

Documentation of phytochemicals, bio-toxicity and haemato-physiological responses at sub-lethal concentrations of *Parthenium hysterophorus* hydroethanolic extract in *Cyprinus carpio*: a concept of waste valorization as a phyto-ichthyotoxin

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Abstract

In the present study, phytoextraction was performed through aqueous, alcoholic and hydroethanolic (80%) solvents followed by phytochemical profiling and evaluation of median lethal concentration (LC₅₀) of hydroethanolic extract in Common carp, *Cyprinus carpio*. The haemato-immunological and biochemical response was evaluated at sub-lethal concentrations (T1: 0.395 mg l⁻¹ and T2: 0.79 mg l⁻¹ and control: devoid of extract) at three intervals (24, 48 and 96 h). The study revealed the presence of toxic constituents in extracts namely saponin, rotenone, tannin and alkaloid. Moreover, phytochemical typing exhibited the superior ability of hydroethanolic solvent and the extract was chosen for further biological activities. The anti-bacterial assay revealed the inhibitory capacity, whereas, phyto-haemagglutination revealed clumping of blood cells due to parthenium extract exposure. The haemagglutination limit test revealed the minimum concentration of extract causes agglutination was 1/96 and the haemolytic activity result exhibited clear lysis of fish RBCs. LC₅₀ of parthenium was found to be 18.99 mg l⁻¹. The study revealed that significant ($p < 0.05$) reduction in haemato-immunological indices alongside serum biochemical parameters. The results presented in the study emphasise locally available weed plant gajar ghas, *P. hysterothorus* as a non-chemical phyto-ichthyotoxin.

1. Introduction

Presently, fisheries and aquaculture are known to be a key source of income for most rural populations particularly people from third-world nations (Ekpenyong et al. 2022). Over the years, negligent fishing practices particularly the application of synthetic ichthyotoxins (cyanide, gammalin 20, etc.) and obnoxious fishing practices (dynamite fishing) by artisanal fishermen have received strong criticism owing to their detrimental effects (Oribhabor et al. 2020). However, phyto-ichthyotoxins have been adjudged to be a safer and more sustainable option in comparison to synthetic piscicides (Adeyemo 2005). Interestingly, ichthyotoxic plants such as *Justicia extensa*, *Derris elliptica*, etc. have been a customary practice in several parts of the world including India in both stagnant and flowing waters (Ekpenyong et al. 2022). Naturally derived substances such as phyto-piscicides are supposed to be less harmful to the environment since they are bio-degradable with less or no residual effects (Wadood et al. 2013). Moreover, intensive and irresponsible application of chemical pesticides or weedicides has resulted in the deterioration of the environment and aquaculture is not an exception (Singh et al. 2008). This scenario has pushed us back towards nature to look for non-chemical alternative sources. Keeping this milieu, the present study was intended to target a well-known and cosmo-available toxic plant, gajar ghas (*Parthenium hysterothorus*), to explore its ichthyotoxic potential.

P. hysterothorus is an extremely invasive, ubiquitous, ruderal weed species with a distribution of about 19 species globally, that has spread beyond its native range, South America and already infested more than 50 countries across the globe (Adkins and Shabbir 2014; Akhtar et al, 2019; Weyl et al, 2021). In India, *P. hysterothorus* is the most common species of the Parthenium family, Asteraceae (Kaur et al. 2021). International Union for Conservation of Nature (IUCN) has recognized the threat caused by the weed and considered it the “7th most devastating weed species” and also included it in the “100 most invasive species” (Roy and Shaik 2013; GISD 2018). Owing to its global distribution, *P. hysterothorus* is known by several names such as skhabootey (Pakistan), Feverfew (Caribbean), ragweed (USA), altamisa’ (Mexico), congress grass or carrot weed or gajar ghas (India) along with several other names including star weed, white top, bitter weed (Hussain et al. 2022). Over time, studies have shown extensive toxico-pathological changes including ulceration of the alimentary tract and

exposed skin alongside cytotoxic effects on liver and kidney tissues, mutagenicity, allergy, *etc.* (Cowie et al. 2020). So, the management of this aggressive weed is an important issue in parthenium-infested countries, including India. The employment of this plant for extracting toxic phytochemicals for multifarious applications might be an option for their management. Despite the toxic attributes and luxuriantly available in many parts of the world including India, its physiological effects on target and non-target organisms including aquatic animals have not been well examined, yet.

Fish is an important bio-indicator to measure the toxicological impacts of diverse xenochemical-induced toxicity (Iheanacho et al. 2021; Iheanacho et al. 2022). Freshwater fish, *C. carpio* is a hardy and disease-resistant organism, and hence can be adopted as a candidate for ecotoxicological assays. Blood-related parameters are important indices in analysing the animal's health status, including fish. Haematological, biochemical and immunological parameters provide extensive information about different physiological phenomena as a consequent result of toxicity causing adverse effects on the cellular or molecular level of the exposed organism (Jain and Kulshreshta, 2000; Svoboda et al, 2001; Kamal Shah et al, 2007). This study is, therefore aimed at evaluating the potential toxicity and effects of *P. hysterophorus* on haemato-physiological indices in a freshwater fish, common carp (*Cyprinus carpio*).

2. Material And Methods

2.1 Experimental fish

Fingerlings of *C. carpio* (mean length of 10.7 ± 1.5 cm and mean weight 17.1 ± 1.5 g) were obtained from Ilfan Fish Farm, Pondi, Kawardha. Fish were transported to the Department of Aquatic Environment and Health Management, Lt. SPN College of Fisheries, Kawardha and acclimatized for the next 20 days in 1000 l FRP tanks. The water in tanks was aerated sufficiently with routine observation. The fish were fed with a commercial diet twice a day @ 2% of body weight.

2.2 Collection of *P. hysterophorus* and phytoconstituents extraction

P. hysterophorus was collected from the college campus (latitude 22.008951° N and longitude 81.224344° E). The plant specimen was identified with the help of SK College of Agriculture, IGKV, Kawardha and further verified at <http://www.theplantlist.org>. The extract was prepared through the method described by Khan et al. (2018) with slight modifications. Briefly, the plants were washed, shed dried (at room temperature for two weeks) and grounded following soaking (1:10; w/v) into three different solvents namely water, absolute ethanol and hydroethanol (80%). The mixture was kept for 48 h and then filtered using Whatman filter paper (No.1) following centrifugation for 5 min at 10,000 rpm. The filtrate was evaporated, and the crude extract was collected followed by storage at -20°C until further use. The percentage yield was calculated by the formula, weight of the extract $\times 100$ / weight of the dry sample.

2.3 Property estimation of extracts

Two different concentrations of each extract were prepared at a uniform concentration of 10 mg of extract ml^{-1} of water for the phytochemical analysis and 50 mg of extract ml^{-1} of water for the anti-bacterial analysis.

2.3.1 Toxic phytochemical profile

The plant extract was screened for the presence of toxic constituents. Where, alkaloid content was determined through the method of Obadoni et al. (2001), where the total alkaloid percentage was calculated as the weight of residue $\times 100$ / weight of extract taken. Whereas, saponin content was evaluated by following the Makkar et al. (2007) method, where saponin content was determined through a standard curve of diosgenin and the content was represented as mg of diosgenin equivalent (mg DE g⁻¹ of extract). Similarly, rotenone content was represented as mg rotenone equivalent (mg RE g⁻¹ of extract) (Pagan 1948) and tannin content was depicted as gallic acid equivalent (GAE g⁻¹ of extract) through the method Schanderl (1970). Based on the results of the quantitative profile, the hydroethanolic extract was chosen for further analyses.

2.3.2 Anti-bacterial assay

To determine the anti-bacterial ability of *P. hysterophorus* extract, ten different indicator bacteria were tested through an agar-well diffusion assay (Khan et al. 2022). Briefly, microorganisms were allowed to grow in their respective media or nutrient agar. For the assay, about 2000 μ l of broth was spread uniformly on nutrient agar plates and let to dry followed by the preparation of wells (about 6 mm) with the help of a gel puncture. All three extracts were poured into the wells along with positive control (oxytetracycline 50 mg ml⁻¹; HiMedia, India) and negative control (distilled water). Bacterial plates were incubated for 24 h at 37°C. After incubation, the inhibitory potential was measured. All three extracts suspension were subjected to filter sterilization (0.22 μ m) before the assay.

