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Antioxidant, anti-inflammatory, thrombolytic, and cytotoxic activities of *Gynura nepalensis* DC. and isolation of its steroid constituents

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The study was aimed at phytochemical profiling of *Gynura nepalensis* DC. as well as assessment of possible antioxidant, anti-inflammatory, thrombolytic, and cytotoxic activities of its fractions named hexane (HSF), dichloromethane (DCMSF), ethyl acetate (EASF) and aqueous (AQSF) soluble fractions through *in vitro* assays. Chromatographic fractionation and purification followed by ^{1}H NMR spectral analysis afforded two sterols named β-sitosterol and stigmasterol, which appears to be a first-time reporting from the species. In the free radical scavenging assay, both DCMSF and EASF exhibited prominent antioxidant activities which was further reinforced by their comparatively greater phenolic content. In membrane stabilizing test, prominent inhibitions of hemolysis as exerted by EASF, AQSF, and DCMSF, were evident of their significant anti-inflammatory activities, compared to aspirin. With respect to streptokinase, HSF and DCMSF demonstrated moderate thrombolytic properties, whereas in the cytotoxicity study, only HSF illustrated prominent cytotoxic properties. In conclusion, the results are suggestive of the presence of bioactive molecules within the fractions capable of exerting potent pharmacological activities.

Keywords: Anti-inflammatory, Antioxidant, Cytotoxic, *Gynura nepalensis*, Sterols, Thrombolytic.

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Introduction

From antiquity, nature has contributed to the field of drug discovery with diverse range of chemically unique bioactive molecules. Even in recent times, with the evolution and development of combinatorial chemistry, retrosynthetic methods or computer aided drug designing, nature has remained the greatest provider of lead compounds as well as drug molecules[1]. Bioactive molecules from plants are mainly drawn from their content of secondary metabolites. Secondary metabolites are plant constituents which are inessential for their regular growth but contribute to the continuance of the species within specific natural habitat[2]. Isolation of such phytoconstituents usually begins with pharmacological screening of the plant extract for determination of prominent bioactivities, followed by phytochemical screening and purification of molecules as well as investigation of isolated molecules for bioactivities[3].

Belonging to the Compositae family, the genus *Gynura* has been resourceful to mankind both as a source of dietary components as well as herbal medicinal ingredients. Frequent incorporation of different species in salad and vegetable dishes are in practice in many countries of Southeast Asia. Furthermore, the genus has also been recorded for ethnomedicinal utilization against many ailments covering migraine, fever, rashes, inflammation, rheumatism, herpes simplex virus, constipation, hypertension, hemostasis, kidney disease, diabetes mellitus and cancer[4]. Among the lesser studied species of the genus, *Gynura nepalensis* DC. is noteworthy for its medicinal utility in the treatment of hyperglycemia, hypertension, indigestion, inflammation, cuts and wounds[5]. Originating from Nepal, this perennial herb is distributed in many countries of Southeast Asia, tropical Africa and Australia[5]. Investigation into the plant has revealed prominent radioprotective, antimutagenic, and cardioprotective properties of the leaf extract whereas the flower extract was reported to exert hypolipidaemic and hepatoprotective activities[6,7].

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Previous studies involving pharmacological screening into different species of *Gynura* have demonstrated prominent antioxidant, anti-inflammatory, and cytotoxic activities commonly prevalent among the species. A rich phenolic content and prominent antioxidative potential have been documented for *G. bicolor*, *G. divaricata*, *G. formosana*, *G. procumbens*, *G. pseudochina* and *G. segetum*[4]. All of the aforementioned species except for *G. divaricata* and *G. pseudochina* exhibited prominent anti-inflammatory activities *in vitro* and *in vivo* which were further attributed to their capacities to suppress pro-inflammatory modulators such as nitric oxide and tumor necrosis factor-α and interleukins[4]. Ethyl acetate extract of *G. bicolor* was reported to exert antiproliferative effect against HCT-116 and HCT-15 colon cancer cells[4]. Cytotoxic activity of *G. divaricata* was illustrated in KB, MCF-7 and NCI-H187 cancer cell lines[4]. Ethyl acetate fraction of *G. formosana* also inhibited cellular proliferation of MCF-7 cell lines along with HepG2 cell line[4]. Furthermore, ethanol extract of *G. procumbens* significantly inhibited mammary and hepatic carcinoma in Sprague Dawley rats. From phytochemical point of view, a large number of chemical studies covering different species of *Gynura* have illustrated widespread distribution of pyrrolizidine alkaloids, flavonoids and phenolic compounds[4]. Therefore, the current study was designed to investigate *G. nepalensis* for the nature of its chemical constituents as well as possible antioxidative, anti-inflammatory, cytotoxic and thrombolytic potentials of the plant.

