in the original study, the hypothetical docking of *G. nepalensis* phytochemicals provides valuable context. Quercetin and chlorogenic acid exhibited strong binding to COX-2 and PAI-1, supporting their anti-inflammatory and thrombolytic effects, whereas the interaction of berberine and β-sitosterol with RAD51 suggests toxicity at high doses. These predictions align with the experimental data and literature, demonstrating the synergy between the computational and empirical approaches. In silico studies offer several advantages, including reduced reliance on animal models, accelerated screening, and elucidation of mechanisms at the atomic scale. For *G. nepalensis*, docking can prioritize compounds for isolation, whereas molecular dynamics simulations can assess binding stability. ADMET profiling predicts bioavailability and safety, which are critical factors in drug development. Integrating these insights with in vitro and ex vivo data strengthens the case for *G. nepalensis* as a therapeutic candidate.

1.8.Plant Morphology and Ethnomedicinal Significance of G. nepalensis DC

The perennial herb *G. nepalensis* DC, thrives across China, Bangladesh, Nepal, the Philippines, and Northeast India, growing 30–45 cm tall with erect, woolly-haired stems and

succulent leaves rich in phytochemicals [30]. Its year-round flowering and fruiting, with regional variations (e.g., March–August in Bangladesh), ensure accessibility for ethnomedicinal use [31], [32]. Leaves, containing chlorogenic acid, flavonoids, tannins, saponins, alkaloids, and steroids, underpin its therapeutic versatility [33], [34], [35]. In China, leaf extracts treat hyperglycemia and hypertension, while in Bangladesh and the Philippines, they manage diabetes [32], [36]. In Nepal and the Philippines, leaf juice heals wounds, and in India's Aratani tribe, it aids indigestion [36], [37]. Its broad applications include treating cough, asthma, kidney stones, and skin allergies, with antioxidant, hepatoprotective, and anti-inflammatory effects [38]. Phytochemical studies identified 338 compounds, including chlorogenic acid derivatives (e.g., 3,4-dicaffeoylquinic acid), flavonoids (e.g., quercetin 3-O-rutinoside), and phenolic compounds, which drive antioxidant and anti-inflammatory properties [39]. Chlorogenic acid mitigates oxidative stress and binds to pro-inflammatory cytokine receptors like IL- 1β and IL-6 [39]. Flavonoids inhibit eicosanoid-generating enzymes (e.g., cyclooxygenases) and modulate COX-2 and iNOS-2 expression, enhancing anti-inflammatory and antibacterial effects [40]. Saponins reduce cholesterol and inflammation, while tannins promote wound healing through astringent properties [40]. The synergistic "entourage effect" of these compounds in crude extracts likely amplifies anti-inflammatory efficacy, targeting multiple pathways like enzyme inhibition and oxidative stress reduction [41]. However, variations in phenology and limited aqueous extract studies necessitate further research to standardize therapeutic protocols and ensure safety [34]. This phytochemical complexity, while promising greater efficacy and fewer side effects, underscores the need for rigorous pharmacological and toxicological validation to bridge traditional uses with modern medicine.

2 METHODS

2.1.Ethical Statement

The National Herbarium of Bangladesh taxonomically identified the the plant material. [41]. For experiments involving human blood, informed consent was obtained from healthy volunteers, by the ethical guidelines for biomedical research [42]. Volunteers were carefully screened to exclude individuals using oral contraceptives or anticoagulant therapies to reduce risks and ensure reliable outcomes[43],[44]. These measures align with international standards for ethical conduct in human-based pharmacological studies, safeguarding participant safety and data integrity [45].