2.3.3 Haemolytic activity and rate of haemolysis

Before the assay, a suspension of red blood cells was prepared. Briefly, collected fish (*C. carpio*) blood was centrifuged for 3 min at 5000 rpm followed by discarding of supernatant and washing of precipitated cells with NSS (normal saline solution; 0.9% NaCl at pH 7.0) at least thrice. At last, a 3% RBC suspension was prepared in NSS for the assay. Similarly, a suspension of *P. hysterophorus* extract was also prepared in NSS.

The haemolytic effect of the extract was determined through the method explained by Ktari et al. (2017). Briefly, 2970 μ l of 3% red blood cells suspension was mixed with 30 μ l extract and kept for incubation at 37°C for about 1 h followed by centrifugation at 5000 rpm for 10 minutes. After centrifugation supernatant was collected and optical density (OD) was recorded at 548 nm. Distilled water served as a positive control whereas NSS was a negative control. Similarly, the haemolysis rate was also determined, where 500 μ l of blood suspension was added with 2500 μ l of distilled water and kept for 1 h. The haemolysis rate was calculated by subtraction of the OD of extract and OD of negative control (NSS) divided by total haemolysis after 1 h.

2.3.4 Phyto-haemagglutination activity

The test is based on the agglutination or precipitation of red blood cells caused by various toxic components present in the extract particularly lectins (Ouattar et al. 2022). The assay was performed in a 96-well round-bottom plate as per the method described by Ouattar et al. (2022). Where, we mixed 25 μ l of extract, 50 μ l of NaCl solution (0.9%), and 25 μ l blood suspension (3%) followed by incubation at 37°C for 30 min and observation of cells under the microscope for any agglutination of RBCs (red blood cells).

2.3.5 Haemagglutination limit test

This study was conducted to find out the minimum concentration which causes agglutination of erythrocytes by following the method described by Ouattar et al. (2022). The assay was also performed in a 96-well round-bottom plate. For this purpose, a range of dilutions of extract starting from 1/3 to 1/3072 was prepared. Briefly, 50 µl of blood cell suspension was mixed with 50 µl of extract and 50 µl of NSS in the first well. Similarly, 10 more wells were prepared without extract in the horizontal direction in a 96-well plate. An aliquot of properly agitated 50 µl was serially transferred to the next well till the dilution reaches 1/3072. 12th well was kept as negative control (NSS). After, 35 minutes of incubation minimum concentration of extract that is causing lattice formation (between phytoconstituents and RBCs) and causing a red diffused coating at the well bottom was observed. Whereas, the unbounded RBCs sink at bottom of the well forming a button shape.

2.4 Determination of LC₅₀ and acute toxicity response

Range finding assay was aimed to determine the lethal concentration (LC₅₀) at 96 h of hydroethanolic *P. hysterothorus* plant extract against *C. carpio* fingerlings. Fish were exposed to a range of concentrations through the non-renewable immersion method in aquariums. Tests were performed in experimental glass aquaria (3'×1.5'×1.5') with 6 fingerlings in each aquarium. Extract concentrations in the assay include 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 mg l⁻¹. The control group deprived of the hydroethanolic extract was observed as a reference for the assessment of any morphological and behavioural changes caused by toxicant exposure. Failure of the fish to respond to push stimuli was used as an indicator of death. Mortality was observed and dead animals were immediately removed. No water exchange and no feeding were done during the entire experiment. Behavioural responses of test animals were monitored regularly according to OECD (OECD, 2014).

Following the determination of LC₅₀, two different sub-lethal concentrations of *P. hysterothorus* extract were chosen for the acute toxicity response for the period of 96 h. The test was performed in glass aquariums (n = 9) containing dechlorinated water with sufficient aeration under a natural photoperiod of 12 h dark/ 12 h light. The LC₅₀ of extract was found to be 18.99 mg l⁻¹ with Log₁₀ (LC₅₀) of 1.278 in static condition. Based on the result, two concentrations of extract *i.e.* T1: LC₅₀/50 (0.395 mg l⁻¹), T2: LC₅₀/25 (0.79 mg l⁻¹) and control: devoid of extract, combined with three exposure times (24, 48 and 72 h) were used for the observation of haemato-biochemical and immunological responses. The test was conducted under a static non-renewal bioassay approach. In total, 81 acclimatized common carp fingerlings were randomly assigned to nine treatment groups (one control and two parthenium extract treated groups; nine fish in each aquarium) followed by a completely randomised sampling design with three replicates for each treatment.

2.4.1 Collection of blood and serum isolation

For blood collection, fish from each tank was drawn randomly and blood was collected from the caudal vein. An aliquot of blood was analysed for haematological parameters and the remaining was kept for some time followed by centrifugation at 3000 rpm for 5 min. Serum was collected in aseptic vials and stored at -20°C until further analyses.

2.4.2 Haematological parameters

Erythrocytes count (EC), total leucocyte count (TLC) and packed cell volume (PCV) or haematocrit (Hct) were evaluated by following Schaperclaus (1991) method. Haemoglobin (Hb) level in blood was determined by using

Sahli Haemometer (Marienfeld, Germany) method. Whereas, mean corpuscular value (MCV), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) were calculated by the method described by Dacie and Lewis (2001).

$$\text{EC mm}^{-3} = \frac{\text{Erythrocytes in 80 small squares}}{80 \times 1/400 \times 1/10 \times 1/200}$$

$$\text{LC mm}^{-3} = \frac{\text{No. of leucocytes in 4 squares of 1 mm}^2}{4 \times 1/10 \times 1/200}$$

$$\text{MCV} = \text{Haematocrit (\%)} \times 10 / \text{RBC count (EC in million mm}^{-3}\text{)}$$

$$\text{MCH} = \text{Haemoglobin (g dl}^{-1}\text{)} \times 10 / \text{RBC count (EC in million mm}^{-3}\text{)}$$

$$\text{MCHC} = \text{Haemoglobin (g dl}^{-1}\text{)} \times 100 / \text{Haematocrit (\%)}$$

2.4.3 Biochemical studies

Serum glucose level was determined through glucose diagnostic kits from Coral Clinical Systems, India. Glutamate-oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) in serum were assayed using Medsource ozone biomedical (India) diagnostic kit.

$$\text{Total glucose (mg dl}^{-1}\text{)} = \text{Absorbance of test} \times 10 / \text{absorbance of standard}$$

$$\text{GPT (IU l}^{-1}\text{)} = \text{Absorbance of test} / \text{min} \times 1732$$

$$\text{GOT (IU l}^{-1}\text{)} = \text{Absorbance of test} / \text{min} \times 1732$$

$$\text{ALP activity (IU l}^{-1}\text{)} = \text{Absorbance of test} / \text{min} \times 2720$$

2.4.4 Immunological studies

The Lysozyme levels of serum were determined by Anderson and Siwicki's (1995) method with slight modification. Free oxygen radical production from phagocytes was estimated using nitro blue tetrazolium (NBT) dye as described by the Anderson and Siwicki (1995) method. Total protein and albumin levels in serum were determined using Medsource ozone biomedical diagnostic kit (India). Serum globulin was evaluated by subtracting the albumin levels from the total protein content in the serum.

$$\text{Total protein (g dl}^{-1}\text{)} = \text{Absorbance of test} \times 5.5 / \text{absorbance of standard}$$

$$\text{Albumin (g \%)} = \text{Absorbance of test} \times 3.5 / \text{absorbance of standard}$$

$$\text{Globulin (g \%)} = \text{Total serum protein} - \text{albumin content}$$

2.5 Statistical Analysis

Data obtained from the present experiment were subjected to analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) IBM version 21 (SPSS Inc, Chicago IL, USA). Differences in the means were compared using Duncan's multiple range test and significance was confirmed at $P = 0.05$. The LC_{50} was determined using probit regression analysis using BioSat v5.3.0 (AnalystSoft Inc. WALNUT, CA, USA). The results of each experiment were completed in triplicate. Results are presented as mean $\pm$ standard error.

3. Results

3.1 Yield percentage of the extracts

Yield percentage helps in assessing the extraction capacity of particular solvents used for the extraction of phytoconstituents. The yield of *P. hysterothorus* extracts for all three solvents including water, ethanol and hydroethanol varies from 6.71, 7.91 and 11.32%, respectively.