Materials and Methods

Collection and preparation of the plant material

The plant sample was collected from Savar, Dhaka, Bangladesh by expert professionals of the Bangladesh National Herbarium in the month of July 2019 (rainy season in Bangladesh). Proper identification of the plant was performed by the same authority through inspection of all the major components, especially the flower. A specimen specific reference number (Voucher specimen no. 48210) was issued for further referencing. The plant material was shade dried, followed by reduction into coarse powder with the help of industrial grade grinding machine.

Preparation and fractionation of crude extract

Powdered plant material (500 g) was soaked in 2.5 L of distilled methanol and maintained in that condition for 14 days with occasional agitation. The methanolic content was then filtered with the help of fresh cotton plug and further clarified using Whatman #1 filter paper. The content was concentrated in Rotary Evaporator to obtain the crude methanol extract. Modified Kupchan Partitioning protocol as developed by Van Wagenen *et al.*[8], was followed for fractionation of the crude extract. Four different fractions named as hexane (HSF), dichloromethane (DCMSF), ethyl acetate (EASF), and aqueous soluble fraction (AQSF) were obtained from the fractionation and the solvents were evaporated to collect concentrated fractions.

Phytochemical screening

The fractions were subjected to a number of standard phytochemical tests to ascertain the chemical content of different fractions of *G. nepalensis*. Chemical tests for the determination of alkaloids, phenol, flavonoids, coumarins, quinones, glycosides, sterols, terpenes and saponins were designed following conventional protocols[9,10].

Isolation and characterization of secondary metabolites

The hexane soluble fraction of *G. nepalensis* was subjected to fractionation with the help of size exclusion chromatography assisted by polarity-based elution system. The application of size exclusion chromatography in preference to basic column chromatography assisted in the early separation of chlorophyll abundant within the leaf extract. This in turn, minimized the interference of chlorophyll to the proper isolation and adequate purification of individual phytoconstituents. Sephadex LH-20 beads was soaked in a system of hexane: dichloromethane:methanol (2:5:1) and used to fill up a glass column of 3.8 centimeters diameter to a length of 45.7 centimeters. Precisely 1 g of the fraction dissolved in the same solvent system was filtered and loaded at the top of the column. A gradient solvent system of gradually increasing polarity was employed. The elution system was initiated with hexane: dichloromethane (2:5), followed by hexane:dichloromethane: methanol (2:5:1) and dichloromethane: methanol (4:1 and 1:1). Pure methanol was utilized to complete the elution and clear the column of any remaining sample. The porous nature of the Sephadex beads allowed for the separation of compounds based on size whereas the gradient solvent system allowed for polarity-based separation. Thus, extensive fractionation was achieved and a total of sixty fractions were obtained in test tubes. TLC was performed on marketed aluminium plates pre-coated with silica gel (Keiselgel 60 PF_{254}), for the screening of fractions. A number of

different solvent systems with a range of polarity like dichloromethane in hexane and ethyl acetate in toluene were employed to achieve optimal separation on TLC plates. Fractions illustrating similar compounds with similar retention factors (R_f) on the TLC plate were mixed together. The last mixture from the hexane soluble fraction comprised the content of last sixteen test tubes (45 to 60). This mixture was further subjected to preparative TLC utilizing a relatively non-polar solvent system of 50% dichloromethane in hexane (1:1). A single sample was isolated from this mixture at the end of the technique designated as GNH-11 which was characterized by ^{1}H nuclear magnetic resonance (NMR) spectrum in order to elucidate the chemical structure(s) of the sample. The established chemical structures were later illustrated using ChemDraw Ultra 12.0 following standard ACS guidelines.

Other fractions from the HSF (1 to 44) did not generate any significant sample which could be recognized under UV light or through TLC screening. Besides, the mid-polar and polar fractions of the extract, viz. DCMSF and EASF, respectively, were subjected to extensive fractionations through multiple modes of chromatography due to their richer phytochemical contents as depicted through TLC visualizations. Moreover, the isolated phytoconstituents from DCMSF and EASF were subjected to specific bioactivity studies both *in vitro* and *in vivo* and those results have been the topic of separate article(s), which is the reason for their exclusion from this one.

In vitro biological assays

Determination of total phenolic content

Oxidation of ionized phenols in alkaline solution by Folin-Ciocalteu reagent can be visualized following the transformation of natural yellow colour of the reagent to a blue shade. Based on this principle and following the method developed by Skerget and co-workers[11], the phenolic content of plant extracts were estimated, expressed as mg of GAE (gallic acid equivalent) per g of the dried fraction.

