NEUTRALIZING ABILITY OF *Andrographis paniculata* (Burm.f.) Wall. ex Nees STEM AND LEAVES ETHANOLIC EXTRACT AGAINST NEUROTOXICITY INDUCED BY *Naja philippinensis* Taylor, 1922 VENOM BASED ON HISTOLOGICAL CHANGES IN *Danio rerio* (F. Hamilton, 1822) GILLS AND LIVER

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Abstract: The need for promising alternative sources of snake antivenin has placed a growing interest in utilizing pharmacologically active compounds derived from medicinal plants capable of inhibiting the deleterious effects of snake venom. This study evaluated the ability of *Andrographis paniculata* (Burm.f.) Wall. ex Nees in neutralizing the toxicity of *Naja philippinensis* Taylor, 1922 venom. Mature stem and leaves of *A. paniculata* were subjected to ethanolic extraction, and the extract was converted to powdered form through lyophilization. Ethanolic fraction was water soluble, and phytochemical analysis revealed the presence of alkaloids, flavonoids, saponins, steroids, tannins and terpenoids. The median lethal concentration (LC_{50}) of *N. philippinensis* venom to *Danio rerio* (F. Hamilton, 1822), more commonly known as zebrafish, was 2.71±0.04 ppm. Seizures, hypolocomotion and paralysis were observed signifying the neurotoxic property of the venom. *In-vitro* neutralization of the venom LC_{50} with increasing concentrations of the extract was performed. The lethality inhibition within 24 hour exposure was used to express the median effective concentration ($EC_{50\text{-}24h}$) of the crude extract. The lethality inhibition within 24 hours upon exposure was used to express the median effective concentration ($EC_{50\text{-}24h}$) of the crude extract. Results showed that venom neutralization by *A. paniculata* ethanolic extract started at 77.64±0.82 ppm ($EC_{50\text{-}24h}$). Histopathological analysis was employed in order to assess and compare the tissue damage induced by raw venom, with those induced by venom treated with various extract concentrations. Raw venom induced irreversible tissue damage in the gills and liver of exposed zebrafish. In contrast, venom treated with extract in higher concentration induced non-lethal alterations. The antivenom potential of *A. paniculata* has been demonstrated. Thorough investigation is highly recommended to elucidate the actual mechanism of its pharmacologically active compounds in neutralizing snake venom.

Keywords: *Andrographis paniculata*, *Naja philippinensis* venom, *Danio rerio*, *in-vitro* neutralization, histopathological analysis

1. INTRODUCTION

Recently, the World Health Organization (WHO) included snakebites in the list of Neglected Tropical Diseases (NTDs) to improve global awareness to this particular disease (Gutiérrez *et al*., 2013). According to the regional office of the World Health Organization in Southeast Asia, there is an estimated 200 to 300 deaths due to snakebites in the Philippines each year, with farmers as the usual victims and cobras as the prominent culprits. Snakebites are commonly treated with monovalent or polyvalent snake antivenin

(Premendran *et al*., 2011). Due to insufficient and poor access to available health care services particularly in rural communities, people tend to rely on medicinal plants as an alternative treatment to snakebite complications (Veronese *et al*., 2005).

The use of plants as cure for diseases is a very old practice and can be traced back to ancient times, since these are readily available in rural areas in many parts of the world. Extract from medicinal plants contains pharmacologically active compounds able to help directly in the cure of ophidian envenomation, or may serve as supplements in traditional serum therapy (Soares *et al*., 2005).

Known as "King of Bitters", *Andrographis paniculata* has been traditionally used as an alternative treatment for common colds, liver disorders, bowel problems among children, colic pain, and upper respiratory tract infection (Chao & Lin, 2010). Its anti-venom potential has also been demonstrated against various snake species like *Daboia russelli* (Meenatchisundaram *et al*., 2009), *Naja naja* (Gopi *et al*., 2011), and *Naja nigricollis* (Kumarapppan *et al*., 2011), and is also found to potentiate the effect of anti-snake venom (ASV) against *Naja naja* and *Vipera russelli* venom (Premendran *et al*., 2011).

To the best of knowledge of the researchers of this study, there is no related work pertaining to its anti-venom potential against the Philippine native cobra species, *Naja philippinensis*. Thus, this study focused on elucidating the potential of this plant in neutralizing the venom of *N. philippinensis*, utilizing zebrafish as model organism. This research showcased the capacity of Philippine plants to be a potential source of alternative snake anti-venom. Furthermore, the use of zebrafish in this particular assay is an attempt to establish the potentiality of this fish species as an alternative to mice as model organism.