3.2 Phytochemical profile

Phytochemical profiling revealed the presence of different active compounds at variable levels in all three extracts (Fig. 1). The alkaloid percentage was maximum in hydroethanolic extract (23.35%) and alkaloid content was significantly ($p < 0.05$) high compared to aqueous extract. Similarly, tannin content was significantly ($p < 0.05$) high in hydroethanolic extract, 19.63 GAE g^{-1} of extract as compared to aqueous extract, 10.44 GAE g^{-1} of extract; however, the tannin content of the hydroethanolic extract was insignificant ($p > 0.05$) as compared to ethanolic extract (11.53 GAE g^{-1} of extract). The hydroethanolic extract also contains the maximum rotenone content, 38.82 mg RE g^{-1} of extract. In the present study, the order of saponin content in extracts was: hydroethanolic extract (394 mg DE g^{-1} of extract) > ethanolic extract (343 mg DE g^{-1} of extract) and > aqueous extract (238 mg DE g^{-1} of extract). Based on the present results, the hydroethanolic extract was chosen for further analyses.

3.3 Anti-bacterial assay

The agar-well diffusion assay revealed the anti-bacterial activity of *P. hysterothorus* extract, as the test exhibited zone of inhibition against ten different indicator bacteria (Table 1). Where the maximum halo zone (23 mm) was observed against *Aeromonas hydrophila* and the minimum (17 mm) was against *Escherichia coli*.

3.4 Haemolytic assay

The haemolytic capacity serves as an indicator of cytotoxicity. The obtained results showed hydroethanolic extract depicted a significant ($p < 0.05$) lytic capacity as compared to a positive control (Fig. 2a). Similarly, the haemolysis rate was also depicted an increasing trend in a dose-dependent manner as the rate was 57% (maximum) at the concentration of 100 mg extract ml^{-1} and minimum (3%) at 1 mg extract ml^{-1} concentration (Fig. 2b).

3.5 Phyto-haemagglutination activity

The obtained results exhibited a substantial agglutination of RBCs when observed under the microscope. This result exhibited that the extract contains lectins, which are responsible for the formation of homogeneous clusters.

3.6 Haemagglutination limit test

The assay is used to assess the minimum extract concentration that causes haemagglutination of red blood cells. The minimum concentration of *P. hysterothorus* hydroethanolic extract causing agglutination was achieved at dilution 1/96.

3.7 Determination of LC₅₀ and acute toxicity response

Acute exposure to hydroethanolic extract elicited toxic effects on *C. carpio* following the noteworthy mortalities observed in the exposed group with reference to the control group. The LC₅₀ of *P. hysterothorus* extract was found to be 18.99 mg l⁻¹ with Log₁₀ (LC₅₀) of 1.278 (Table 2; Fig. 3). Upon exposure to extract fish exhibited certain unnatural behavioural changes, particularly at elevated dosages such as excessive mucus secretion, gulping, irregular swimming, fainted upside-down movement, change in colour of skin and haemorrhage over the body surface particularly on the ventral side of the body.

3.7.1 Changes in haematological parameters during acute toxicity

As shown in Fig. 4, it can be noted that exposure to sub-lethal concentrations caused substantial changes in the haematological parameters of fish. The present study revealed that upon exposure to *P. hysterothorus* extract, WBCs count, RBCs count, PCV, and Hb concentration exhibited a significant ($p < 0.05$) decreasing trend with increasing concentration of extract at all sampling points. Whereas haematology-based indices, MCV showed significant ($p < 0.05$) increasing levels when exposed to extract at both the concentrations, T1: 0.395 mg l⁻¹ and T2: 0.79 mg l⁻¹ up to 96 h. However, the MCH level was varying at different observation points, where the levels exhibited a significantly increasing trend only at the first sampling point, 24 h. Moreover, the MCHC levels in the present experiment were insignificant ($p > 0.05$) as compared to the control.

3.7.2 Changes in serum biochemical response during acute toxicity

Serum biochemical parameters such as glucose, GPT, GOT and ALP levels of *C. carpio* in the present study increased significantly ($p < 0.05$) in comparison to control upon exposure to hydroethanolic parthenium extract (Fig. 5).

3.7.3 Changes in immunological response during acute toxicity

The immunological parameters in the present study including lysozyme activity, NBT level, total protein content, albumin and globulin levels were significantly ($p < 0.05$) down in comparison to the control in a dose-dependent manner (Fig. 6). However, the albumin and globulin ratio in the present experiment was variable. As the ratio was significantly ($p < 0.05$) high in T1 at 24 h and 96 h.

4. Discussion

Employment of different plant-based products for a range of purposes such as treatment, growth, immune-enhancement, *etc.* has been widely accepted owing to different phytochemicals present in them. However, their

toxic effects are yet to be explored in different target and non-target species, since they also contain toxic botanicals in them it becomes an inherent part to explore such phenomenon (Okoro et al. 2019).

In the present study, the extraction yield was higher for the hydroethanolic solvent (80% ethanol) as compared to the aqueous and ethanolic solvents. The present results corroborate the findings of Senguttuvan et al. (2014) and Khan et al. (2022) as they also reported the superior ability of hydroethanolic solvent. The possible reason for the present outcome could be the cumulative effect of both polar (ethanol) and non-polar (water) solvents especially the polarity of ethanolic solvent which leads to better solvating of phyto-constituents (Vanitha et al. 2016; Akhtar et al. 2018; Nigam et al. 2021). For a plant extract, the exertion of toxic effects on fish depends on the presence of bioactive compounds, which could vary significantly depending on several factors, particularly on the type of solvent (Tung et al. 2007; Shahi and Singh 2011). For this reason, three different solvents were employed in the present study.

The study revealed presence of different toxic phytochemicals (alkaloids, saponin, tannin and rotenone) in all three *P. hysterophorus* extracts. In corroboration with to present findings, Nigam et al. (2021) reported alkaloids and saponin in aqueous and ethanolic extracts of parthenium. Whereas, Krishnavignesh et al. (2013), Deshpande et al (2017) and Tarekegn et al. (2021) reported the presence of alkaloids, tannin and saponin in different solvent-derived extracts including hexane, acetone, ethanol, methanol and petroleum ether. Similarly, Gupta et al. (1977) also reported saponin in the aqueous extract of gajar ghas. Moreover, present findings also revealed the superior ability of hydroethanolic solvent as compared to water and ethanolic solvent in extracting all four toxic botanicals. The reason for the hydroethanolic solvent's superior ability could be a combined effect of both polar and non-polar solvents (Vanitha et al. 2016; Akhtar et al. 2018; Nigam et al. 2021). Moreover, reports are suggesting the better solubility of the bio-active components in organic solvents such as ethanol which could also help in explaining the present finding (De Boer et al. 2005; Salama and Marraiki 2010). Based on the quantitative phytochemical profile hydroethanolic extract was selected for further examination.

Like many other plants, *P. hysterophorus* extract also showed anti-bacterial activity against ten different indicator bacteria. Where the zone of inhibition varies from 17–22 mm. The present result is in agreement with the findings of earlier researchers who have also reported the anti-bacterial response of parthenium against *E. coli*, *Bacillus subtilis*, *B. cereus*, *Enterococcus* spp, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes* (Barsagade and Wagh 2010; Fazal et al. 2011; Khan et al. 2011; Kumar et al. 2012). Moreover, there are reports where parthenium was reported to exhibit an anti-fungal response against *Aspergillus niger*, *Aspergillus flavus* *Candida albicans* (Kumar et al. 2012; Malarkodi and Manoharan, 2013; Krishnaveni et al. 2015; Kaur et al. 2016; Bajwa et al. 2020; Sharif et al. 2021). The antimicrobial efficacy of plants including parthenium is largely attributed to secondary metabolites such as essential oils, flavonoids, phenolics, and tannins as well as sugars, amino acids and phenolic derivatives (Sukanya et al. 2009; Rahmat et al. 2011; Khaket et al. 2015; Veena and Shivani 2012; Malarkodi and Manoharan 2013; Shashank and Abhay 2014). Recently a non-substituted cyclopentenone moiety, parthenin was characterized which exhibited an antimicrobial response (Lalita 2018).

