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Article

Investigation of In-Vitro Antioxidant, Antimicrobial, Thrombolytic and In-Vivo Anti-Inflammatory and Antidepressant Potential of *Holigarna Longifolia* Leaf Extract

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Abstract: Discovery of genus *Holigarna longifolia* and its traditional uses were obtained by living in forest or semi forest areas by close observations of the indigenous populations. The main aim of this study was to evaluate phytochemical screening, antioxidant and anti-microbial activity, thrombolytic activity, in vivo anti-inflammatory and neuropharmacological activity of *H. longifolia* leaf extract. In vitro antioxidant was evaluated by DPPH and ferric reducing power assay, Disc diffusion method was used to evaluate anti-microbial activity, thrombolytic activity was evaluated by clot lysis, determine the ability of the extracts to suppress inflammation was done using the formaldehyde-induced paw oedema test influence of the sedative activity of the drug on the forced swimming and tail flick-suspension tests were studied by using diazepam. The phytochemical screening of *H. longifolia* extract revealed the presence of reducing sugars, tannins, gums, steroids, glycosides, xanthoprotein and terpenoids. In DPPH the extract exhibited strong antioxidant with an IC_{50} of 0.877 µg/mL, comparable to ascorbic acid IC_{50} 0.839 µg/mL and in ferric reducing power assay the extract showed significant IC_{50} value about 43.736 µg/mL. In anti-microbial tests, the highest inhibition, 19 mm was observed against *Escherichia coli* at 500 µg/disc, while *Bacillus magaterium* showed the lowest inhibition 9 mm at 250 µg/disc. In the MIC test, the extract leaves showed highest activity against *E. coli* (MIC = 4 mg/mL) and lowest against *B. cereus*, *B. megaterium*, and *A. niger* (MIC = 128 mg/mL). The extract demonstrates clot-lytic activity of 20.45 ± 1.42% versus 31.17 ± 0.65 for streptokinase. *H. longifolia* extract was determined to be safe in the acute toxicity trial, with no toxicity, mortality, or behavioral abnormalities in the mice. In the anti-inflammatory test, paw thickness was significantly reduced with inhibition rates of 54.22% by the dose of 250 mg/kg and 60.27% by 500 mg/kg) compared to 71.71% for the standard. Neuropharmacological tests revealed reduced immobility periods of 126.2 s in forced swimming and 145.8 s in tail suspension tests at dose of 200 mg/kg. Further studies will be needed to evaluate the full pharmacological potential *H. longifolia.*

Keywords: phytochemicals; anti-oxidant; anti-microbial; thrombolytic; anti-inflammatory; antidepressant

1. Introduction

The plant *Holigarna longifolia* grows natively across South and Southeast Asia as it occurs in India together with Bangladesh and many other nearby locations. *H. longifolia* extends its growth best within semi-evergreen and evergreen forests of hilly environments under warm and humid climatic conditions. Evaluations show that this species exists in Western Ghats plus northeastern India areas and particular sections of forest located in Bangladesh [1]. The plant is from the Annonaceae family and has been used for ages in traditional medicine because of its wide vast therapeutic effects. Several studies have been reported on the occurrence of a number of bioactive compounds in the *H. longifolia*, namely alkaloids, flavonoids, terpenoids and phenolic compounds, probably being the causative of its therapeutic potential. Recalling this work, the present research aimed at exploring the phytochemical composition and determining its antioxidant, antimicrobial, thrombolytic and neuropharmacological activity, in order to explore *H. longifolia* potential as a good source for discovery of drugs [2].

Phytochemical screening is important as a first step in identifying the bio active constituents that may be responsible for medicinal value in plants. *H. longifolia*, was reported to possess, among others, flavonoids, tannins,

glycosides, and alkaloids. Many biological activities such as antioxidant, antimicrobial as well as anti-inflammatory have been reported to these compounds. To understand the plant's therapeutic mechanisms, the identification and quantification of these bioactive compounds are important. Previous studies have shown that in *H. longifolia*, several phytochemicals are present and therefore it should be subjected to further pharmacological investigations [3].

This property of plants regarding its antioxidant actions is import in eliminating reactive oxygen species (ROS) and preventing oxidative stress, which is linked to the pathogenesis of diverse chronic diseases such as cancer, diabetes, or cardiovascular disorders. Studies on *H. longifolia* have shown its antioxidant activity as scavenging of free radical and reducing oxidative damage. To measure the plant's antioxidant capacity using various in vitro assays, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assays have been used. Results from these studies indicate that *H. longifolia* may be a natural source of antioxidants and could be valuable in avoiding oxidative diseases resulting from oxidation stress [4].

Drug resistant diseases have aroused great interest as a global health problem and the search for 'natural alternatives' to synthetic antibiotics is one of them. *H. longifolia* has been screened for its antimicrobial activity against different bacterial and fungal strains. However, extracts from the plant showed strong inhibitory effects against both Gram positive and Gram-negative bacteria and fungal species. It is believed that antimicrobial properties of the plant are due to its bioactive compounds present in the plant, i.e., flavonoids and alkaloids. *H. longifolia* can also be a promising natural solution to fight against microbial infections, especially due to increasing rate of antibiotic resistance [5].