2. METHODOLOGY

2.1 Collection and preparation of stem and leaves of Andrographis paniculata

Mature shoots of *A. paniculata* collected from Poblacion, Teresa, Rizal, and authenticated by the Botany Division of the National Museum, were shed-dried for 40 days. Dried plant parts were cut into pieces and pulverized further. A total of 410 g of dried plant material were stored in a clean and dry place away from direct sunlight, prior to extraction.

2.2 Extraction and preparation of concentration fractions of A. paniculata

The extraction procedure follows Darbar *et al*. (2009). Dried plant materials weighing 410 g were placed inside the percolator followed by the addition of one liter of 95% ethyl alcohol as menstruum. After 24 hours, the percolate is allowed to drip slowly through the outlet and the process was repeated thrice. The percolate was then filtered using Whatman filter paper No. 1 and was evaporated under reduced pressure. Finally, the extract was converted to solid form through freeze drying or lyophilization. The powdered extract was placed in an air-tight container and kept inside the freezer prior to use. Stock solutions of the extract with 100, 150, 200, 250 and 300 ppm concentrations were prepared in reconstituted dilution medium and kept refrigerated until needed.

2.3 Phytochemical screening

Qualitative tests were performed in order to detect alkaloids, flavonoids, saponins, steroids, tannins and terpenoids in the extract (Biradar *et al*., 2014; Gond, 2014). The presence of the mentioned phytoconstituents was further confirmed through thin layer chromatography (TLC).

2.4 Acquisition and preparation of N. philippinensis venom solution

Lyophilized *Naja philippinensis* venom was obtained from the Research Institute for Tropical Medicine (RITM) and stored at -20°C prior to use. A stock solution was prepared by dissolving 950 mg of venom in 200 mL reconstituted dilution medium that contains 10.42% dimethyl sulfoxide (DMSO). The solution was kept inside the refrigerator at 2°C until use.

2.5 Zebrafish acute toxicity test

Adult *D. rerio* were purchased from Cartimar pet shop in Pasay City, Manila. All the fish were acclimatized for 12 days prior to the test. Venom solution of concentrations 2.25, 2.71, 3.16, 3.61 and 4.10 ppm were freshly prepared by diluting the venom stock solution using reconstituted dilution medium. Eight (8) individuals of zebrafish were transferred to a transparent container of 1L capacity containing the venom concentrations. Mortalities after 24-hour static exposure test were counted and utilized for the calculation of median lethal concentration (LC_{50}) of *N. philippinensis* venom. Calculation of LC_{50} was performed using GraphPad Prism 6.0 software.

2.6 Neutralization of venom toxicity and determination of effective concentration (EC_{50}) of the A. paniculata ethanolic extract

The calculated value of venom LC_{50} from zebrafish lethality test was used as standard concentration for the assessment of effective concentration (EC_{50}) of the two crude extracts. Freshly prepared extracts with concentrations of 140, 150, 160, 170, and 180 ppm were mixed in the venom solution in a 1:1 mixture ratio (Okonogi *et al*., 1979) for 1 hour at room temperature. The resulting solution (1 L) contains the calculated LC_{50}

value of the venom, and at the same time contains 70, 75, 80, 85 and 90 ppm of the extracts.

One (1) hour after incubation, eight (8) individuals of zebrafish were transferred carefully in 1.0 L of the prepared solution. Mortalities after 24-hour static exposure were counted and utilized in the calculation for the effective concentration (EC_{50}) of the extract. Calculation of EC_{50} was performed using GraphPad Prism 6.0 software.

2.7 Preparation of D. rerio for histopathological analysis

After 24 hours of exposure in each treatment, fishes were immediately euthanized by means of hypothermal shock or rapid cooling (Matthews & Varga, 2012). One fish per replicate was dissected in order to isolate the gills and liver. Isolated organs were washed with phosphate buffered saline (PBS) solution and fixed immediately by 10% neutral buffered formalin (Sabaliauskas *et al*., 2006). Zebrafish that died prior to the end of 24 hours of exposure were immediately removed in the water and processed according to the protocol mentioned above.

Organs contained in vials were submitted to HI-PRECISION Diagnostics (East Avenue, Quezon City Branch) for histological processing and histopathological analysis.