Erythrocyte-based lytic assays have been adopted as a measure of toxicity particularly, cell membrane-targeted toxicity for various formulations and drug interactions (Zohra et al. 2014; Hussain et al. 2022; Khan et al. 2022). Additionally, the advantage of erythrocytes model is easy availability of blood cells, which resemblance with other cells and are easy to handle as per the necessity (Litman et al. 1976; Robertis and Robertis 1995). In the

present study, the *P. hysterophorus* extract causes substantial lysis of fish red blood cells. Additionally, the haemolysis rate exhibited dose-dependent lytic potential. The possible reason for the lysis of cells could be the destruction of the lipid bilayer of plasma membrane caused by the toxic components present in the extract. Saponin present in the extract is a major compound responsible for haemolysis of blood cells because of lipid-soluble moieties present in saponin, sapogenin which destabilize the RBCs membrane (Amini et al. 2014). Additionally, other phytochemicals (rotenone, alkaloid, tannin) may also lead to cellular toxicity (Khan et al. 2022). In agreement with the present findings, Hussain et al. (2022) reported haemolysis of human RBCs upon exposure to parthenium leaf extract. The authors also reported cells treated with different extract concentrations starting from 80, 120, 160 and 200 µg were capable to cause lysis ranging from 6.09 to 76.90%. Furthermore, Bajwa et al. (2020) and Hussain et al. (2022) suggested the reason for lysis caused by parthenium extract may be because of sesquiterpene lactone, 'parthenin' in the extract, particularly in parthenium leaves. Parthenin content in the extract is very toxic to cells as they prevent the synthesis of important cellular enzymes and DNA and RNA (Bajwa et al. 2020). Additionally, several other botanicals present including sesquiterpene lactones (coronopilin, ambrosin, hysteron D and tetraeurin A) α-methylene-γ-lactone and some acetylated pseudoguaianolides have also associated with the cytotoxic response of the *P. hysterophorus* extract (Das et al. 2007; Bajwa et al. 2020). Recently Jiménez et al. (2021) also reported the haemolytic activity of ethanolic parthenium extract on human red blood cells at 200 to 800 µg ml⁻¹ of concentration where they reported absolute haemolysis at a concentration of 800 µg ml⁻¹.

Phyto-haemagglutination activity and haemagglutination limit test in the present study also revealed the toxicity caused by the extract by causing clumping of the cells. Phytolectins in the extract are majorly responsible for the clumping of erythrocytes which ultimately hinders the normal functioning of the cells (Senthilganesh et al. 2021; Ouattar et al. 2022). A similar kind of agglutination was also reported by Abdali et al (2021) while working on *Linum usitatissimum* extract. Irrespective of the reason, agglutinated cells will eventually be neutralised by the organisms' immune system which may also roots to malfunctioning of physiological functions.

The findings of the present study confirmed the toxicity of *P. hysterophorus* hydroethanolic extract in *C. carpio*. The 96-h median lethal concentration of extract was found to be 18.99 mg l⁻¹ with Log₁₀ (LC₅₀) of 1.278. In alignment with the present study, Tarekegn et al. (2021) have also reported LC₅₀ of parthenium petroleum ether extract derived from different parts leaf, stem and root with three different solvents namely hexane, acetone and petroleum ether. Where, the LC₅₀ ranges from 10.7 ppm (for root) to 478 ppm (for leaf) in mosquito larvae, *Anopheles Arabiensis*. Similar larvicidal activities of parthenium extract were also reported in *Culex quinquefasciatus* (Bansode et al. 2016) and *Aedes aegypti* (Kumar et al. 2012). Additionally, present study has reported the presence of different toxic phytochemicals in extract followed by cytotoxic/ haemolytic effects. Possibly, exposure to active compounds present in the extract starts interfering with the normal physiological and metabolic processes of cells and ultimately leading to the hampered health status of fish or subsequent mortality as also suggested by Vikhar and Jadhao (2018), Cowie et al. (2020) and Tarekegn et al. (2021). Bioactive compounds present in the extract cause moderate to severe toxicity. These responses can be measured by behavioural response of the organism (Okoro et al. 2019). Behavioural expressions produced in response to toxicants were duration- and dose-dependent. As with the increasing concentration and exposure time, severe symptoms such as copious mucus production, excessive haemorrhage on the ventral body surface, etc. exhibited by the intoxicated *C. carpio*. In agreement with the present behavioural response Oribhabor et al (2020) and

Ekpenyong et al. (2020) also reported such response in African catfish, *Clarias gariepinus* to *Lonchocarpus cyanescens* and *Justicia extensa*, respectively.

In determining the toxic intensity of various compounds haematological, biochemical and immunological indices play a central role as an indicator of xenobiotic compound intrusion (Nostro et al. 2000; Wu et al. 2018).

Parthenium exposure in the present study at a sub-lethal concentration (T1: 0.395 mg l⁻¹ and T2: 0.79 mg l⁻¹) caused a significant reduction ($p < 0.05$) in haematological parameters including RBCs count, WBCs count, haemoglobin content and haematocrit value in comparison to extract devoid control group. In agreement with the present findings, Hussain et al. (2022) also reported a significant ($p < 0.05$) reduction in haematological parameters upon exposure to parthenium extract. Additionally, Hussain et al. (2022) also reported a significant reduction in differential leucocyte count (neutrophils and lymphocytes).

The decline in erythrocyte count in extract-treated groups could be the consequence of kidney and liver injuries leading to a decline in erythropoiesis and subsequent anaemic condition as a result of phytochemical intoxication (Saha et al. 2013; Wu et al. 2018). Saha et al. (2013) reported a 20% reduction in red blood cell count and a 30% reduction in haemoglobin content after the oral treatment of methanolic parthenium extract @ 20 mg 100⁻¹ g body weight in Wistar albino rats (*Rattus norvegicus*). Another possible reason may be the phytochemical-induced internal haemorrhages which were very prominent in the fish body, particularly on the ventral surface of the body during range finding assay. The decline in Hb content in the present study may be due to impaired oxygen supply resulting in a slow metabolic rate and hampered energy production because of lack of oxygen (Ahmad et al. 1995). Reduction in RBC count and haemoglobin levels can be correlated with the induction of anaemic conditions in fish (Cella and Watson 2000). Additionally, Saha et al. (2013) suggested that parthenin content induces anaemic condition either by interfering with haemoglobin synthesis or by fading RBCs survival. However, in contradiction to the present finding, Hussain et al. (2022) reported a significant increase in haemoglobin content after parthenium treatment.

Leucocytes are regarded as the major component of first-line fish defence which plays a central role in innate immune response (Menezes et al. 2006). The present study revealed a reduction in WBCs count at all sampling points. Similar to hampered RBCs levels, WBCs levels may also be correlated as a consequence of potential environmental stressor (dissolved extract), which causes an immunosuppressive condition in fish as also reported by Khedkar (2022) while studying parthenium extract effect on haematology of rohu. The possible reason could be the faulty physiological activities and subsequent vulnerable leucopoiesis. However, there are several contradictory results where reports suggested a significant rise in the leucocyte counts upon exposure to plant extract as a countering mechanism of fish's non-specific immune response to re-establish homeostasis (Shahi and Singh 2011).

The hydroethanolic parthenium extract exposure in the present experiment also caused noteworthy changes in haematology-based indices including MCV, MCH, and MCHC. Similarly, Hussain et al. (2022) also reported deviation in haematology-based indices following exposure to leaf extract of parthenium (10, 20, 40, and 80 mg kg⁻¹ body weight). Where a significant ($p < 0.05$) decline in MCV levels and an increase in MCH and MCHC were observed. The present elevation of MCV and MCH levels may be an indicator of erythrocyte swelling and resultant macrocytic anaemia upon toxicant exposure (Kavitha et al. 2012). Similar results were also reported by Suely et al. (2015) where they exposed *Heteropeneustes fossilis* to a sub-lethal concentration of *Terminalia arjuna* bark extract. MCHC levels in the present study showed fluctuating levels in common carp. Fluctuation of

MCHC level might be the toxic result of the extract which results in malfunctioning of normal physiological activities such as a drop in iron constitute of blood and ultimately affects MCV and MCH levels (Hodson et al. 1978). Similar fluctuations in MCHC levels were also reported by Shahi and Singh (2011) and Suely et al. (2015).

The PCV level in all the treatments upon exposure to hydroethanol-based extract showed a decreasing trend. The present finding was in collaboration with Ayotunde et al. (2004) and Anju et al. (2013) who noticed a decreasing trend in haematocrit levels in *Oreochromis niloticus* when exposed to aqueous extracts of *Moringa oleifera* seeds and in *Heterobranchus longifilis* upon *Tephrosia vogelii* extract exposure, respectively. The reduction in PCV levels may be the result of toxic components affecting erythropoiesis and causing the depletion of erythrocytes (Ahmad et al. 1995; Atamanalp and Yanik 2003). However, in contradiction Hussain et al. (2022) reported increased Hct value in experimental rabbits following parthenium leaf extract treatment.