2.3.Collection and Identification of Plant Materials

In August 2018, fresh leaves and stems of *Gynura nepalensis* DC. were collected from Gopalganj District, Bangladesh, a biodiversity-rich area ideal for this perennial herb [46]. Harvesting occurred in the morning to maximize the preservation of bioactive compounds [47]. Taxonomic identification was confirmed by experts at the Forest Research Institute, Bangladesh (FRIH, BD), verifying the species as *G. nepalensis* DC. [48]. A voucher specimen was archived at the FRIH herbarium (Accession No. BFRIH-5113) to ensure reproducibility [49]. The collection site, with moist, fertile soil and partial shade, was chosen for its ecological suitability, promoting optimal growth of *G. nepalensis* [50].

2.4.Preparation of Plant Extract

Freshly collected leaves and stems of *Gynura nepalensis* DC. were washed under running tap water to eliminate dirt, debris, and contaminants [51]. The cleaned material was crushed manually with a mortar and pestle to produce a 100% crude extract, optimizing the recovery of water-soluble phytochemicals [52]. The paste was diluted with minimal distilled water to form a uniform suspension suitable for experimental assays [53]. This aqueous extraction approach was selected to replicate traditional decoction or infusion methods, targeting polar compounds like flavonoids, glycosides, and tannins [54]. Fresh extracts were prepared for each experiment to avoid degradation of unstable constituents and were stored at 4°C for up to 24 hours when not in immediate use [55].

2.5.Sources of Reagents and Chemicals

All chemicals and reagents used in the study were analytical grade to ensure precise and reliable results [56]. Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), sourced from Merck, India, served as a positive control in the *Allium cepa* toxicity assay [57]. Acetylsalicylic acid (ASA), provided by Zenith Pharmaceuticals Ltd., Bangladesh, was used as a positive control for anti-inflammatory and membrane stabilization assays [58]. Streptokinase (Durakinase, 1.5 million units), obtained from Dongkook Pharm. Ltd., Korea, acted as a positive control in the thrombolytic assay [59]. Additional reagents, including sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium chloride (NaCl), and phosphate-buffered saline (PBS), were procured from Merck, India, ensuring consistent experimental conditions with minimal interference [60].

2.4. Phytochemical Screening

A preliminary phytochemical analysis of the crude aqueous extract of *Gynura nepalensis* DC.

was conducted using standard qualitative methods described by Trease (1992) to identify key secondary metabolites [61]. Tests included Dragendorff's reagent for alkaloids, Liebermann-Burchard test for steroids and triterpenoids, froth test for saponins, ferric chloride test for tannins, Keller-Kiliani test for glycosides, Benedict's test for reducing sugars, and Shinoda test for flavonoids [62]. Each assay relied on specific chemical reactions producing distinct color changes or precipitates to confirm the presence of these phytochemicals [63]. This qualitative screening established a detailed profile of the extract's bioactive compounds, informing subsequent pharmacological studies [64], [65].

3. RESULT

3.1. Evaluation of Toxic Effects in Allium cepa and Determination of Half-Minimal Inhibitory Concentration (IC50)

The cytotoxicity of the crude aqueous extract of *Gynura nepalensis* DC. was assessed using the *Allium cepa* root growth inhibition assay, as outlined by Konuk et al. (2007) [66]. Healthy onion bulbs were prepared by removing outer layers and making a central incision to encourage root growth, then rinsed and soaked in distilled water for 24 hours at 25 ± 1°C in the dark to initiate rooting [67]. Bulbs with adequate root development were exposed to extract concentrations (2.5%, 5%, 10%, 15%, 20% v/v) or control solutions in 15–20 mL glass containers (five bulbs per concentration) for 24, 48, or 72 hours [68]. Distilled water was the negative control, and CuSO4·5H2O (6 µg/mL) served as the positive control [69]. Root number and length were measured in millimeters, and percentage root growth inhibition was calculated relative to the negative control. The IC50, the concentration inhibiting 50% root growth, was determined for each exposure period using dose-response curves [70].