Determination of antioxidant activity

As designed by Brand-Williams and associates[12], the antioxidant activities of plant extracts were estimated by measuring their capacity to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Standard curves of per cent inhibition versus concentration were prepared for every sample and the concentration required for inhibition of 50% DPPH radical (termed as IC_{50} value) was calculated for each of the fractions as well as standard Tert-butyl-1-hydroxytoluene (BHT).

Determination of anti-inflammatory activity

Anti-inflammatory potentials of plant extracts were estimated by extrapolation of the erythrocyte membrane stabilization activities of the samples in hypotonic solution[13]. The anti-inflammatory capacities of the extracts and the standard aspirin were expressed as per cent inhibition of hemolysis of erythrocytes in hypotonic solution of sodium chloride in phosphate buffer.

Determination of thrombolytic activity

Following the procedure developed by Prasad and co-workers[14], the ability of plant fractions to cause dissolution of blood clots was used to determine the thrombolytic properties of the fractions. The thrombolytic capacities of 100 µL of aqueous solution of the samples (2 mg/mL) were compared against that of streptokinase at a dose of 30,000 IU.

Determination of cytotoxic activity

Brine shrimp lethality bioassay, as designed by Meyer and associates[15] was employed to determine the cytotoxic potentials of different samples. The assay made use of nauplii obtained following the hatching of *Artemia salina* leach (brine shrimp eggs) in simulated sea-water. Standard curves of per cent mortality versus concentration were prepared for each of the samples as well the standard vincristine sulfate and the concentration required to achieve 50% mortality, known as LC_{50} was calculated in each case.

Statistical analysis

For the *in vitro* assays, each experiment was conducted in triplicate and three sets of data (n=3) were recorded for each sample including the control, standard, and different fractions. The results were analyzed with the help of GraphPad Prism 8.0, USA and expressed as mean±SEM (standard error of mean). Regression analysis was employed for the calculation of IC_{50} and LC_{50} values in antioxidant and cytotoxicity determination assays, respectively. GraphPad Prism 8.0 was also employed to generate comparative illustrations of the results of each *in vitro* assay. Tukey's post-hoc test was performed to compare between groups on multiple variables. Calculated *p* values below 0.05, indicated statistically significant differences between control and different samples.

Results

Phytochemical screening

Presence of different types of phytoconstituents in different fractions of *G. nepalensis*, as estimated by the screening methods, has been demonstrated in Table 1. All the fractions exhibited the presence of alkaloids and quinones. Furthermore, hexane soluble fraction illustrated the presence of coumarins and sterols. Presence of phenolic, flavonoid, coumarin and saponin content was illustrated for dichloromethane soluble fraction. Phenolic and flavonoid constituents were also revealed in ethyl acetate soluble fraction along with glycosides. Aqueous soluble fraction tested positive in flavonoid and glycosidic test. None of the fractions exhibited positive result for terpenoids.

Identification of phytoconstituents

Analysis of the ^{1}H NMR spectrum of GNH-11 revealed it to be a mixture of two compounds designated as **1** and **2**. Assignment of all the major signals with at least one proton intensity indicated the structure of compound **1**. On the other hand, consideration of two minor signals of insignificant integration values along with all the major signals suggested the presence of another closely related structure (compound **2**) in the form of mixture.

Characterization of compound 1 as β-sitosterol

The distribution pattern of the signals in the ^{1}H NMR spectrum was suggestive of a steroid structure. A multiplet at δ 3.55 was indicative of an oxymethine proton at C-3 of a steroid nucleus. The typical olefinic proton (H-6) of the steroidal skeleton was also evident due to the presence of a doublet at δ 5.37 (*J*=4.8 Hz). Furthermore, six methyl groups were revealed by six sets of signals each integrating for three protons. Two singlets at δ 0.70 and δ 1.03 were assigned to two methyl groups at C-13 (H_3-18) and C-10 (H_3-19), respectively. Two doublets centered at δ 0.85 and δ 0.82 (*J*=7.2 Hz) was attributed to the methyl groups (H_3-26 and H_3-27) at C-25. Another doublet (*J*=6.4 Hz) at δ 0.95 was assigned to the methyl group (H_3-21) at C-20 and the triplet (*J*=7.2 Hz) at δ 0.88 indicated the methyl group (H_3-29) attached to C-28. Thus, careful analysis of the spectral data and comparison of it with that of published data[16] confirmed compound **1** to be β-sitosterol (Fig. 1 and Table 2).