Thrombolytic drugs are a well-established medical therapy for thromboembolic diseases, including stroke and myocardial infarction. In recent days, investigations have been conducted on the ability of *H. longifolia* to promote clot dissolution in vitro. Collectively, the plant's extracts have been demonstrated to promote fibrinolytic activity (the ability to break down blood clots) and to inhibit the formation of new clots. It is likely that this thrombolytic activity results, in part, by virtue of the presence of various bioactive compounds (including flavonoids and tannins) affecting the coagulation cascade. With an increasing need for natural thrombolytic agents, *H. longifolia* could be a potential resource for the development of new cardiovascular diseases therapies [6].

Researchers have gained interest in neuropharmacological investigations of *H. longifolia* on basis its possible role as a neuroprotective and cognitive enhancing agent. The plant's extracts have been shown to modulate neurotransmissions of the cholinergic, adrenergic and dopaminergic pathways. Importantly, these effects may be an avenue to its potential therapeutic application in neurodegenerative diseases e.g., Alzheimer's disease or Parkinson's disease. Additionally, the plant's role in maintaining mental health has been added as well, as it has anxiolytic and sedative properties. *H. longifolia* contains rich chemical composition, including alkaloids, flavonoids and other secondary metabolites, and the neuropharmacological properties associated with it are possibly due to these ingredients [7].

Finally, pharmacological activities of various nature have been described for *H. longifolia:* antioxidant, antimicrobial, thrombolytic and neuropharmacological. The rich phytochemical profile presents the potential of this species as a source of bioactive compounds for future development of novel therapeutic agents. It is necessary to further research these compounds and more fully understand the mechanisms of action for these compounds. For this study, the findings may pave the way to the use of *H. longifolia* for the modern medicine in the treatment of various diseases and health conditions [8].

Future aims of this research include further isolating and characterizing the bioactive compounds of *H. longifolia* to attempt to identify their specific mechanisms of action. The in vivo validations include studies in life, to confirm antioxidant, antimicrobial, thrombolytic and neuropharmacological activities noted in vitro. Furthermore, it will examine the possibility of synergy when combining bioactive compounds found in the plant for enhanced therapeutic efficacy. The study also evaluates the safety and toxicity profile of *H. longifolia* and evaluates its potential feasibility for clinical applications.

2. Materials and Methods

2.1. Chemicals and Reagents

Analytical grade chemicals, including 2,2-diphenyl-1-picryl hydrazyl (DPPH) from Sigma, St. Louis, MO, USA, $AlCl_3$, NaOH, $Na_2HPO_4 \cdot 2H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ from Loba, Mumbai, India, as well as H_2O_2 from Merck, Darmstadt, Germany, and phenazine methosulfate (PMS) from Sigma, USA, were utilized. All required chemicals were acquired from Department of Pharmacy, Dhaka International University, Bangladesh

2.2. Collection and Preparation of Plant Extract

Plants of *H. longifolia* were collected from Bhola, Barisal, Bangladesh. The identification of the plant specimen was conducted by Khondokar Kamrul Islam, a senior scientific officer at the national Herbarium in Mirpur, Dhaka, Bangladesh and the authentication number was DACB-87893. With the exception of the root, the entire plant was collected, thoroughly cleaned with fresh water to get rid of any dirt, and then allowed to dry in the shade for a few days. Next, a grinding mill was used to break the plant into a coarse powder. To remove moisture, the plant's leaves sample were dried at room temperature. Then leaves were subjected to size reduction to get coarse powder was then stored in a clean dry airtight container. Next, in an amber glass container, 350 g of the dried-crushed materials were steeped in approximately 1150 mL of ethanol. For seven days, this mixture was periodically shaken and agitated before being filtered through a piece of fresh white cotton. Next, Whatman No. 1 filter paper was used to filter it once again. With constant air flow, the filtrate was evaporated and dried at room temperature. Then, the extract was stored in refrigerator (4 °C) for further use.

2.3. Animals

Swiss albino mice of four to six weeks old with an average weight of twenty to twenty-five grams were bought from Jahangirnagar University, Bangladesh. Due to the need for acclimatization, the animals were housed in the pharmacology lab of the Pharmacy Department's animal house immediately after purchase for two to three weeks. This was done in Bangladesh in Dhaka International University. Every trial took place in an isolated, reduced acoustic, and reverberant environment. All animals used in this study were handled strictly following the ethical principles endorsed by the Department of Pharmacy, Dhaka International University, Satarkul, Badda, Dhaka-1212, Committee of Clinical Pharmacy & Pharmacology [Source: EC/009/CPP/DIU].

2.4. Phytochemical Screening

Phytochemical Screening as described by Ghani [9]. A few positive control techniques were used to establish the chemical constituents of the plant extract. Chemical reagents used for the classification include Fehling's Solution and Benedict's solution for Reducing sugar, Mayer's and Dragendroff's reagents for alkaloids, saponins reagent for saponins reactivity test, glycoside reagent for glycosides, and steroid and H_2SO_4 for steroids, tannin with ferric chloride and potassium dichromate solution, and Molish's reagent Specifically, the chemical constituents of *H. longifolia* were classified and analyzed qualitatively through these methods [10].