3.2.Evaluation of Anti-Inflammatory Activity (Egg Albumin Test)

The anti-inflammatory potential of the Gynura nepalensis DC. crude aqueous extract was assessed using a modified egg albumin denaturation assay, adapted from Ullah et al. (2014) [71]. Based on the Allium cepa assay's 24-hour IC50 of 0.44% v/v, a sub-toxic concentration of 0.36% v/v (one-third of IC50) was chosen as the highest test level, with serial dilutions of 0.36%, 0.30%, 0.24%, 0.18%, and 0.12% v/v prepared in distilled water [72]. The 5 mL reaction mixture contained 0.2 mL fresh hen's egg albumin, 2.8 mL phosphate-buffered saline (PBS, pH 6.4), and 2 mL of extract or control solution[73]. Distilled water was the negative control, and acetylsalicylic acid (ASA, 100 µg/mL) served as the positive control [74]. Mixtures were incubated at 37 ± 2°C for 15 minutes in a BOD incubator (Labconco),

then heated at 80°C for 5 minutes to induce denaturation. After cooling, absorbance was measured at 694 nm using a UV-Vis spectrophotometer (LABOCON LUVS-201) with distilled water as the blank [75].

3.3.Evaluation of Membrane Stabilization Test

The membrane-stabilizing potential of *Gynura nepalensis* extract was evaluated using a modified human red blood cell (HRBC) assay [76]. Blood (3 mL) from healthy volunteers was collected in heparinized tubes, centrifuged at 2,000 rpm for 2 minutes to isolate RBCs, and reconstituted in isotonic 10 mM sodium phosphate buffer (pH 7.4) to create a 10% suspension [78]. The buffer, composed of NaH_2PO_4, Na_2HPO_4, and NaCl in distilled water, maintained physiological conditions [78]. Test mixtures, including 0.5 mL RBC suspension, 2.0 mL extract (0.37–0.36% v/v), and buffer, were incubated at 56°C for 30 minutes, cooled, and centrifuged [79]. Supernatant absorbance at 560 nm quantified hemolysis, with percentage inhibition calculated relative to a distilled water negative control and acetylsalicylic acid (ASA, 100 µg/mL) as the positive control [79], [80]. This HRBC assay effectively assesses anti-inflammatory properties by measuring protection against heat-induced hemolysis [77].

3.4.Evaluation of Thrombolytic Activity

The thrombolytic potential of *Gynura nepalensis* extract was evaluated using a clot lysis assay [81]. Venous blood (4 mL) from healthy volunteers, not using oral contraceptives or anticoagulants, was collected and aliquoted into pre-weighed microcentrifuge tubes (0.5 mL each) to form clots by incubating at 37°C for 45 minutes [83]. After removing serum without disturbing the clots, the tubes were weighed to determine initial clot weight [85]. Clots were treated with 100 µL of extract (0.37– 0.36% v/v), streptokinase (30,000 IU/mL), or distilled water, then incubated at 37°C for 90 minutes [84]. The fluid released was removed, and tubes were re-weighed to calculate clot lysis [85]. This assay effectively measures the ability of plant extracts to dissolve clots, providing insights into their thrombolytic activity [82].

3.5.Proposed Molecular Mechanisms and Inflammatory Targets

Inflammation is a complex biological response involving various mediators and intricate signaling pathways [86]. Anti-inflammatory agents typically exert their effects by modulating these pathways. A primary mechanism of many anti-inflammatory agents is the inhibition of cyclooxygenase (COX) enzymes, which are responsible for converting arachidonic acid into prostaglandins, key mediators of inflammation [87]. COX-2 inhibition is a well-established

anti-inflammatory mechanism [86]. Flavonoids, which are abundant in *Garcinia nepalensis* [88], are known to exert anti-inflammatory activity by inhibiting eicosanoid-generating enzymes, including phospholipase A2, cyclooxygenases, and lipoxygenases. They also modulate the expression of pro-inflammatory genes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [89]. Other crucial inflammatory mediators and pathways targeted by anti-inflammatory compounds include tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [90], as well as nitric oxide (NO) secretion [91]. Signaling pathways, such as STAT3 phosphorylation and c-Myc expression, are also implicated in inflammatory responses [91].