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

Qualitative analysis revealed the presence of all the metabolites in the extract was tested for, which include alkaloids, flavonoids, saponins, steroids, tannins, and terpenoids (Table 1). This is also confirmed through thin layer chromatography (TLC). The aerial parts of the plant are the most commonly used in various researches because these contain large number of phytochemical constituents (Akbar, 2011). According to Chao & Lin (2010) and Okhuarobo *et al*. (2014), flavonoids, terpenoids and polyphenols (including tannins) are the major bioactive components of this herb. The presence of these metabolites has a significant impact on the potentiality of *A. paniculata* extract as anti-venom.

3.2 Zebrafish lethality and neutralization of venom toxicity by the extract

The LC_{50} of *N. philippinensis* venom for zebrafish was found to be 2.71±0.04 ppm. Seizure behavior which includes ataxia, corkscrew swimming, hyperactivity, and increased erratic movements (Kalueff *et al*., 2013) was clearly observed approximately 30 minutes to one (1) hour after exposure to each concentration of the venom.

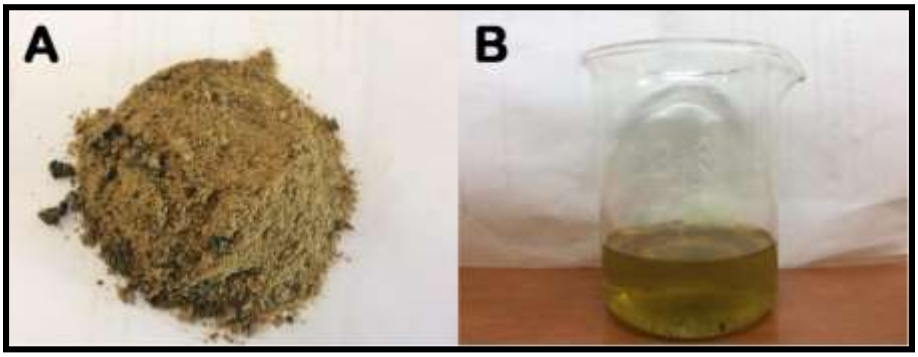

Figure 1. *Andrographis paniculata* stem and leaves ethanolic extract in (**A**) powdered form and (**B**) in aqueous solution.

Table 1. Summary of secondary metabolite composition in *Andrographis paniculata* stem and leaves ethanolic extract.

Secondary Metabolites	Qualitative Screening	Thin Layer Chromatography
Alkaloids	+	+ Rf= 0.81± 0.008
Flavonoids	+	+ Rf= 0.91± 0.014
Saponin	+	+ Rf= 0.87± 0.007
Steroids	+	+ Rf= 0.69± 0.011
Tannin	+	+ Rf= 0.86± 0.011
Terpenoids	+	+ Rf= 0.96± 0.011

Legend: (+) Present; (-) Absent; (Rf) Retention factor

The percentage mortality of zebrafish exposed in LC_{50} of the venom (positive control), tap water (negative control), and venom treated with increasing extract concentration was summarized in Table 2. It was observed that the mortality of zebrafish decreased dramatically with respect to increasing concentration of plant extract being incubated to the venom. Mortality is thus inversely proportional to the concentration of the plant extract.

The calculated EC_{50} for the *A. paniculata* ethanolic extract was 77.64±0.82 ppm. This suggests that venom neutralization by *A. paniculata* ethanolic extract started at this concentration and this was evident on the drastic decrease in percentage mortality beyond this concentration.

Snake venoms are complex cocktails of enzymes, polypeptides, non-enzymatic proteins, nucleotides, and other substances, many of which may have different neurotoxic properties (Ranakawa *et al*., 2013). Phospholipase A_2, metalloproteinase, and L-amino oxidase (LAAO) are some of toxic components present in *Naja* venom (Leon *et al*., 2011; Guo *et al*., 2012; Gasanov *et al*., 2014). Pre-incubation of *N. philippinensis* venom to varying concentrations of the extract before introducing to zebrafish showed that ethanolic extract from *A. paniculata* has the ability to deactivate various enzymes present in the venom and cause their decreased biological activities.