2.5. 1,1-Diphenyl-2-Picrylhydrazyl-Free Radical Scavenging Activity (DPPH)

Plant extract and ascorbic acid in ten test tubes with the following concentrations: 500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953, and 0.977 μg/mL. The plant extract and ascorbic acid were measured three times before being dissolved in ethanol to create these amounts. The positive control in this case was ascorbic acid. To create a 0.004% (*w*/*v*) solution, a precise amount of DPPH was weighed and dissolved in ethanol. A Sonicator was then used to mix the solution. The sonicator was sourced from Shenzhen Boda Ultrasonic Engineering Co. Ltd., headquartered in Shenzhen, Guangzhou, China. One milliliter of the different ascorbic acid and plant extract concentrations was added to each test tube. The 0.004% DPPH solution was then pipetted into each test tube in an amount of 3 mL. The flasks were then moved to incubator and kept at room temperature and in dark for 30 min in order to enhance the intensity of the reaction. Moreover, control test tubes, including only ethanol with DPPH, were also prepared. Subsequently, after this incubation, the absorbance of each test tube was measured at 517 nm using UV Spectro-photometer [11]. The percentage of inhibition was determined with the following formula: % inhibition = [(Blank absorbance − Sample absorbance)/Blank absorbance] × 100.

2.6. Ferric Reducing Antioxidant Power Assay

To perform the FRAP assay, the same working reagent, standards, controls, and test samples are used; reagent and sample volumes are simply increased pro rata to give a volume large enough for manual handling/transfer of reaction mixtures. For example, 3.0 mL of working FRAP reagent is mixed with 100/mL. test sample or standard in a test tube; this is vortex mixed, and the absorbance at 593 nm is read against a reagent. Aqueous ascorbic acid solutions at 100, 250, 500, and 1000/mM (equivalent to 200, 500, 1000, and 2000/mM FRAP) prepared fresh daily and aged QC serum freshly spiked with ascorbic acid are recommended as quality control samples. These should be run in parallel with test samples to actively monitor the performance of the test and to ensure comparability with previous results. A standard solution or 0.2 mL of plant extract incorporate with 3 milliliters of FRAP reagent. For 30 min, reactions are incubated at 37 °C in an H_2O bath. Measured the absorbance at 593 nanometers [12].

2.7. Determination of Antimicrobial Activity

To evaluate the extract's antimicrobial activity disc diffusion method was used which was outlined by Fakruddin et al. [13]. Tests were performed on the extract against a range of eight gram-negative microorganisms, which included *Shigella boydii*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Salmonella typhi*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, and *Escherichia coli*. Additionally, there were eight gram-positive bacteria tested as well, *comprising Sarcina lutea*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger*, and *Staphylococcus aureus*. To prepare test plates, Mueller-Hinton agar medium was used. Five-millimeter filter paper discs (Whatman No. 1) were loaded with 25 and 50 micrograms per microliter of crude leaf extract. The discs were completely dry after that. The discs that had been imbued were incubated on sterilized agar plates for 24 h at a temperature of 37 °C, during which they were covered with 100 µL of the culture. For the positive control, discs containing ciprofloxacin (5 µg) were utilized. After the incubation period, diameter of the inhibition zone was measured in millimeters. A sterilized blank disc was used as a control reference.

2.8. Determination of Minimum Inhibitory Concentration (MIC)

The agar dilution method was chosen to identify the MIC [14]. Sensitization was done in 10 cm experimental tubes containing SD broth for fungi and Muller Hilton broth for bacteria in different concentrations (20, 40, 80, 160, 320, 640, 1280, 2560 mg/mL). Nine milliliters of autoclave sterilized SDA for fungi and Muller Hilton for bacteria are put in each tube. To prepare the final concentrations of 2, 4, 8, 16, 32, 64, 128, 256 and 512 mg/mL, 1 mL of each extract concentrations was added on each tube after it had cooled. In laminar flow cabinet, the SDA/Muller Hilton and extract combination was aseptically placed into plates. Therefore, when the agar medium had solidified, 2 µL of the modified spore suspension was inoculated onto each plate by micropipette and plates were placed in incubators at temperature optimal for fungi, at 27 °C and bacteria at 35 °C. In order to control, the SDA and Muller Hilton had no addition of any herbal extract in their formulation. The MIC of the extract was describe after 12 days of incubation, as the lowest concentration of the extract at which no appreciable growth was observed as compared to the control.

2.9. Thrombolytic Activity

This test was conducted in accordance with the procedure outlined by Prasad et al. [15]. Following the addition of 5 mL of sterile distilled water, the commercially available lyophilized streptokinase vial (1,500,000 IU) was thoroughly mixed. An adequate dilution was made from this suspension, which served as the stock solution. To create the clot, 5 mL of venous blood was drawn from 10 healthy volunteers who had never taken oral contraceptives or anticoagulants. The blood was then divided into 10 sterile micro centrifuge tubes, each 0.5 mL in size, and incubated for 45 min at 37 °C. Following this clot formation, the serum was extracted entirely without causing any disruption to the clot, and the weight of the clot was determined by weighing each tube containing the clot once more. Every micro centrifuge tube containing a pre-weighed clot received 100 µL of ethanol extract (10 mg/mL). To the designated control tube, 100 µL of streptokinase was added as a positive control, and 100 µL of distilled water was added as a negative control [16]. After 90 min of incubation at 37 °C, each tube was examined for clot lysis. Following incubation, the discharged fluid was withdrawn, and tubes were weighed once more to determine the weight difference following clot disruption. The percentage of clot lysis was determined by comparing the weight acquired before and after clot lysis.