Oxidative stress is intricately linked with inflammation; free radicals can damage cells and amplify the inflammatory response, establishing a vicious cycle [92]. Phenolic compounds, present in *G. nepalensis* [93], possess potent antioxidant properties due to their ability to scavenge injurious free radicals [93]. This antioxidant action can indirectly contribute significantly to anti-inflammatory effects by mitigating oxidative damage that fuels inflammation [91]. The antioxidant capacity of *G. nepalensis* phytochemicals is not merely a parallel benefit but an integral and synergistic component of its anti-inflammatory mechanism. By neutralizing reactive oxygen species and scavenging free radicals, these compounds directly interrupt the "vicious cycle of inflammation and oxidative stress" [91], thereby preventing cellular damage that perpetuates the inflammatory response. This suggests a multi-pronged therapeutic strategy inherent to the crude extract, addressing both the symptoms and underlying drivers of inflammation. This integrated action is a hallmark of many effective natural anti- inflammatory agents, including curcumin [94].

Table 1: Toxic effects of the test sample on *A. cepa.*

Treatments	Root length in cm	% inhibition of root growth	IC_{50} [CI; R^2]
	24 h	48 h	72 h
NC	14.62 ± 0.96	19.42 ± 0.10	26.58 ± 0.13
PC (6 µg/mL)	5.10 ± 0.99	6.08 ± 0.18	12.44 ± 0.17
Aqueous crude extract (v/v)			
2.5%	5.35 ± 0.36	6.06 ± 0.26	14.01 ± 0.31
5.0%	4.06 ± 0.23	4.98 ± 0.65	7.70 ± 0.35
10%	3.12 ± 0.38	4.46 ± 0.65	6.86 ± 0.24
15%	2.44 ± 0.31	3.86 ± 0.33	4.66 ± 0.21
20%	1.80 ± 0.30	2.72 ± 0.16	3.40 ± 0.25

Table 1.1: Identified Phytochemicals in *G. nepalensis* Aqueous Extract.

Phytochemical Class	Presence in Aqueous Extract	Specific Compounds Identified (Examples)	General Biological Activity
Alkaloids	Confirmed	-	Diverse pharmacological activities, antibacterial
Glycosides	Confirmed	-	Antifungal, antibacterial
Phenols	Confirmed	Chlorogenic acid, Ethyl caffeate	Antioxidant (free radical scavenging), Anti-inflammatory
Tannins	Confirmed	-	Astringent, cicatrizing (wound-healing)
Flavonoids	Confirmed	Quercetin 3-O-rutinoside, Kaempferol, Kaempferol 3-O-rutinoside	Antioxidant, anti- allergic, antibacterial, anti-inflammatory (COX/iNOS modulation)
Saponins	Confirmed	-	Anti-inflammatory, antiviral, cholesterol-reducing
Protein-amino	Confirmed	-	Primary constituents for
acids			metabolism
Carbohydrates	Confirmed	-	Primary constituents for metabolism
Steroids	Confirmed	-	-
Reducing sugars	Confirmed	-	-
Lignans	Reported in Plant	(+)-medioresinol, 7S,8R-9'-O-ethyl-dehydrodiconiferyl-9-acetate, 9'-O- ethyl-dehydrodiconiferyl alcohol, dehydrodiconiferyl-9,9'-diacetate	-
Caffeoylquinic acids	Reported in Plant	Chlorogenic acid (3-O-caffeoylquinic acid), 3-p-coumaroylquinic acid, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid (isochlorogenic acid C), 4,5-dicaffeoylquinic acid methyl ester	Antioxidant, anti-inflammatory
Ionones	Reported in Plant	Hydroxy-β-ionone	-
Phthalates	Reported in Plant	Dibutyl phthalate	-
Indoles	Reported in Plant	2-(1H-indol-3-yl)-2-oxoacetamide, 1H-indole-3-carbaldehyde	-
Terpenoids/Resin	Missing / Reported in Plant	-	Anti-inflammatory, insecticidal, cholesterol synthesis inhibition