According to Lindahl & Tagesson (1997), the hydroxyl group in 5-position along with the double bond and the double-bonded oxygen in the tetrahydropyran (oxane) ring are accountable for the overall capability of flavonoids in inhibiting PLA_2 activity. Studies of Gopi *et al*. (2011) elucidate the interaction of andrographolide, which is a diterpene derivative and the major bioactive phytoconstituent of *A. paniculata*, with several venom toxins through molecular docking technique. They found that andrographolide from *A. paniculata* exhibits a significant binding activity against six toxins, namely disintegrins, aggretin, echicetin, acutolysin C, denmotoxin, and haditoxin, which are derived from different species of venomous snakes. Moreover, a negative binding energy was documented between ligand (andrographolide) and the six toxins mentioned after the docking experiment, suggesting that the ligand bounds effectively to the toxins; thus, neutralization is more likely to happen.

Table 2. Percentage mortality of zebrafish exposed to venom treated with increasing concentration of *Andrographis paniculata* ethanolic extract.

	Percentage Mortality (n=8)
Positive Control (Venom LC_{50})	100.00^{b}
Negative Control (dechlorinated tap water)	0.00^{a}
Treatments	
LC_{50} + 70.32 ppm plant extract	100.00^{b}
LC_{50} + 75.61 ppm plant extract	90.28 ± 2.78^{b}
LC_{50} + 80.15 ppm plant extract	66.67 ± 3.61^{c}
LC_{50} + 85.44 ppm plant extract	30.56 ± 4.71^{d}
LC_{50} + 90.73 ppm plant extract	0.00^{a}

**values with the same superscripts within the same column are not significantly different at 95% confidence limit*

***values reported as mean percentage mortality with S. E. after 24 hours*

3.3 Histopathological analysis of gills and liver of zebrafish

Histopathology is known as an important and widely accepted tool to assess the injury induced in fish after exposure to a certain or a variety of xenobiotics (Olarinmoye *et al*., 2009; Hassaninezhad *et al*., 2014). Histopathological changes of the gills and liver (Table 3) were used as biomarkers in this study because these are the sites where toxicants are mainly absorbed (Perera & Pathiratne, 2012). Static bioassay allows the entry of the cobra venom most probably through gills when fishes were exposed to its median lethal concentration (LC_{50}), and then diffuses into the bloodstream as the fish respires. The toxin inside the body of zebrafish travels to the liver through the hepatic portal vein and hepatic artery (Wolf & Wolfe, 2005). The same route of entry is expected when the zebrafish were exposed to the venom LC_{50} neutralized *in-vitro* by increasing concentrations of *A. paniculata* ethanolic extract.

Fish gills are involved mainly in respiration, osmoregulation, nitrogenous waste excretion, and acid-base balance (Hassaninezhad *et al*., 2014). Its structure provides a large surface area that has direct contact with the external environment making it a vulnerable target organ for waterborne xenobiotics (Flores-Lopes & Thomaz, 2011). The most common aberration found in all groups was lamellar blood congestion. Damaged pillar cell system causes dilation of lamellar capillary allowing increased blood flow inside the lamellae promoting blood congestion (Elshaer *et al*., 2013). This can possibly be attributed to the increased blood flow itself or the cell disruption property of the toxin (Hassaninezhad *et al*., 2014).

Aneurysm that was found in the positive control group as well as in experimental groups that received lower concentration of *A. paniculata* extract is a severe irreversible type of lesion characterized by balloon-like lamellae filled with erythrocytes due to blood extravasations into dilated blood vessels (Nascimento *et al*., 2012).

Clubbing of lamellar tips was another prevalent alteration found in all groups. Club shaped appearance of filaments is due to fusion of secondary lamellae as a result of an abnormal increase in the number of cells (hyperplasia) derived from primary lamellae and proliferates distally accumulating in inter-lamellar space towards the edge of secondary lamellae (Girija *et al*., 2014). Epithelial lifting is characterized by the elevation of epithelial cells that cover gill lamella which increases the distance between water and blood circulation in response to acute exposure to toxic substances (Flores-Lopes & Thomaz, 2011; Elshaer *et al*., 2013).

N. philippinensis venom induces serious irreversible damage in the major respiratory organ of the fish. This was still observed in experimental groups that were exposed to treatments A and B which contain lower concentrations of the extract. However, histopathologic alterations found in the succeeding groups that received treatments with higher extract concentrations (treatments C, D and E) are reversible and less fatal. Clubbing of lamellar tips due to hyperplasia, blood congestion and epithelial lifting that were found in the rest of the experimental groups belong to Stage I on the severity of alterations, and these do not impair the normal physiology of the gill tissue (Camargo & Martinez, 2007).