2.10. Evaluation of Acute Toxicity

All the animals were kept at overnight fasting before to the experiment with free access to water. Group I was administered orally normal saline solution (0.9% NaCl). The mice of this group were administered (0.01 × body weight) ml solution on 1st day. Groups II and III, IV were orally administered 300, 2000 and 5000 mg/kg body weight of the extract (dissolved in distilled water). The mice of these group were administered (0.01 × body weight) ml solution on 1st day. The animals were observed for any toxic effect for first 4 h after the treatment period. Further animals were investigated for a period of 3 days for any toxic effect [17]. Behavioral changes and other parameters such as body weight, urinations, food intake, temperature, changes in eye and skin colors were noted.

2.11. Evaluation of Anti-Inflammatory Activity

Reducing inflammation potential of *H. longifolia* was also tested by Garrido et al. using formaldehyde induced paw edema method in mice [18]. The positive control group of four animal groups was administered with 100 mg/kg body weight of the routine drug ibuprofen. The doses of the plant material were 250 and 500 mg/kg body weight, given per oral route. Linear circumference of right hind paw was measured after 30 min by placing two slide calipers at the proximal and distal ends of paw. After that, the mice were injected with a 0.2% formaldehyde solution in to the right hind paw. Paw volume of the injected limb was measured with a plethysmometer at one, two, three, and four hours after the formaldehyde injection with paw size differences determined.

2.12. Neuropharmacological Test

2.12.1. Forced Swimming Test

This approach was described by Rd Bertin [19]. As a result of random sampling five groups of animals were selected for observation. Each mouse was acclimatized for 30 min, daily in an open cylindrical container measuring 45 cm long by 20 cm in diameter, with 17 cm deep water at 25 °C for five minutes. The mice were then administered with distilled water (0.1 mL/mouse, oral), extract in different concentrations (Low = 50 mg/kg/Kg, Medium = 100 mg/kg, High = 200 mg/kg), diazepam (1 mg/kg, intraperitoneally) or regular drug. Any mice which simply bobbed on the water or those that swam only slightly to keep their heads elevated were considered to be "still".

2.12.2. Tail Suspension Test

This technique was named by (Steru Raymond; Thierry, Bernard; Simon, Pierre, 1985). All together five groups of mice were prepared. After 30-min test groups were administered oral doses of 50, 100 and 200 mg/kg of the extract. The control group was treated with distilled water (0.1 mL/mouse orally); the standard treatment was diazepam (1 mg/kg/case, IP). Sterile adhesive tape was applied one centimeter from the extremity of tails of mice and these were hanged at 50 cm height above the floor [20].

2.13. Statistical Analysis

All experimental results were reported using the means ± standard errors of the means. Statistical significance was determined by one-way analysis of variance using Dunnett's test. Analysis of the data was done using Prism 6.0 (Graph Pad Software Inc., San Diego, CA, USA), and Microsoft Excel. The findings of the research were considered statistically significant if $p < 0.05$.

The least squares mean ± standard errors of the means were used to report all experimental results. To compare for significance, the ANOVA test was conducted at 0.5 level of significance using Dun net test. The results were analyzed statistically using computer basics soft wares that include, Prism 6.0 (Graph Pad Software Inc., San Diego, CA, USA) and Microsoft Excel. All statistical tests used employed significance level of 5% was used, that is the *p* value must be below 0.05.

3. Results and Discussion

3.1. Phytochemical Screening

The phytochemical analysis of the *H. longifolia* extracts is presented in Table 1. The extracts contained reducing sugar, tannin, flavonoids, saponins, alkaloids, glycosides, terpenoids and acidic compounds.

Phytochemical screening of the prepared extracts was conducted to identify the presence of various chemical constituents with various qualitative tests. The tests were performed by different reagents for different chemicals were also used. These were identified by characteristic color changes using standard procedures [9]. Phytochemicals found in plant leaves may have a role in their therapeutic properties. The therapeutic effect of medicinal plants is attributed to their secondary metabolites, or phytochemicals, as well as other chemical elements, according to Varadarajan et al. [21] Saponins, for instance, are glycosides of steroids and triterpenes with hypotensive and cardiodepressant effects. Congestive heart failure and cardiac arrhythmia are treated with naturally cardioactive medications called cardiac glycosides [22]. Numerous studies suggested that different types of polyphenolic chemicals, such as tannins, flavonoids, and phenolic acids, have a wide range of biological effects, including antioxidant activity [23].