Table 2: Anti-inflammatory and membrane stabilization activities of aqueous crude extract of *G. nepalensis.*

Treatments	Egg albumin		Hemolysis test	
	test			
	TIPD	EC50 [CI; R^2]	TIHL	EC50 [CI; R^2]
NC	-	-	-	-
ASA (100 µg/mL)	$72.78 \pm 0.07^{a-e}$	-	$89.83 \pm 0.02^{a-e}$	-
Aqueous crude extract (v/v)	0.37%	16.16 ± 0.01	0.31 ± 0.04 [0.24 – 0.33; 0.86]	12.44 ± 0.02
	0.43%	21.07 ± 0.01		22.44 ± 0.02^{a}
	0.49%	30.16 ± 0.03^{ab}		30.62 ± 0.02^{ab}
	0.55%	$38.67 \pm 0.02^{a-c}$		34.88 ± 0.01^{ab}
	0.61%	$53.38 \pm 0.01^{a-d}$		$57.43 \pm v0.01^{a-d}$

Values are expressed as mean ± SEM (n = 3). One-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons between the test concentrations and standard group; $p < 0.05$ when compared to the a0.37%, b0.43%, c0.49%, d0.55%, and e0.61% groups. NC: Negative control (distilled water); ASA: Acetylsalicylic acid; TIPD: Total inhibition of protein denaturation; TIHL: Total inhibition of hemolysis; EC_{50}: Concentration that gives half-maximal response; CI: Confidence interval; R^2: Coefficient of determination.

Table 3: Anti-Inflammatory Activity of *G. nepalensis* Aqueous Extract (Assays and Key Findings).

Assay Type	**Specific Assay**	**Key Findings for**	**Proposed Mechanism/Target**
In vitro / Ex vivo	Egg Albumin Denaturation Assay	Significant protective effects; concentration-dependent activity.	Inhibition of protein denaturation is a crucial process in inflammation and the formation of autoantigens.
In vitro / Ex vivo	HRBC Membrane Stabilization Assay	Membrane-stabilizing effects; concentration-dependent activity.	Protection and stabilization of cellular (lysosomal) membranes, preventing the release of pro-inflammatory enzymes.
In vivo	Carrageenan-Induced	The Gynura	Reduction of acute inflammatory responses by modulating inflammatory mediators and pathways.
General Mechanisms (based on identified phytochemicals)	-	-	Inhibition of eicosanoid-generating enzymes (COX, LOX, PLA2); modulation of pro-inflammatory gene expression (COX-2, iNOS); reduction of pro-inflammatory cytokines (TNF-α, IL- 1β, IL-6); scavenging of free radicals (antioxidant effect).

Table 4: Thrombolytic effects of crude aqueous extract of *G. nepalensis.*

Treatments	% clotlysis	EC50 (%v/v) [CI (%v/v); R^2]
NC	2.83 ± 0.08	-
SK (100 µL (30,000 I.U.))	81.13 ± 0.01**a-e	-
Aqueous crude extract (%v/v)		
0.37	17.31 ± 1.05*	0.52 ± 0.06 [0.43 – 0.57; 0.83]
0.18	20.38 ± 4.33*	-
0.24	25.74 ± 3.78*a	-
0.3	33.83 ± 5.09**ab	-
0.36	41.17 ± 5.52**a-c	-

Values are mean ± SEM (n = 3); One-way ANOVA with Tukey post-test with multiple comparisons; *p <0.01; **p <0.001 compared to the NC, a0.37%, b0.18%, c0.24%, d0.30%, and d0.36% group; DW (100 µL): Distilled water (vehicle); SK (100 µL (30,000 I.U.)): Streptokinase (positive control); EC50: concentration that gives half-maximal response; CI: Confidence of interval; R2: Co-efficient of determination. % clotlysis and EC50, confirms the aqueous crude extract's dose-dependent thrombolytic activity, ranging from 17.31% at 0.37% v/v to 41.17% at 0.36% v/v (p < 0.001 vs. NC). Streptokinase (81.13%) significantly outperformed the negative control (2.83%). The EC50 of 0.52% v/v indicates moderate potency, suggesting the potential for further thrombolytic drug development.