Blood biochemical indices have exhibited significant ($p < 0.05$) variations in levels of blood glucose, GPT, GOT and ALP upon extract exposure in common carp. Glucose is the most important source of energy for any organism which can be affected in response to an unusual condition caused by myriads of pollutants hampering the physiological homeostasis of organisms (Manush et al. 2005). In the present study, it was observed that the glucose level followed an increasing trend from 24 h to 96 h in both treatments with a positive relation to the sub-lethal concentration of extract. Glucose serves as a vital indicator of stress caused by various irritants (Manush et al. 2005). In indirect agreement with the present finding, several studies reported the stress (gulping, copious mucus production, erratic movement, etc.) (Vikhar and Jadhao 2018; Ekpenyong et al. 2020; Oribhabor et al. 2020) caused by parthenium extract which might have rooted the increased levels of glucose in test animal. A similar type of result was also obtained by Suely et al. (2015) in *H. fossilis* due to exposure to *T. arjuna* extract. The elevation in the blood glucose found in the present study can be ascertained due to the conversion of hepatic glycogen into glucose to counter the elevated energy demand as also reported by Datta and Kaviraj (2003). However, in contradiction to the present finding, Patel et al. (2008) reported a decrease in glucose levels after the oral treatment with an aqueous extract of *P. hystrophorus* @ 100 mg kg⁻¹ body weight in adult albino Wistar rats.

Alkaline phosphatase (ALP) is a marker enzyme which plays an essential role in membrane transport as well as is a significant bio-indicator of stress in fish. ALP level was found to be significantly increased in fish from 24 h to 96 h in all the treatments. And the probable reason could be an acceleration of transphosphorylation activity as a result of the toxic effect of phytochemicals present in the hydroethanolic extract as also reported by Sharma (1990). Similarly, Hussain et al. (2022) reported a significant ($P < 0.05$) increase in serum ALP level with the increasing concentration (10, 20, 40 and 80 mg kg⁻¹ body weight) of parthenium extract. These variations in serum ALP levels are indicators of liver (chief detoxification organ) damage, as this enzyme is a recognized marker associated with hepatocyte damage (necrosis and cytosol drip) caused by the accumulation of toxic compounds (Cho et al. 2006; Ozer et al. 2008; da Silva et al. 2020).

Glutamate-oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) are other vital enzymes which play a central role in carbohydrate and protein metabolism and liver function (Nemcsok and Benedecsky 1990). In the present study, GPT and GOT levels increased significantly ($p < 0.05$). In corroboration to the present finding, Hussain et al. (2022) also reported a significant increase in GPT level with the increasing extract concentration. A similar type of finding was reported by Jessa et al. (2015) due to exposure to the extract of *Derris elliptica* stem extract on *Clarius gariepinus*. Gabriel et al. (2009) also suggested elevation may be the

consequence of a disturbed Krebs cycle and also indicates hepatic cell damage caused by the toxic components present in the plant extracts.

Serum immunological parameters such as lysozyme activity, NBT, total protein, albumin and globulin levels have recorded significant ($p < 0.05$) decline in the levels following extract exposure. Lysozyme is a major immune factor which aids as an indicator of innate immunity (Tort et al. 2003). Lysozyme activity in the present study has shown a decreasing trend with an increasing concentration of crude extract. However, contradictory results were obtained by Huet and Wu (2004) in Jian carp while working on Astragalus Root (*Radix astragalin seu Hedysari*) and Chinese Angelica Root (*Angelicae Sinensis*) extract and observed the positive influence in the lysozyme level due to exposure of herbal extract. NBT assay helps to determine production of free oxygen radicals by phagocytes especially macrophages or monocytes (Dalmo et al. 1997). Respiratory burst activity is considered a vital indicator of innate immunity, which is a measure of increased oxidation level against foreign compounds. Similar to lysozyme activity, respiratory burst response also revealed a significant decrease in the levels following extract exposure at all the sampling hours. The reduction in the overall immune response of fish observed in the present study, may be due to reduced levels of leucocytes, which serves as a critical compromising factor of the immune system (Misra et al. 2006a, b) as also reported in the present study as a consequence of extract exposure.

Protein could be an alternative source of energy for carbohydrates in fish under certain conditions, which may result in hypoproteinaemia (Martinez et al. 2004). The present study revealed a marked decline in serum protein levels upon extract exposure in *C. carpio* at every sampling point. The present findings may be the result of protein utilization to meet the energy demand under the stressed condition created by the experimental condition as also reported by Tiwari and Singh (2003). Omoniyi et al. (2002) also reported significant hypoproteinaemia possibly due to cellular injuries or necrosis with subsequent impairment of protein synthesis while working on the effect of Tobacco leaf extract in *C. gariepinus*. Similar results were also observed by Adamu et al. (2009) under the influence of tobacco leaf dust on Catfish (*C. gariepinus*) and by Shahi and Singh (2011) while working on euphorbious plants on *C. punctatus*. However, on contrary, there are several reports suggesting hyperproteinaemia in fish exposed to plant extracts may be because of water loss from plasma, elevated *de novo* synthesis or malfunctioning of blood protein mobilization (Al-Attar 2005; Abalaka et al. 2011). Similarly, albumin and globulin content also depicted declining levels in a dose-dependent manner. The decreasing levels of different protein constituents in serum can be negatively correlated with the disturbed homeostasis caused by external stress stimuli, gajr ghas hydroethanolic extract.

Conclusions

The results from the present study suggested that the crude extracts contained toxic phytochemicals which were responsible for their toxicological attributes. The qualitative analyses of the parthenium extracts demonstrated the presence of saponin, tannin, alkaloids and rotenone. The results of this study also pave the way for further investigation to recognize the compound/s that are responsible for its claimed haemotoxic response and antimicrobial action. This study has revealed that hydroethanolic extract of *P. hysterothorus* is toxic to a freshwater fish, *C. carpio*, since the 96-h LC_{50} value is 19.67 mg l^{-1} . Acute toxicity response employing a range of sub-lethal concentrations showed that they had brought significant modulation in the haematological, immunological and biochemical parameters. Variations in these indispensable parameters of fish caused by extract indicate its toxicity and its piscicidal activity. Therefore, *P. hysterothorus* whole plant hydroethanolic

extract could be a probable phyto-ichthyotoxin to control the infestation of unwanted fishes from aquaculture ponds.

Declarations

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Competing Interests

The authors declare no conflict of interest.

Author's contribution

M.I.R.K.: Conceptualization and designing of the experiments, data analysis and curation, drafting & editing of the manuscript; M.S, P.S, P.K. and S.K.: Involved in the investigation and conducting the experiments and P.B.: Review of the draft.

Conflict of Interest

The authors declare no conflict of interest.

Ethical Statement

All experiments were performed in accordance with the standard guidelines and policies suggested by the ethical committee of the institute.

Data availability

Data generated during this study are included in this present manuscript.

Consent for publication

All authors have approved the manuscript and declare that this is an original contribution and none of the material in this manuscript is under consideration for publication elsewhere.

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Tables

Table 1

Anti-bacterial activity of *Parthenium hysterophorus* hydroethanolic extract and positive control, oxytetracycline (zone of inhibition in mm)

S.N.	Indicator bacteria (strain/ code)	ATCC/NCBI GenBank number	<i>P. hysterophorus</i> extract	Oxytetracycline
1.	<i>Aeromonas veronii</i> (COFKWD_AV22)	ON248036	19	24
2.	<i>A. jandaei</i> (COFKWD_AJ22)	ON248037	21	26
3.	<i>A. hydrophila</i> (COFKWD_AH22)	ON248038	22	24
4.	<i>A. caviae</i> (COFKWD_AC22)	ON248039	21	25
5.	<i>Vibrio cholerae</i> (COFKWD_VC22)	ON248040	18	20
6.	<i>Shewanella xiamenensis</i> (COFKWD_SX22)	ON248041	19	21
7.	<i>S. baltica</i> (COFKWD_SB22)	ON248042	22	24
8.	<i>Escherichia coli</i>	ATCC 10536	17	19
9.	<i>Vibrio parahemolyticus</i>	ATCC 17802	21	22
10.	<i>Pseudomonas aeruginosa</i>	ATCC 27853	19	20

Table 2

Effective concentrations of *Parthenium hysterophorus* extract at an exposure period of 96 h

Organism	Effective LC ₅₀ dose (mg/l)	Log ₁₀ [LC ₅₀]	Limits (mg/l)		Chi-square (χ^2)	Degrees Of Freedom	p-level	Beta	Intercept
			LCL	UCL					
Common carp (Cyprinus carpio)	18.99	1.278	11.255	25.989	5.237	8	0.731	4.72	-1.115
Note: Alpha value (for confidence interval: 0.001), LCL lower confidence limit, UCL upper confidence limit									