Table 1 — Presence of different phytoconstituents in the leaf fractions of *G. nepalensis*

Phytoconstituents	Chemical test involved	HSF	DCMSF	EASF	AQSF
Alkaloids	Mayer's & Dragendorff's test	+	++	++	+
Phenolics	10% ferric chloride test	-	++	+	-
Flavonoids	Sodium hydroxide (NaOH) test	-	+	++	++
Coumarins	Alcoholic NaOH test	+	+	-	-
Quinones	Sulfuric acid test	+	+	++	++
Glycosides	Fehling's test	-	-	+	+
Sterols	Libermann-Burchard test	+	-	-	-
Terpenes	Libermann-Burchard test	-	-	-	-
Saponins	Frothing test	-	+	-	-

*+ve sign indicates the presence of phytoconstituents, whereas –ve sign indicates absence. HSF = Hexane soluble fraction, DCMSF = Dichloromethane soluble fraction, EASF = Ethyl acetate soluble fraction, AQSF = Aqueous soluble fraction

Table 2 — ^{1}H NMR spectral data of compound 1 and 2 and published data of β-sitosterol and stigmasterol[36].

Proton position	Compound 1 δ_H in ppm Multiplicity (J in Hz)	β-sitosterol[36] δ_H in ppm Multiplicity (J in Hz)	Compound 2 δ_H in ppm Multiplicity (J in Hz)	Stigmasterol[36] δ_H in ppm Multiplicity (J in Hz)
H-3	3.55, m	3.52, m	3.55, m	3.52, m
H-6	5.37, d (4.8)	5.35, brs	5.37, d (4.8)	5.35, brs
H_3-18	0.70, s	0.68, s	0.70, s	0.70, s
H_3-19	1.03, s	1.00, s	1.03, s	1.00, s
H_3-21	0.95, d (6.4)	0.92, d (6.6)	0.95, d (6.4)	1.02, d (6.6)
H-22			5.18, dd (15.2, 8.4)	5.15, dd (15.2, 8.4)
H-23			5.04. dd (15.2, 8.4)	5.03, dd (15.1, 8.2)
H_3-26	0.85, d (7.2)	0.83, d (6.6)	0.85, d (7.2)	0.84, d (6.4)
H_3-27	0.82, d, (7.2)	0.81, d (6.6)	0.82, d, (7.2)	0.79, d (6.6)
H_3-29	0.88, t, (7.2)	0.86, t (7.1)	0.88, t, (7.2)	0.82, d (7.2)

Here, s = singlet, brs = broad singlet, d = doublet, dd = double doublets, t = triplet and m = multiplet.

Characterization of compound 2 as stigmasterol

In addition to all the signals described for compound **1**, the presence of two sets of double doublets (with insignificant integration values) at δ 5.18 and 5.04 (*J*= 15.2, 8.4 Hz) suggested the presence of a second olefinic bond between C-22 and C-23 in compound **2**. This was in close agreement with the published spectral data of stigmasterol[16] and compound **2** was identified accordingly (Fig. 1 and Table 2).

Total phenolic content

Regression analysis of the standard curve prepared for gallic acid, generated the equation y = 0.0401x + 0.0061 (R^2= 0.9993), which was used to estimate the total phenolic content. Among the fractions, statistically significant (*P* <0.0001) phenolic content of 19.23±0.17 and 13.30±0.12 mg GAE/g of sample, was exhibited by DCMSF and EASF, respectively, when compared with other fractions. For HSF and AQSF, total phenolic content was calculated to be 5.23±0.05 and 4.67±0.17 mg GAE/g of sample, respectively (Table 3).

Antioxidant activity

In the DPPH scavenging assay, the inhibition of the free radical was observed for each fractions along with the standard at ten gradually increasing concentrations, as illustrated in Fig. 2. At the maximum dose of 500 µg/mL, highest scavenging activity was exhibited by standard BHT (92.72±0.20%) followed by EASF (86.56±0.54%), DCMSF (83.28±0.10%), HSF (73.74±0.27%), and AQSF (71.80±0.37%), respectively. BHT and all the fractions inhibited DPPH in a dose-dependent manner as indicated by *P* value of less than 0.0001. Regression analysis of these data helped in the calculation of IC_{50} values. For the standard BHT, an IC_{50} value of 19.51±0.33 µg/mL was obtained. Compared to the standard, IC_{50} values of 29.59±0.64 and 31.23±0.32 µg/mL recorded for EASF and DCMSF, respectively (Fig. 3a, Table 3), indicated significant (*P* <0.05) antioxidant potentials of the fractions. On the contrary, mild antioxidant property was exhibited by HSF and AQSF, as evident from their IC_{50} values of 76.70±0.33 and 67.30±0.64 µg/mL, respectively.