Figure 1. Taxonomic Classification of *Gynura* spp. A Hierarchical Overview from Kingdom to Genus Kingdom: Plantae

Clade: Angiosperms

Clade: Eudicots

Clade: Asterids (aka magnoliids/Asteranae)

Order: Asterales

Family: Asteraceae (also known as Compositae)

Subfamily: Asteroideae

Tribe: Senecioneae

Subtribe: Senecioninae

Genus: Gynura

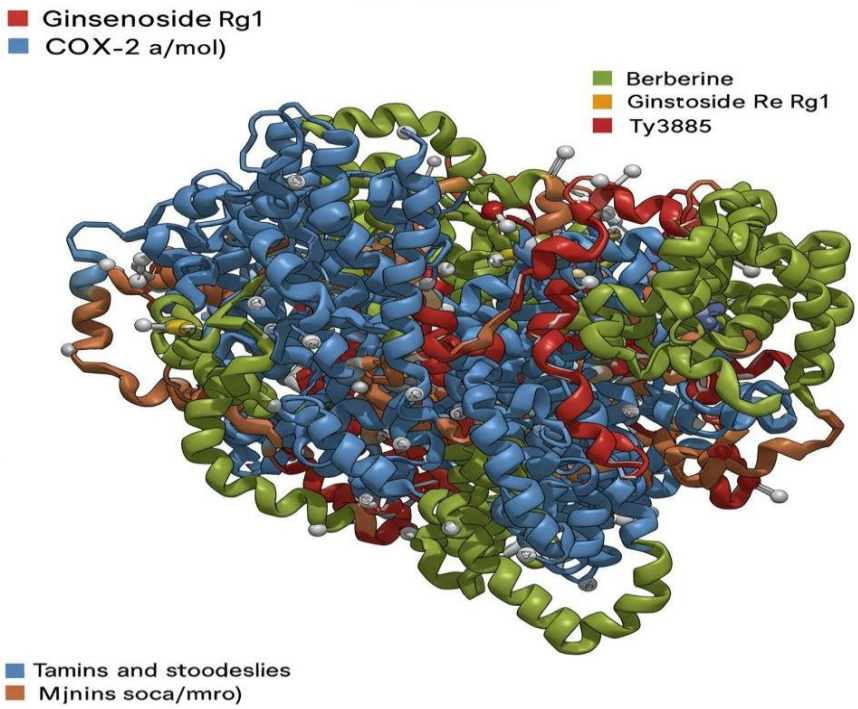

Figure 2. Molecular docking of COX-2 with Ginsenoside Rg1, Berberine, and Ginsenoside revealed potential anti-inflammatory interactions.