Table 1. Phytochemical activity of ethanol extract of *H. longifolia.*

Phytochemicals Group	Result
Reducing sugar (Benedict's test)	−
Reducing sugar (Fehling's test)	+
Combined reducing sugar	−
Tannins (Ferric chloride test)	+
Tannins (Potassium dichromate test)	+
Flavonoids	+
Saponins	+
Gums	−
Steroids	−
Alkaloids	+
Glycoside	+
Xantho Proteins	−
Terpenoids	+
Acidic compounds	+

Note: Present = **+,** Absent = −.

3.2. 1,1-Diphenyl-2-Picrylhydrazyl-Free Radical Scavenging Activity (DPPH)

The DPPH free radical scavenging assay was conducted to assess the quantitative antioxidant activity, revealing that the IC_{50} values for the ethanol crude extract of *H. longifolia* was measured at 517 nm. the IC_{50} values for the ethanol crude extract of *H. longifolia* 0.877 µg/mL, while ascorbic acid, the standard reference, had a value of 0.839 µg/mL (Figure 1). *H. longifolia*, plant extract was evaluated for its capacity to neutralize free radicals by reacting with DPPH free radicals. The extract's effectiveness in scavenging DPPH was compared to that of ascorbic acid, which is recognized as a potent antioxidant. The ability to act as an antioxidant is a crucial pharmacological characteristic of plants. DPPH is commonly used to evaluate the free radical scavenging or antioxidant potential of plant extracts since it is easily neutralized by antioxidants [24]. This indicates that the crude extract has strong antioxidant properties compared to the standard. Additionally, the findings imply that the plant contains phytoconstituents that can donate hydrogen, offering protection to cells against potential harm.

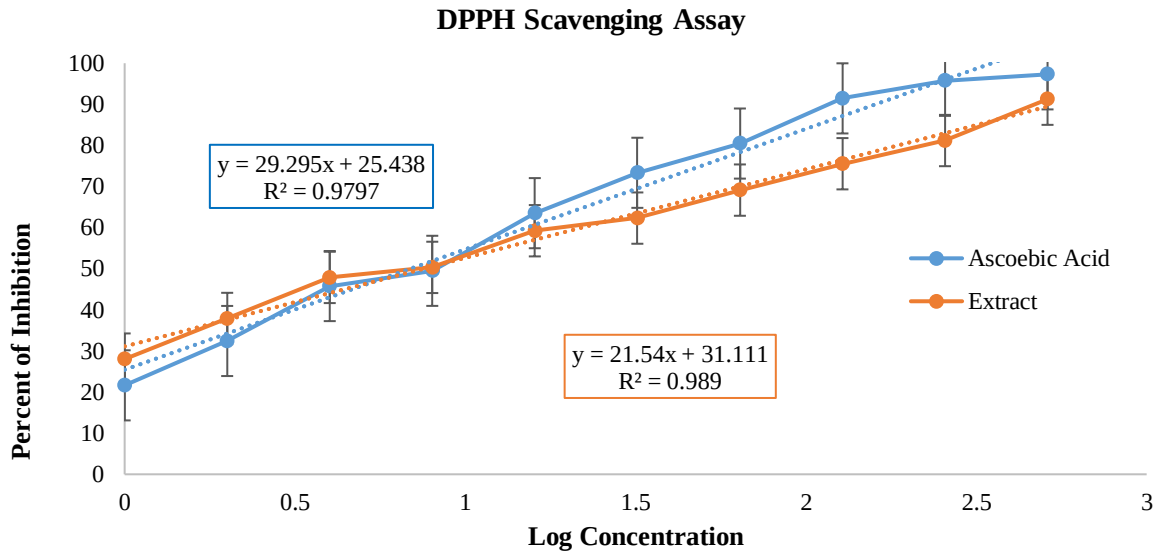

Figure 1. Comparison of absorbance vs. log concentration graph for ascorbic acid vs. *H. longifolia.*

3.3. Ferric Reducing Power Assay

The IC_{50} of ascorbic acid (standard) was found to be 12.763 µg/mL, while the IC_{50} of *H. longifolia* (extract) was 43.736 µg/mL (Figure 2). Observations were conducted to determine the ferric ion reducing activities of extracts at different concentrations. The results showed that extract has a high ferric ion reducing activity compared to the standard Ascorbic acid. This suggests that the extract has the ability to donate hydrogen. During the FRAP assay, a deep blue color was observed, which occurs due to a non-specific reaction that converts the ferric tripyridyl

triazine (Fe III TPTZ) complex to the ferrous form. This assay is commonly used for determining hydrophilic antioxidants [25].