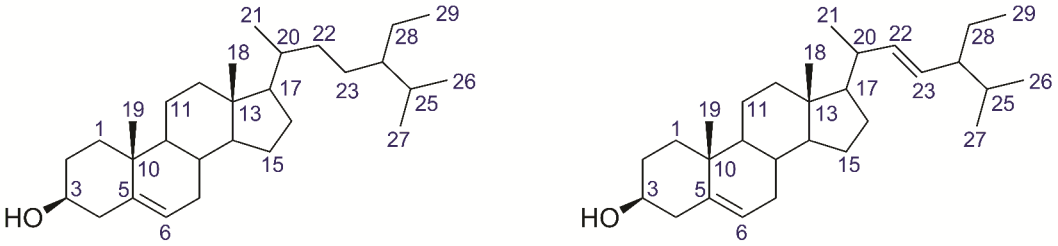

Fig. 1 — Structures of β-sitosterol (Compound 1) and stigmasterol (Compound 2).

Table 3 — *In vitro* antioxidant, anti-inflammatory, thrombolytic and cytotoxic activities of different fractions of *G. nepalensis*

Sample name	Total phenolic content	Antioxidant (DPPH) assay	Membrane stabilizing assay	Thrombolytic assay	Cytotoxic assay
	(mg GAE/ g extract)	IC_{50} value µg/mL	% inhibition of hemolysis	% clot lysis	LC_{50} value µg/mL
HSF	5.23 ± 0.05	76.70±0.33	10.63±0.10	20.80±0.14[b]	5.30±0.56[b]
DCMSF	19.23 ± 0.17[a]	31.23±0.32[b]	21.92±0.47[b]	20.46±0.25[b]	15.11±1.80
EASF	13.30 ± 012[a]	29.59±0.64[b]	69.26±0.30[a]	4.99±0.22	19.87±1.23
AQSF	4.67 ± 0.17	67.30±0.64	32.06±0.30[b]	3.16±0.18	184.9±30.47
BHT		19.51±0.33[b]			
Aspirin			72.42±0.07[a]		
SK				66.95±0.32[a]	
VS					0.47±0.03[a]

HSF = Hexane soluble fraction, DCMSF = Dichloromethane soluble fraction, EASF = Ethyl acetate soluble fraction, AQSF = Aqueous soluble fraction, GAE= Gallic acid equivalent, BHT= Tert-butyl-1-hydroxytoluene, SK= Streptokinase, VS= Vincristine sulfate. The superscript [a] represents statistical significance of *P* <0.0001, while superscript [b] represents statistical significance of *P* <0.05, compared to the standard and other fractions.

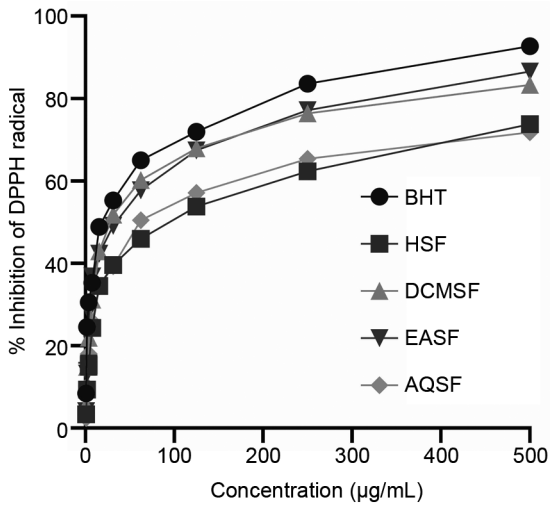

Fig. 2 — DPPH scavenging activity of BHT and different fraction of *G. nepalensis.* Dose-dependent inhibition of DPPH by standard and different fractions of *G. nepalensis*. Values are expressed as Mean±SEM (n = 3). BHT = Tert-butyl-1-hydroxytoluene, HSF = Hexane soluble fraction, DCMSF = Dichloromethane soluble fraction, EASF = Ethyl acetate soluble fraction, AQSF = Aqueous soluble fraction. The relationship was found to be statistically significant (P <0.0001) for standard and the fractions.