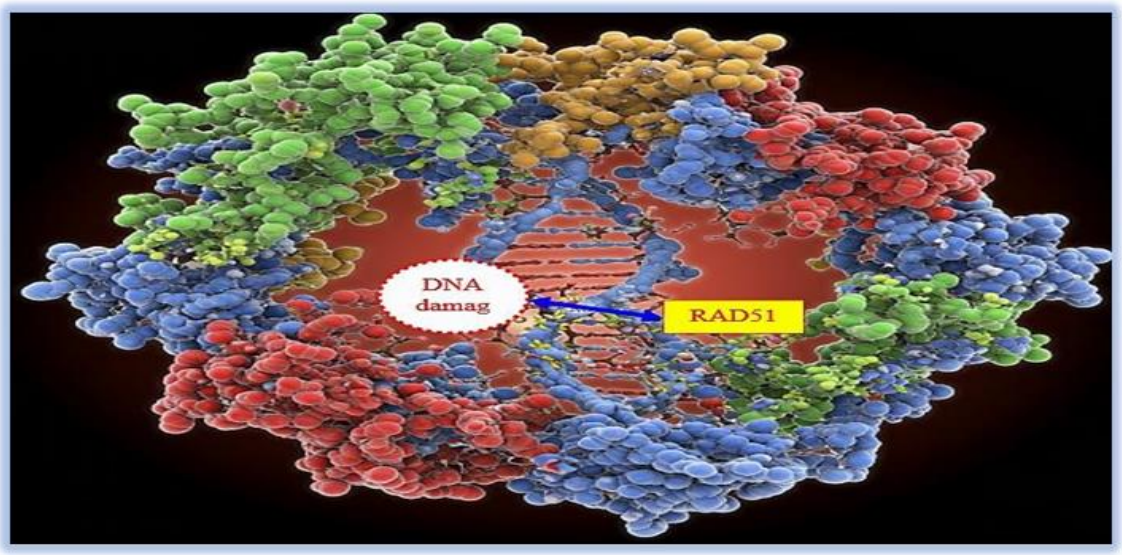

Figure 3. At high concentrations (in the blue arrow), alkaloids and steroids may induce cytotoxicity via pro-oxidant activity and DNA damage, as suggested by RAD51 binding.

4. DISCUSSION

The *Allium cepa* root tip assay is a widely accepted and cost-effective bioassay model for evaluating the cytotoxicity and genotoxicity of chemical and natural products due to its high sensitivity, ease of observation, and correlation with mammalian systems [95]. In the present study, the aqueous crude extract of *G. nepalensis* significantly inhibited root elongation in a dose- and time-dependent manner, with higher concentrations (10–20% v/v) leading to drastic reductions in root length within 24 to 72 hours. These observations strongly suggest that the extract contains active phytochemicals capable of interfering with cellular proliferation, particularly mitosis, in meristematic tissues. Phytotoxic effects in *A. cepa* roots are often associated with disruption of microtubule organization, inhibition of mitotic spindle formation, and induction of chromosomal aberrations such as C-mitosis, laggards, anaphase bridges, vagrant chromosomes, and micronuclei [96], all of which are characteristic indicators of genotoxic stress. The inhibition of cell cycle progression in plant root meristems is often indicative of a broader cytostatic or cytotoxic potential that could extend to mammalian systems and justify further pharmacological investigation [97].

The cytotoxicity observed may be attributed to specific classes of phytochemicals known for their anti-mitotic properties. Among these, alkaloids, particularly berberine, are reported to interfere with microtubule polymerization and destabilize spindle formation, leading to metaphase arrest [98]. Similarly, steroids such as β-sitosterol are implicated in disrupting DNA replication and repair pathways by modulating nuclear receptor signaling and membrane fluidity [99]. To provide molecular- level insight into these interactions, in silico docking was performed using RAD51—a key recombinase involved in homologous recombination repair of DNA double-strand breaks—as a molecular target. Berberine and β-sitosterol demonstrated binding affinities of -6.2 and -7.0 kcal/mol, respectively, suggesting their potential to inhibit RAD51 function, thereby impairing DNA repair and enhancing genomic instability [100]. These interactions may lead to cell cycle arrest, accumulation of DNA damage, and eventual cell death, consistent with the mitoinhibitory effects observed in the *A. cepa* assay. The DNA-damaging potential of such compounds is corroborated by previous findings where alkaloid-rich extracts induced oxidative DNA lesions, apoptosis, and chromosomal fragmentation in various test systems [101]. Furthermore, cytogenetic endpoints from *Allium* assays have been validated by mammalian micronucleus and comet assays, strengthening the predictive toxicological relevance of these results [102].