Figures

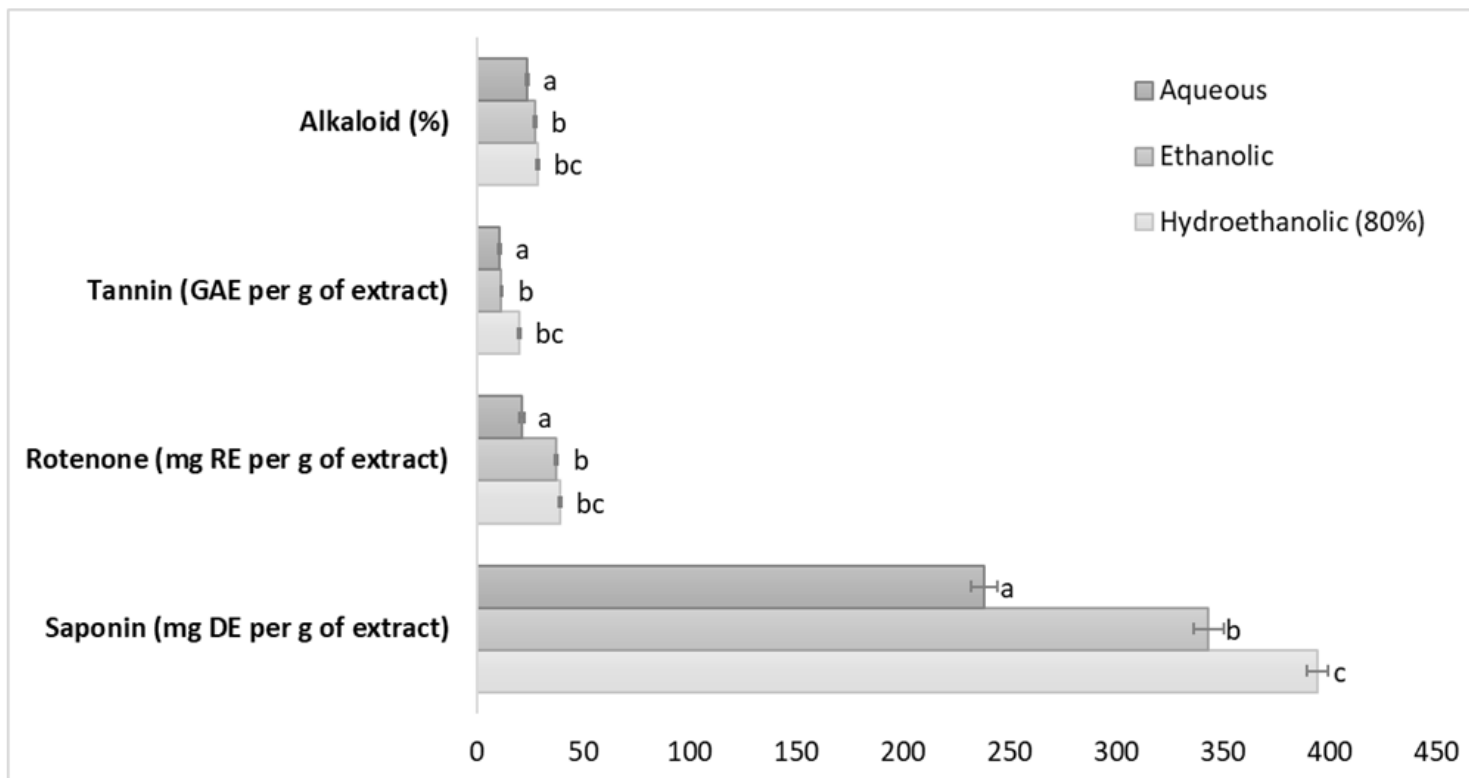


Figure 1

Toxic phytochemical profile of *P. hysterophorus* extracts. Values are presented as mean $\pm$ SE. Significant differences are indicated by different superscripts (P < 0.05).

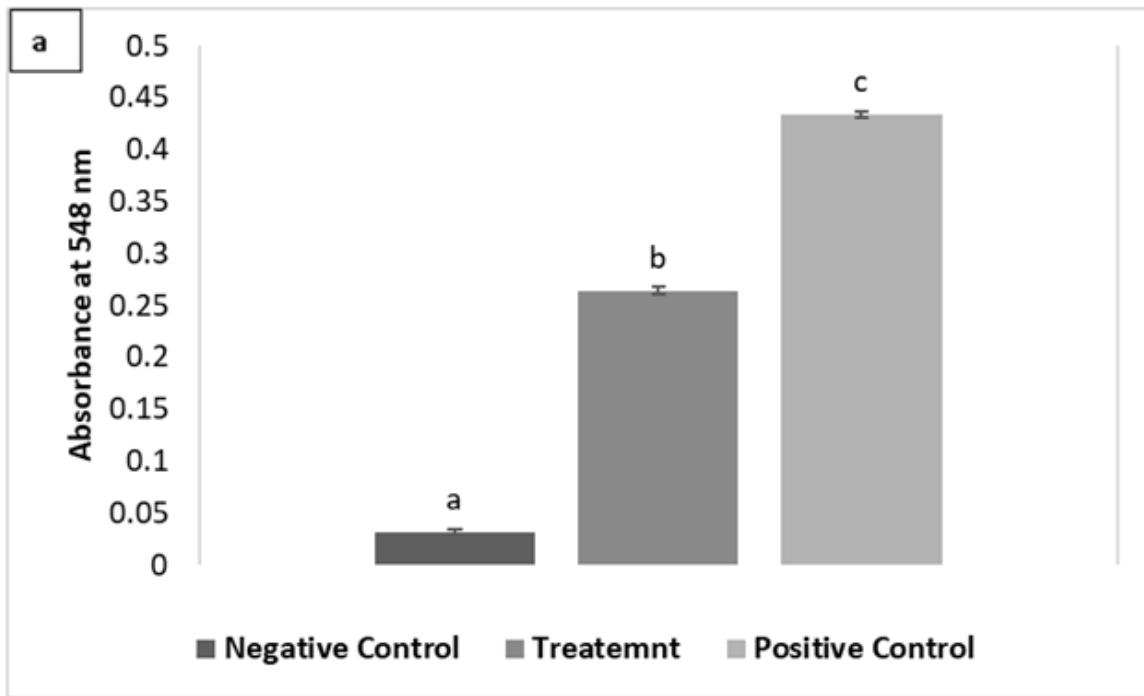
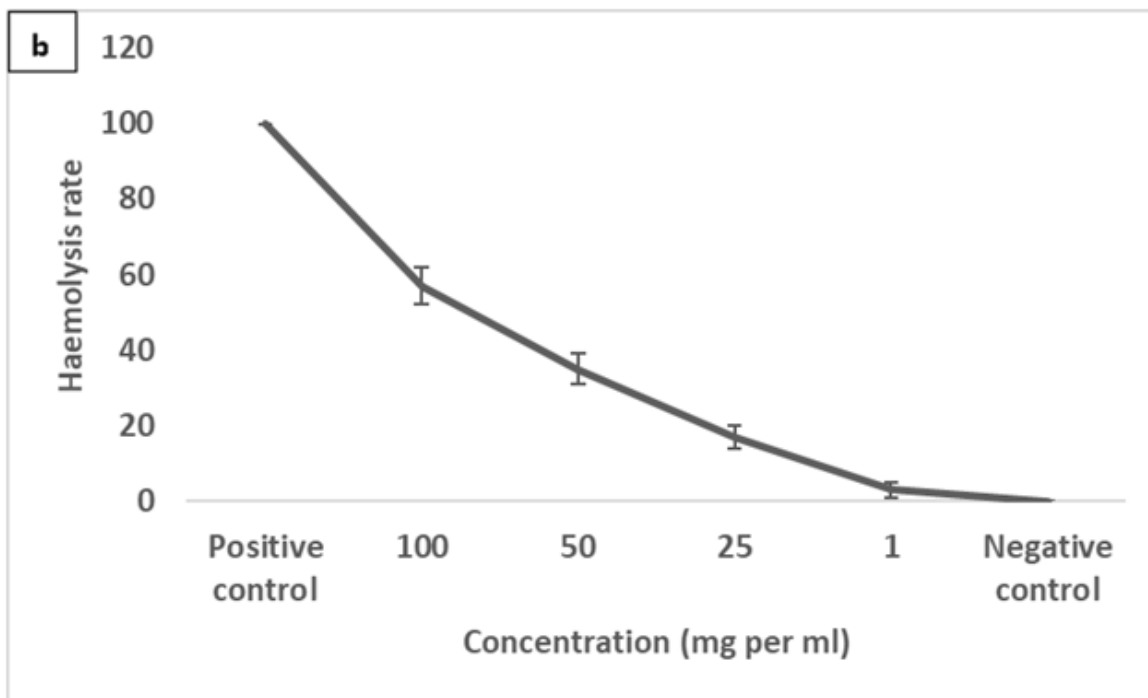


Figure 2

Haemolytic capacity (a) and haemolysis rate (b) of *P. hysterophorus* extract against common carp RBCs. Values are presented as mean $\pm$ SE. Significant differences are indicated by different superscripts ($P < 0.05$). Where positive control is water and negative control is normal saline solution.