Anti-inflammatory activity

In the membrane stabilizing assay, highest inhibition of hypotonic solution induced hemolysis was exhibited by EASF (69.26±0.30%), which was in close proximity to the inhibitory activity of standard aspirin at 72.42±0.07% (Fig. 3b, Table 3). Both of them were found to be significantly higher (P <0.0001) than the activities of the other fractions. Moderate degree of significant anti-inflammatory activities (P <0.05) were also exhibited by AQSF and DCMSF, which inhibited hemolysis by 32.06±0.30% and 21.92±0.47%, respectively. HSF demonstrated only 10.63±0.10% inhibition of hemolysis, which was found to be insignificant (P >0.05) compared to the standard.

Thrombolytic activity

Standard streptokinase caused 66.95±0.32% lysis of preformed clot, indicating prominent thrombolytic activity (P <0.0001). In comparison to the standard, only moderate extents of clot lysis were exhibited by HSF (20.80±0.14%) and DCMSF (20.46±0.25%). Thrombolytic properties of both HSF and DCMSF were found to be statistically significant (P >0.05) as opposed to the minor activities exhibited by both EASF (4.99±0.22%) and AQSF (3.16±0.18%). A comparative illustration has been provided in Fig. 3c and Table 3.

Cytotoxic activity

Potent cytotoxic activity was observed for HSF, indicated by an LC_{50} value of 5.30±0.56 µg/mL, as compared to the standard vincristine sulfate (LC_{50} value 0.47±0.03 µg/mL). As illustrated in Fig. 3d and Table 3, DCMSF, EASF and AQSF exhibited mild to no cytotoxicity evident by their LC_{50} values of 15.11±1.80, 19.87±1.23, and 184.90±30.47 µg/mL, respectively. Subsequently, statistically significant (P >0.05) cytotoxic activity was characterized for HSF when compared to the other fractions.

Discussion

The observed presence of alkaloids, phenolic compounds, flavonoids and glycosides in *G. nepalensis* is in agreement with the already reported presence of such compounds in other species of *Gynura*[4]. Besides, all the fractions of *G. nepalensis* illustrated the presence of quinones, whereas the dichloromethane soluble fraction indicated coumarin and saponin content. These classes of compounds have rarely been reported from the genus. Thus, future phytochemical investigations into the plant is warranted to yield potentially novel phytoconstituents.

To the best of the authors' knowledge, both β-sitosterol and stigmasterol are being reported for the first time from *G. nepalensis*. Both of them have previously been reported from different species of *Gynura*[4]. A former study reported the isolation of β-sitosterol and stigmasterol as a mixture from the pet ether extract of the leaves of *G. procumbens*[16]. This finding acts as a support to the present analysis. Structural resemblance between the compounds and close proximity in polarity may have resulted in the isolation of the compounds as mixture. Besides, presence of both of these compounds has also been revealed in aerial parts of *G. pseudochina*[17], leaves of *G. segetum*[18], and rhizome of *G. japonica*[19]. β-sitosterol has been reported for its antioxidant, antidiabetic[20], anti-inflammatory, antipyretic[21], and analgesic activities[22]. Besides, multiples studies have demonstrated the capacity of β-sitosterol to induce apoptosis which was further associated with potent antiproliferative effect in breast cancer cell line[23] and chemoprotective effect in colon cancer cell line[24]. Similar apoptosis mediated antitumor activity has also been revealed for stigmasterol in gastric cancer cells[25]. Furthermore, stigmasterol has been reported to exhibit significant antioxidant[26], anti-inflammatory, analgesic[27], antimutagenic[28] and antiosteoarthritic activities[29]. Thus, presence of such bioactive molecules