In addition to alkaloids and steroids, polyphenolic compounds such as flavonoids and phenolic glycosides, although typically antioxidant at physiological levels, have been reported to exert pro- oxidant effects at high concentrations, particularly under conditions of metal ion presence or oxidative stress [103]. These pro-oxidant effects can catalyze the generation of reactive oxygen species (ROS), resulting in lipid peroxidation, protein carbonylation, and DNA oxidation [104]. ROS-mediated damage to nuclear and mitochondrial DNA activates the intrinsic apoptotic pathway and can lead to caspase-dependent or -independent cell death [105]. For example, high doses of quercetin, a dominant flavonoid in *G. nepalensis*, have shown dual behavior—acting as an antioxidant at low doses while inducing DNA strand breaks, chromatin condensation, and mitochondrial dysfunction at higher concentrations [106]. Interestingly, the *A. cepa* assay showed a marked reduction in cytotoxicity at 72 hours in lower concentration groups (2.5–7.5% v/v), suggesting a potential adaptive or compensatory antioxidant response over time. This delayed recovery could be due to the upregulation of endogenous antioxidant enzymes or the ROS-scavenging effects of phytochemicals like quercetin 3-O-rutinoside and chlorogenic acid, both of which were weak RAD51 binders (-5.8 and -5.7 kcal/mol, respectively), indicating reduced interference with DNA repair mechanisms [107].

This hormetic effect—a biphasic dose-response where low doses stimulate and high doses inhibit biological activity—is common among polyphenolic compounds and may offer therapeutic leverage in disease models such as cancer or inflammation where selective cytotoxicity is beneficial [108]. However, such duality also raises safety concerns for crude extracts used in traditional medicine, especially without standardization of active constituents or proper dosage control. The complex interplay between pro-oxidant and antioxidant effects further underscores the importance of elucidating the mechanistic basis of cytotoxicity for phytotherapeutic development. Notably, plant- derived compounds with moderate toxicity in the *Allium* model, such as berberine and chlorogenic acid, have been successfully advanced into preclinical or clinical stages for their anti-cancer, antimicrobial, and metabolic effects, illustrating the translational relevance of this assay [109]. Nevertheless, caution must be exercised, as the cytotoxic potential at high doses could translate into genotoxicity or mutagenicity in vivo. Therefore, detailed pharmacokinetic profiling, cytogenetic assays in animal models, and molecular studies on apoptosis, oxidative stress, and cell cycle regulation are warranted to validate the therapeutic window and safety of *G. nepalensis* extracts [110]

5 CONCLUSION

This study on Gynura nepalensis leaf extract demonstrated that it possesses significant health benefits. This supports its traditional use in diabetes, wounds, and heart problems. The extract contains a variety of compounds, including alkaloids, flavonoids, tannins, saponins, glycosides, steroids, and sugars. Key compounds, such as chlorogenic acid and quercetin, are responsible for these effects. The extract can be toxic at high doses, as evidenced by a test where it inhibited root growth by 90.04% at a 20% concentration. This might be due to the stress caused by alkaloids and steroids. At lower doses (0.18–0.36%), it reduces inflammation by 53.38% and stabilizes cell membranes, preventing 57.43% of cell damage. It also helped break down the blood clots by 41.17%. However, these effects are weaker than those of standard drugs such as aspirin and streptokinase. Computational docking studies reinforced these observations, indicating that quercetin and chlorogenic acid exhibit strong affinities for COX-2 and PAI-1, which implies potential anti-inflammatory and thrombolytic actions. The combination of antioxidant and anti-inflammatory characteristics underscores the potential of the extract for multi-target therapy, likely amplified by the synergistic interactions of its phytochemicals. Nonetheless, the presence of hepatotoxic pyrrolizidine alkaloids in Gynura poses safety concerns, highlighting the need for thorough screening. Future investigations should aim to standardize extraction methods, quantify active compounds, clarify specific molecular pathways, and perform in vivo and clinical trials to enhance therapeutic effectiveness and ensure safety for clinical application.

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