Table 3. Summary of histopathological alterations in the gills and liver of exposed zebrafish per treatment and in positive control.

	Gills	**Liver**
Positive Control	Congested lamella Multifocal clubbing of lamellar tips Some aneurysm and microthrombi	Sinusoidal congestion Severe hepatocellular lytic necrosis Prominent vacuolar degeneration
Group 01: Treatment-A	Congested lamella Multifocal clubbing of lamellar tips Occasional lamellar hyperplasia Some microthrombi	Sinusoidal congestion Severe hepatocellular lytic necrosis Prominent vacuolar degeneration
Group 02: Treatment-B	Congested lamella Multifocal to diffuse epithelial lifting and clubbing of lamellar tips Some aneurysm and microthrombi	Sinusoidal congestion Moderate to severe hepatocellular lytic necrosis Prominent random vacuolar degeneration
Group 03: Treatment-C	Congested lamella Multifocal to diffuse clubbing of lamellar tips Occasional lamellar hyperplasia Some microthrombi	Prominent sinusoidal congestion Moderate hepatocellular lytic necrosis Random vacuolar degeneration
Group 04: Treatment-D	Congested lamella Multifocal to diffuse epithelial lifting and clubbing of lamellar tips Microthrombi	Prominent sinusoidal dilation and congestion Diffuse mild hepatocellular lytic necrosis
Group 05: Treatment-E	Congested lamella Multifocal to diffuse epithelial lifting and clubbing of lamellar tips Some microthrombi	Sinusoidal congestion Mild hepatocellular lytic necrosis Prominent random vacuolar degeneration

Legend:

Positive Control: Venom LC_{50}
Treatment-A: Venom LC_{50} + 70 ppm *A. paniculata* extract
Treatment-B: Venom LC_{50} + 75 ppm *A. paniculata* extract
Treatment-C: Venom LC_{50} + 80 ppm *A. paniculata* extract
Treatment-D: Venom LC_{50} + 85 ppm *A. paniculata* extract
Treatment-E: Venom LC_{50} + 90 ppm *A. paniculata* extract

Primary metabolic activities such as processing and storage of nutrients, enzyme synthesis, bile synthesis and excretion, detoxification, and most specially the biotransformation of xenobiotic compounds are performed by fish liver (Wolf &Wolfe, 2005). Due to physiological similarity of mammalian liver to fish liver, snake venom-induced alterations in fish hepatocytes could possibly be seen in mammalian model in case of envenomation.

Sinusoidal congestion, vacuolar degeneration, and hepatocellular lytic necrosis are the histopathological alterations found in liver samples of zebrafish exposed to LC_{50} of *N. philippinensis* venom, as well as in all experimental groups that were exposed to venom treated with different concentrations of the extract. Sinusoidal dilation and congestion is considered as a circulatory disturbance alteration in the liver (Agamy, 2012) that happens when there is increased blood volume in sinusoids (Naeemi *et al*., 2013). Vacuolar degeneration or cellular swelling that was found in all exposed zebrafish is possibly linked to the action of phospholipase A_2 of the cobra venom wherein PLA_2 hydrolyzes phospholipids of plasma membranes and causes disturbance in the permeability of cell membrane to some ions such as sodium ions and water.

Hepatocellular necrosis is the most prominent cellular reaction induced by toxic substances, and is often irreversible (Wolf & Wolfe, 2005). A remarkable regression in the severity of hepatocellular lytic necrosis was observed from the experimental groups which received treatments containing increasing concentrations of the *A. paniculata* extract, up to the positive control group. Only the zebrafish exposed to raw cobra venom suffers severe hepatocellular lytic necrosis. Although sinusoidal congestion and vacuolar degeneration were still present in all groups, it must be noted that hepatocellular necrosis is the core indicator of toxicity. The observed remarkable decline of hepatocellular lytic necrosis among the groups is a clear indication of the weakened effect of the venom as a result of the intervention of *A. paniculata* ethanolic extract.

4. CONCLUSIONS

Based on the results obtained, ethanolic extract derived from dried stem and leaves of *A. paniculata* was found to contain alkaloids, flavonoids, saponins, steroids, tannins, and terpenoids. The extract was able to neutralize the LC_{50} of *N. philippinensis* venom. Venom treated with higher concentrations of *A. paniculata* extract induced non-lethal alterations in the gills and liver of exposed zebrafish.