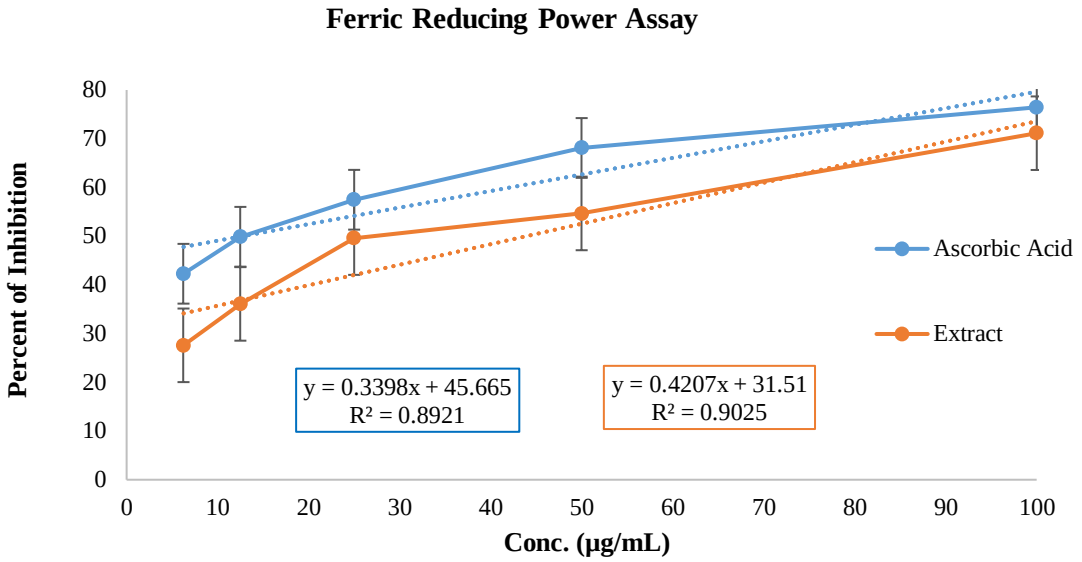

Figure 2. Comparison of percent inhibition vs. concentration graph for ascorbic acid and extract.

3.4. Determination of Antimicrobial Activity

H. longifolia shown antimicrobial efficacy against both gram-positive and gram-negative by utilizing the disk diffusion method (*Bacillus subtilis, Shigella dysenteriae, Pseudomonas aureus, and Sarcina lutea*) etc. in disk diffusion assay.

The results showed in that the extracts from *H. longifolia* inhibited the growth of those microbes with 19-mm zone of inhibition and an amount of extract employed of 500 µg/disc, *Escherichia coli*, a gram-negative bacterium, had the highest inhibition rate. *Aspergillus niger* a gram-positive bacterium, has the lowest inhibition rate; the zone of inhibition is just 6 mm, and 250 µg/disc of extract is utilized (Figure 3).

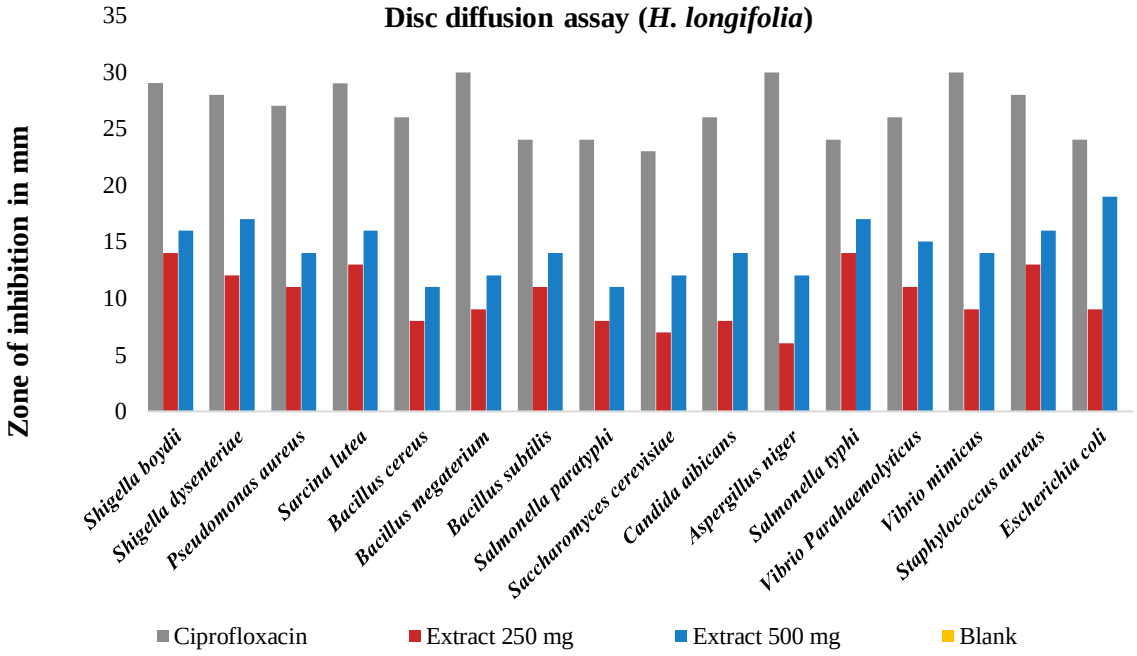

Figure 3. In vitro antibacterial activity of ethanolic extract of *H. longifolia* by disc diffusion assay.

3.5. Evaluation Minimum Inhibitory Concentration (MIC)

Table 2 displays the MIC values of the ethanolic extract of *H. longifolia* leaves, which show differing degrees of efficacy against various bacteria. With a MIC of 4 mg/mL, E. coli showed the maximum sensitivity. The lowest

sensitivity was shown by the MIC of 128 mg/mL for *B. cereus*, *B. megaterium*, and *A. niger*. The MIC for *Salmonella typhi* was 8 mg/mL, and the MIC for *V. parahaemolyticus* was 16 mg/mL. These results imply that *H. longifolia* leaf ethanolic extract is least effective against *B. cereus*, *B. megaterium*, and *A. niger* and most effective against *E. coli*.