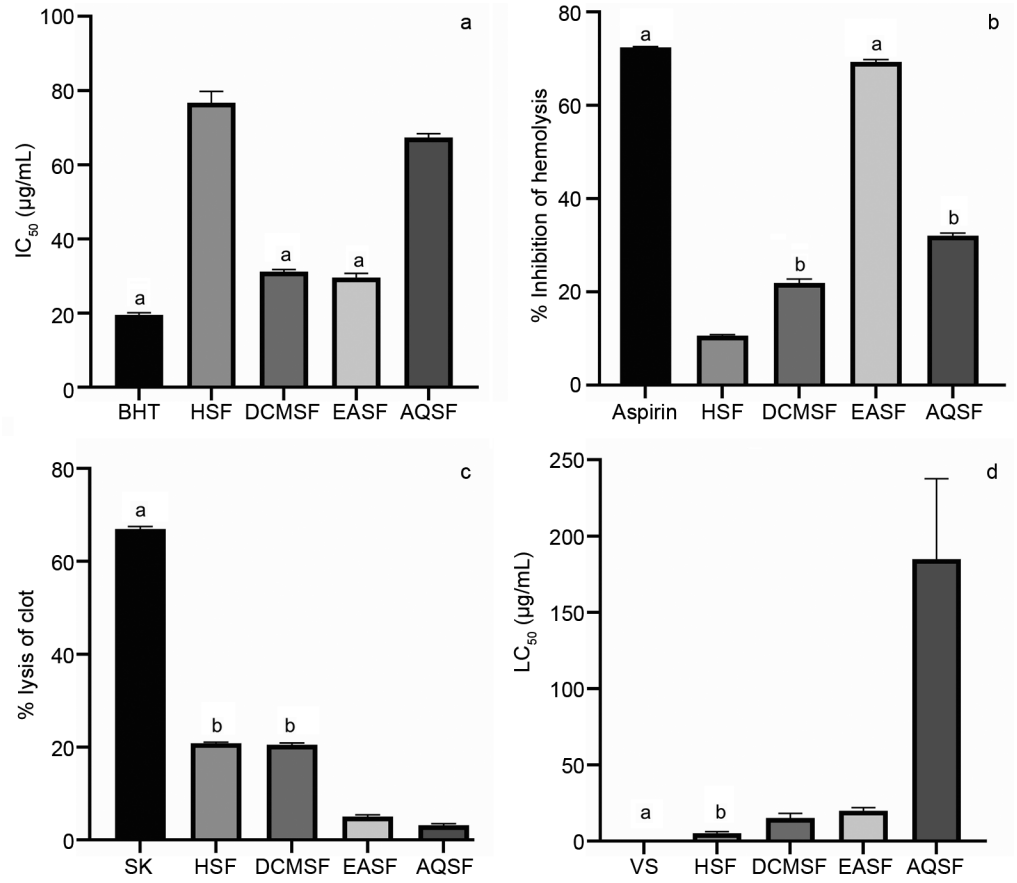

Fig. 3 — a) IC_{50} values of BHT and different fraction of *G. nepalensis.* The letter a represents statistical significance (P <0.05) compared to the IC_{50} of HSF and AQSF; b) Membrane stabilizing activity of aspirin and different fraction of *G. nepalensis.* The letter a represents statistical significance of P <0.0001 compared to the inhibition exhibited by HSF, DCMSF and AQSF, while b represents statistical significance of P <0.05 compared to the inhibition exhibited by Aspirin, HSF and EASF; c) Thrombolytic activity of streptokinase and different fraction of *G. nepalensis.* The letter a represents statistical significance of P <0.0001 compared to the inhibition exhibited by HSF, DCMSF, EASF and AQSF, while b represents statistical significance of P <0.05 compared to the inhibition exhibited by SK, EASF and AQSF; d) Cytotoxic activity of streptokinase and different fraction of *G. nepalensis.* The letter a represents statistical significance of P <0.0001 compared to the inhibition exhibited by HSF, DCMSF, EASF and AQSF, while b represents statistical significance of P <0.05 compared to the inhibition exhibited by VS, DCMSF, EASF and AQSF. Values are expressed as Mean±SEM (n = 3). BHT = Tert-butyl-1-hydroxytoluene, HSF = Hexane soluble fraction, DCMSF = Dichloromethane soluble fraction, EASF = Ethyl acetate soluble fraction, AQSF = Aqueous soluble fraction, SK = Streptokinase, and VS = Vincristine sulfate.

within *G. nepalensis* may provide scientific basis for ethnomedicinal and potential pharmacological uses of the plant.

Generation of excessive free radicals have been associated with a large number of chronic pathological conditions including inflammation, degenerative brain diseases, heart diseases, diabetes, cancer and atherosclerosis[30]. Thus, exogenous supply of antioxidant molecules from dietary ingredients, supplements or herbal preparations, may assist in the minimization of oxidative stress-related diseases. The significant antioxidative potential (P <0.05) of different fractions of the methanol extract of *G. nepalensis*, especially the dichloromethane and ethyl acetate soluble fractions could be of utmost importance in this regard. Phenolic compounds have been reported to possess superior capacity for free radical stabilization, compared to other phytoconstituents and a rich phenolic content within plant extracts may render them with significant antioxidative properties[31,32]. This postulation supported the prominent antioxidant activity of the dichloromethane and ethyl acetate soluble fractions of *G. nepalensis* (IC_{50} values of 31.23±0.32 and 29.59±0.64 µg/mL, respectively) as both of them illustrated high contents of phenolic compounds both in the phytochemical test and *in vitro* assay. In a previous study[4], 45% aqueous ethanol extract of