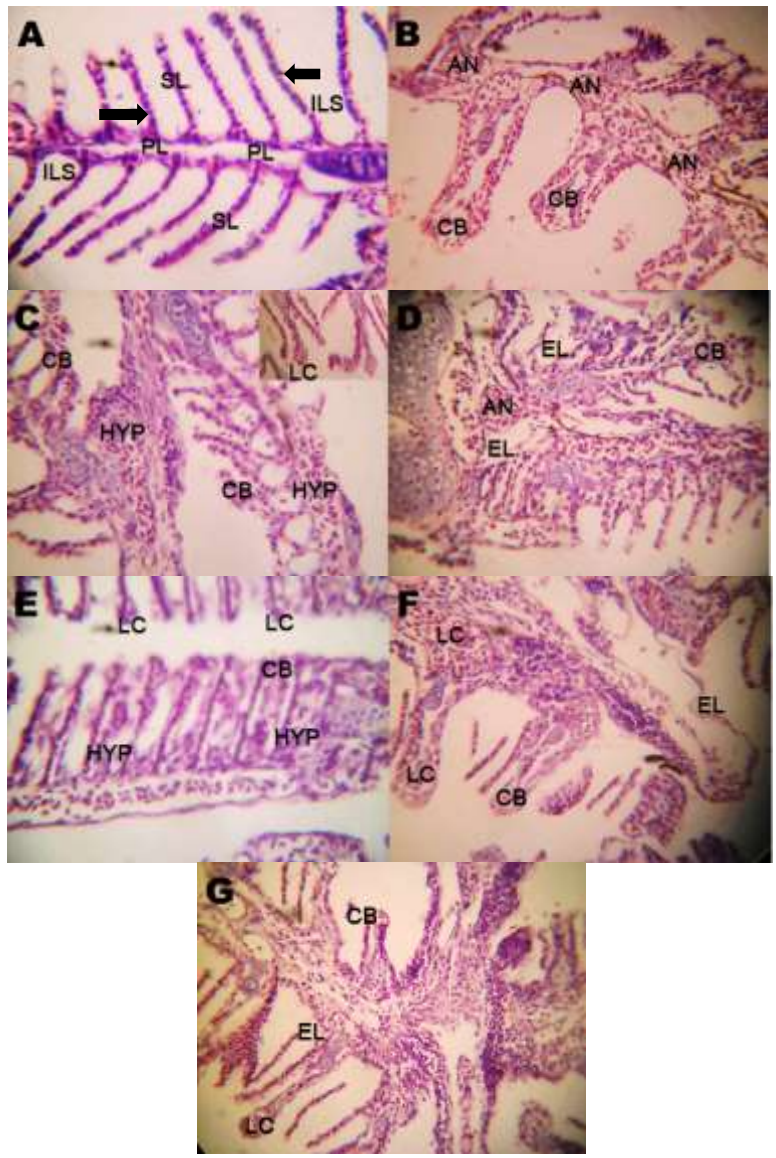

Figure 2. Representative photomicrographs of the normal gill histology of *Danio rerio* (zebrafish), and of gills containing histological aberrations after 24-hour exposure to *Naja philippinensis* venom (positive control) and to *N. philippinensis* venom treated with increasing concentrations of *Andrographis paniculata* ethanolic extract. (**A**) Normal aspect of zebrafish gill showing the primary lamella (PL), secondary lamella (SL), inter-lamellar space (ILS) and pillar cells (arrow); (**B**) Zebrafish gill after exposure to lethal concentration of *N. philippinensis* venom showing aneurysm (AN) and clubbing of lamellar tips (CB); (**C**) Zebrafish gill after exposure to Treatment-A showing lamellar congestion (LC), hyperplasia (HYP) and CB; (**D**) Zebrafish gill after exposure to Treatment-B showing AN, epithelial lifting (EL) and CB; (**E**) Zebrafish gill after exposure to Treatment-C showing LC, HYP and CB; (**F**) Zebrafish gill after exposure to Treatment-D showing LC, EL and CB; (**G**) Zebrafish gill after exposure to Treatment-E showing LC, EL and CB.