Gram-positive bacteria have more mucopeptide in their cell wall composition than gram-negative bacteria, which have a thin layer of mucopeptide and a cell structure primarily composed of lipoprotein and lipopolysaccharides. This is because the two types of bacteria differ in their cell structure. Gram-negative bacteria have more resistance as a result [26]. As Kotzekidou et al. [27] hypothesized, this data add credence to the notion that antimicrobial substances from plant extracts alter bacterial membrane permeability and consequently release intracellular biomolecules. Cell wall and membrane disruption is highlighted in many investigations as a way through which bacterial survival is compromised due to disruptions in electron transport, enzyme activity in food uptake.

Table 2. Minimum inhibitory concentration (MIC) of *H. longifolia* extract.

Microorganisms	Concentration mg/mL									
	512	**256**	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**Control**
Shigella boydii	+	+	+	+	+	−	−	−	−	−
Shigella dysenteriae	+	+	+	+	+	−	−	−	−	−
Pseudomonas aureus	+	+	+	+	−	−	−	−	−	−
Sarcina lutea	+	+	+	+	+	−	−	−	−	−
Bacillus cereus	+	+	+	−	−	−	−	−	−	−
Bacillus megaterium	+	+	+	−	−	−	−	−	−	−
Bacillus subtilis	+	+	+	+	+	−	−	−	−	−
Salmonella paratyphi	+	+	+	+	−	−	−	−	−	−
Saccharomyces cerevisiae	+	+	+	+	−	−	−	−	−	−
Candida aibicans	+	+	+	+	−	−	−	−	−	−
Aspergillus niger	+	+	+	−	−	−	−	−	−	−
Salmonella typhi	+	+	+	+	+	+	+	−	−	−
Vibrio Parahaemolyticus	+	+	+	+	+	+	−	−	−	−
Vibrio mimicus	+	+	+	+	+	−	−	−	−	−
Staphylococcus aureus	+	+	+	+	+	+	−	−	−	−
Escherichia coli	+	+	+	+	+	+	+	+	−	−

(+): Positive inhibition; (−): Negative inhibition.

3.6. Thrombolytic Activity

In Figure 4, clot lysis was demonstrated by 37.42% in the ethanolic extracts and 64.17% by streptokinase. Distilled water, on the other hand, demonstrated very little clot lysis (3.05%). There was a significant difference (p = 0.0013) in the mean clot lysis percentage between the extract and negative control.

Generally, thrombin forms blood clots from fibrinogen. Medication classified as thrombolytic or antithrombotic can obstruct the thrombus formation process. Plasmin, which can be activated by activators from inactive plasminogen, is the primary mechanism via which thrombolytic therapy breaks down fibrin [28]. Staphylokinase and streptokinase, two cofactor molecules involved in bacterial plasminogen activator, help generate exosite and improve the enzyme's ability to provide substrate to the enzyme. In addition to destroying the extracellular matrix (ECM) and fibrin fibers that keep cells together, staphylokinase stimulates plasminogen to dissolve clots. When the clots were treated with aqueous and ethanol extract, a considerable thrombolytic activity was seen when compared to this positive and negative control. However, the results of the *H. longifolia* ethanol extract indicates less potential to lyse the clot. The extract from *H. longifolia* may have thrombolytic activity (Clot lysis) due to the individual compounds or to the combined action of all the active compounds present.