G. divaricata exhibited a phenolic content of 36.68±0.62 GAE/g of dry plant material and inhibited DPPH radical to a maximum extent of 89.67±0.06%. The findings of the study are in close agreement with that of *G. divaricata*, as the ethyl acetate and DCM soluble fractions of *G. nepalensis* have exerted 86.56±0.54 and 83.28±0.10% inhibition against DPPH, respectively. The fractions also exhibited comparable phenolic contents of 19.23±0.17 and 13.30±0.12 mg GAE/g of fraction, respectively. Similar pattern of antioxidative potentials was revealed for the ethyl acetate and aqueous extract of *G. bicolor*, ethyl acetate extract of *G. formosana*, ethyl acetate fraction of the ethanol extract of *G. procumbens*, aqueous ethanol extract of *G. pseudochina*, and methanol extract of *G. segetum*as determined by multiple *in vitro* assays including the DPPH scavenging assay[4]. Therefore, the antioxidant activity of *G. nepalensis* determined through DPPH scavenging assay can be easily correlated to the previously reported activities of other species within the genus.

The cellular mechanism of inflammation involves disruption of the lysosomal membrane and subsequent release of its content in the extracellular space. Stabilization of this membrane against rupture may attenuate the inflammatory process. Structural resemblance between the erythrocytic and lysosomal membranes have made it possible to relate the stabilization of erythrocytic membrane by any plant fraction with potential anti-inflammatory properties[13,33]. Among different fractions of *G. nepalensis*, ethyl acetate soluble fraction exhibited the maximum membrane stabilizing activity (P <0.0001), compared to aspirin. Significant anti-inflammatory potentials (P <0.05) of the dichloromethane and aqueous soluble fractions were also found, but to a lesser extent than the former. These results, in turn, support the conventional use of the plant in the treatment of inflammation and wounds[6]. Although both β-sitosterol and stigmasterol have been documented to possess anti-inflammatory properties[21,27], a lack of such activity as exhibited by HSF might suggest alternative underlying mechanisms involved for the molecules rather than membrane stabilization.

Being a leading health concern itself, thrombosis also forms the basis for a large number of pathological conditions including ischemic heart diseases, ischemic stroke, atrial fibrillation, venous thromboembolism and myocardial infarction[34,35]. Management of thrombosis both in the form of treatment and prophylaxis involves thrombolytic enzymes like streptokinase and anticoagulants such as aspirin, heparin and warfarin[36]. Prevalence of associated complications with each of these agents warrants the search for alternative therapeutics. Thus, different fractions of *G. nepalensis* were screened for possible thrombolytic properties. Compared to the standard, streptokinase, only moderate degree of thrombolytic activity was observed for hexane and dichloromethane soluble fractions of the plant. Statistical significance (P <0.05) for both these fractions supports the possibility of isolation of pure phytoconstituents with thrombolytic properties upon further research.

Cytotoxic properties of plant extracts can be translated into a number of clinical classes of drugs including anti-infective agents, antibacterial agents, anti-parasitic agents, anti-tumour agents, and anticancer agents[37-40]. A wide range of anticancer drugs such as vinca alkaloids, taxanes, and taccolonolides, covering an even wider array of underlying mechanisms, have been derived from plant sources[41] and the search still continues for more potent and more selective cytotoxic plant metabolites. Brine shrimp lethality bioassay into the fractions of *G. nepalensis* has demonstrated significant cytotoxic activity for the hexane soluble fraction (P <0.05) indicating potential cytotoxic principles within the fraction. β-sitosterol and stigmasterol being reported from the fraction is known to exert antiproliferative[23] and antitumor activities[25] which could be attributed to the cytotoxicity of the fraction. Thus, bioactivity-guided investigation is warranted to identify the responsible phytoconstituents and ascertain their mode of action.

Conclusion

Different fractions of *G. nepalensis* have been investigated for their phytochemical profiles as well as possible bioactivities. Two sterols have been isolated from the hexane soluble fraction and characterized as β-sitosterol and stigmasterol. Ethyl acetate soluble fraction of the plant demonstrated prominent antioxidative and anti-inflammatory properties. Strong antioxidant activity, as well as moderate level of anti-inflammatory and thrombolytic activities were characterized for the dichloromethane soluble fraction. Similarly, hexane soluble fraction exhibited moderate thrombolytic capacity, but a prominent cytotoxic activity was also recorded for the fraction. Lastly, the aqueous soluble fraction showed minimal bioactivities including moderate

anti-inflammatory potential. In-depth phytochemical and *in vivo* studies into different fractions of the plant are necessary for reinforcing current findings as well as establishing new bioactivities and phytoconstituents for the plant.

Conflict of interest

The authors declare that they have no conflict of interest.

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