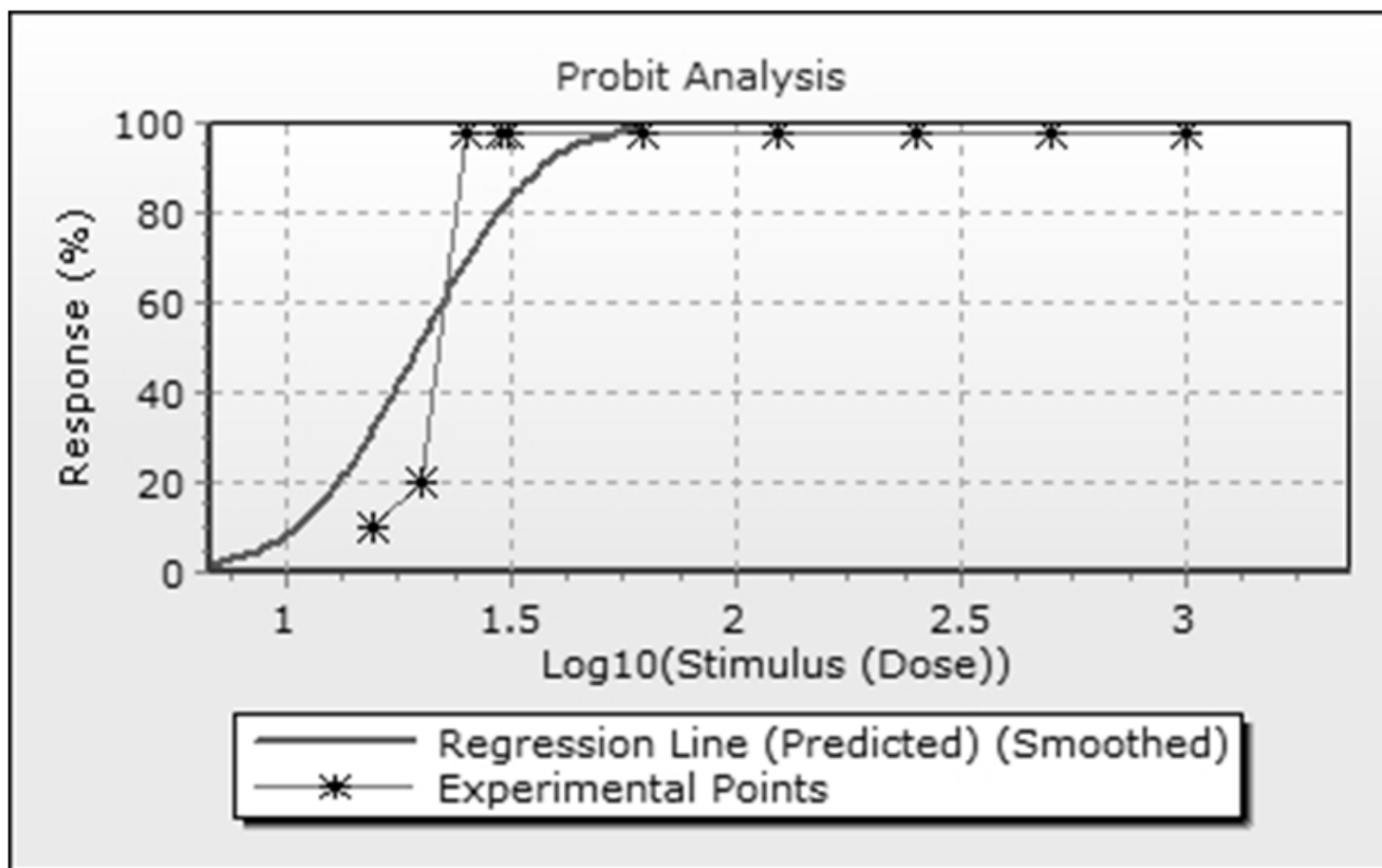


Figure 3

The median lethal concentration (LC_{50}) of *P. hysterophorus* extract at 96-h in Common carp, *C. carpio*. Probit analysis was performed through BioSat software.

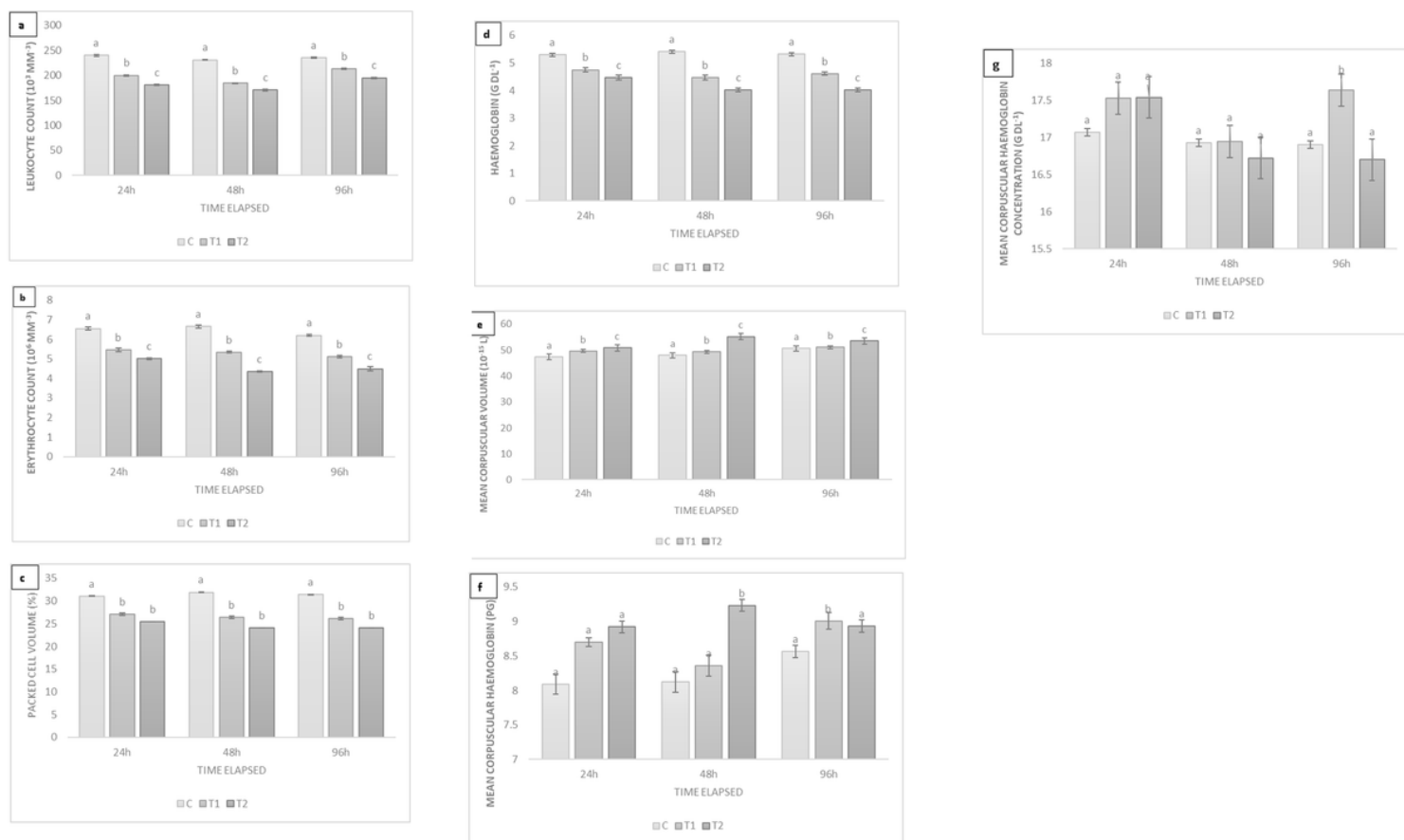


Figure 4

Effects of sub-lethal concentration (T1: 0.395 mg l⁻¹ and T2: 0.79 mg l⁻¹ and control: devoid of extract) of *P. hysterophorus* extract on haematological parameters of Common carp, *C. carpio*. [leukocyte count (a), erythrocyte count (b), packed cell volume (c), haemoglobin (d), mean corpuscular volume (e), mean corpuscular haemoglobin (f), and mean corpuscular haemoglobin concentration (g)]. Values are presented as mean ± SE. Significant differences are indicated by different superscripts (P < 0.05).