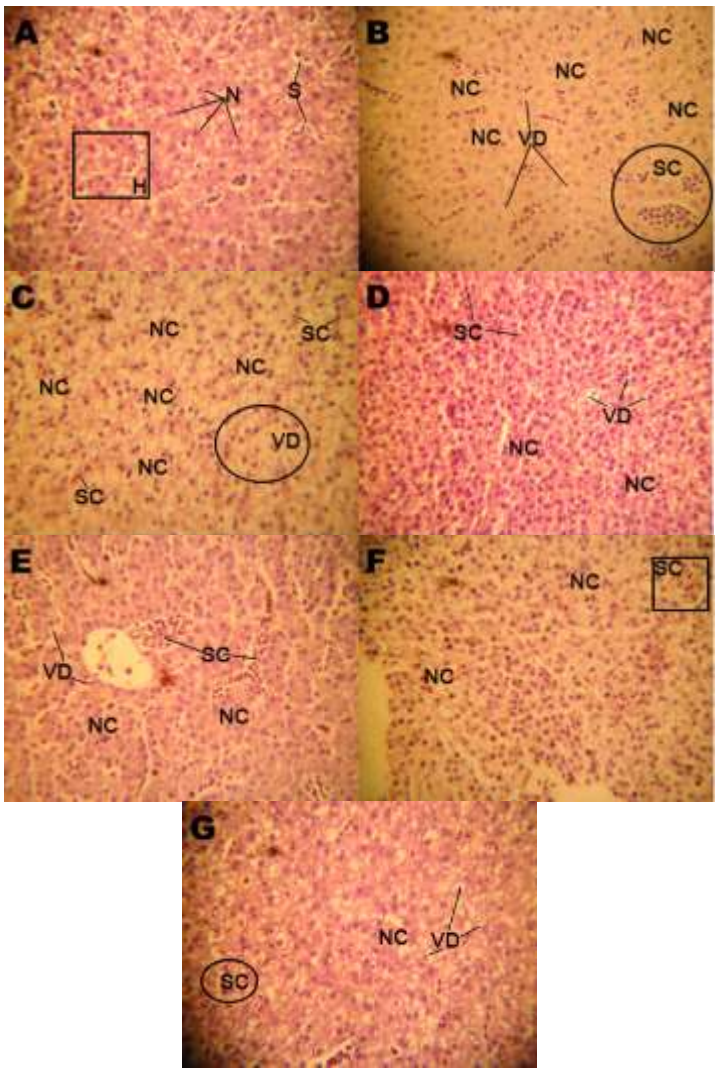

Figure 3. Representative photomicrographs of the normal liver histology of *Danio rerio* (zebrafish), and of livers containing histological aberrations after 24-hour exposure to *Naja philippinensis* venom (positive control) and to *N. philippinensis* venom treated with increasing concentrations of *Andrographis paniculata* ethanolic extract. (**A**) Normal aspect of zebrafish liver showing the hepatocytes (H), nuclei (N) and sinusoid (S); (**B**) Zebrafish liver after exposure to lethal concentration of *N. philippinensis* venom showing sinusoidal congestion and severe hepatocellular lytic necrosis with prominent vacuolar degeneration; (**C**) Zebrafish liver after exposure to Treatment-A showing sinusoidal congestion and severe hepatocellular lytic necrosis with prominent vacuolar degeneration; (**D**) Zebrafish liver after exposure to Treatment-B showing sinusoidal congestion, moderate to severe hepatocellular lytic necrosis with prominent random vacuolar degeneration; (**E**) Zebrafish liver after exposure to Treatment-C showing sinusoidal congestion, moderate hepatocellular lytic necrosis with random vacuolar degeneration; (**F**) Zebrafish liver after exposure to Treatment-D showing sinusoidal congestion and diffuse mild hepatocellular lytic necrosis; (**G**) Zebrafish liver after exposure to Treatment-E showing sinusoidal congestion and mild hepatocellular lytic necrosis with prominent vacuolar degeneration. Legend: NC= necrosis; SC= sinusoidal congestion; VD= vacuolar degeneration.

5. RECOMMENDATIONS

Upon concluding this study, the following can be recommended: (1) quantify and isolate the specific metabolite(s) in *Andrographis paniculata* that has the neutralizing activity on cobra venom; (2) compare the activity of the extract with standard Philippine cobra anti-venom; perform molecular techniques to scrutinize the antagonistic property of individual plant metabolites against snake venom enzymes at the molecular level; and (3) test the neutralizing activity of the extract in higher test organisms such as mice or rabbit.

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