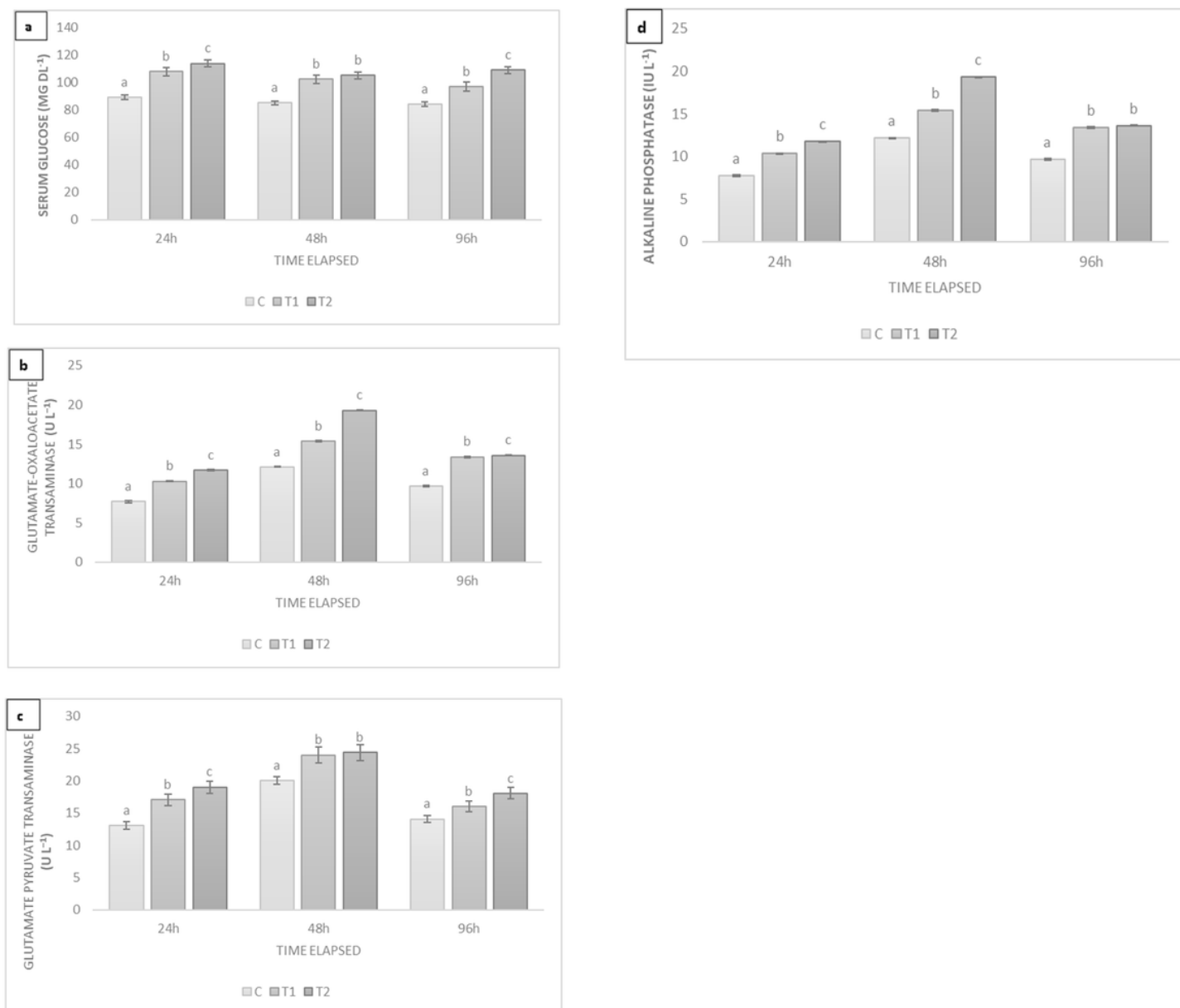


Figure 5

Effects of sub-lethal concentration (T1: 0.395 mg l⁻¹ and T2: 0.79 mg l⁻¹ and control: devoid of extract) of *P. hysterophorus* extract on biochemical parameters of Common carp, *Cyprinus carpio*. [serum glucose (a), glutamate oxaloacetate transaminase (b), glutamate pyruvate transaminase (c), and alkaline phosphatase (d)]. Values are presented as mean $\pm$ SE. Significant differences are indicated by different superscripts ($P < 0.05$).

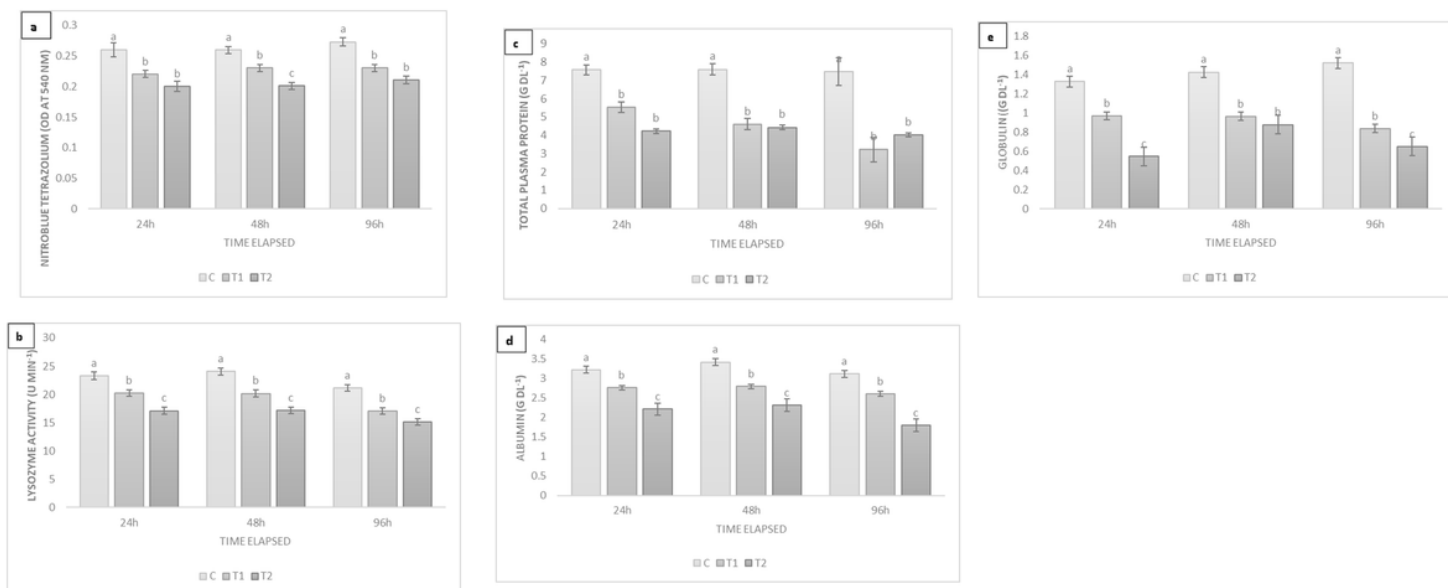


Figure 6

Effects of sub-lethal concentration (T1: 0.395 mg l⁻¹ and T2: 0.79 mg l⁻¹ and control: devoid of extract) of *P. hysterophorus* extract on immunological parameters of Common carp, *Cyprinus carpio*. [nitroblue tetrazolium (a), lysozyme activity (b), total plasma protein (c), albumin (d), and globulin (e)]. Values are presented as mean ± SE. Significant differences are indicated by different superscripts (P < 0.05).