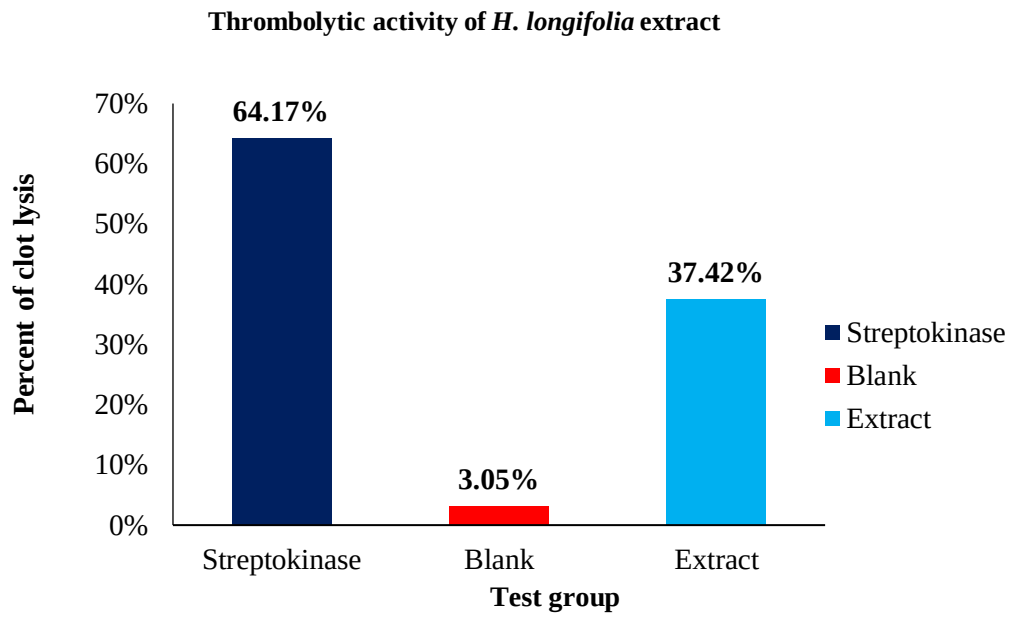

Figure 4. Percent of clot lysis of ethanol extracts of *H. longifolia.*

3.7. Evaluation of Acute Toxicity

In this acute toxicity investigation, the extract of *H. longifolia* leaves had no effects on mice's appearance or behavior, nor did it show any indications of toxicity or mortality. By the end of the experiment, every rat was in good health and continued to exhibit its typical behaviors and gait. Given the assumption that *H. longifolia* is a safe medical plant with no harmful side effects, its LD_{50} was calculated to be greater than 5000 mg/kg b.wt (Figure 5 and Table 3).

Table 3. Effects of ethanolic extract of *H. longifolia* leaves on body weight of mice.

Group	Avg. Weight ± SEM	Avg. Weight ± SEM
Group-I (control)	26.6 ± 1.76	28.3 ± 1.82
Group-II (300 mg/kg)	18.2 ± 1.67	21.2 ± 1.56
Group-III (2000 mg/kg)	15.8 ± 0.92	21.8 ± 0.82
Group-IV (5000 mg/kg)	22 ± 1.48	24.45 ± 1.51

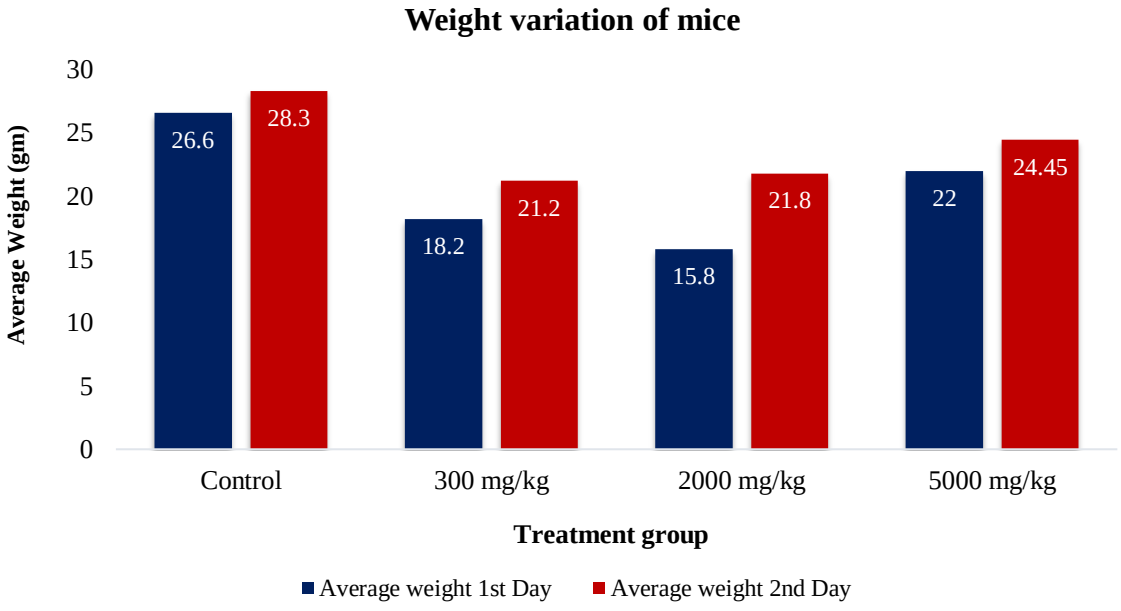

Figure 5. Effects of ethanolic extract of *H. longifolia* leaves on body weight of mice.

3.8. Anti-Inflammatory Activity

Inflammation, pain and pyrexia produce similar compulsive environments in our body [29]. So, following the evaluation of the analgesic activity of the plant, we have tested its anti-inflammatory potential. In the anti-inflammatory test, the *H. longifolia* extract showed significant inhibition in the paw edema in mice within the observation period at both doses of 250 mg/kg and 500 mg/kg b.wt. The values are shown in (Figure 6). This tissue paw thickness measurements revealed that the animals have shown dose- dependent anti-inflammatory effects. The mice of the ibuprofen group (100 mg/kg) exhibited the highest decrease in paw thickness that reduce by 0.56 ± 0.110 mm after 4 h and the inhibition percentage was 71.71%. The extract at 250 mg/kg had the III% of inflammation reduced at 54.22% on 4 h whiles the 500 mg/kg had more marked activity with III% of inflammation reduced at 60.27% after 4 h of administration. Substantial inhibition of paw edema demonstrated by the extract can be due to the presence of anti-inflammatory compounds like 4H-Pyran-4–one, catechin, gallocatechin, etc., [30]. The study also justifies the use of plant parts as an anti-inflammatory agent in folklore medicine [30].

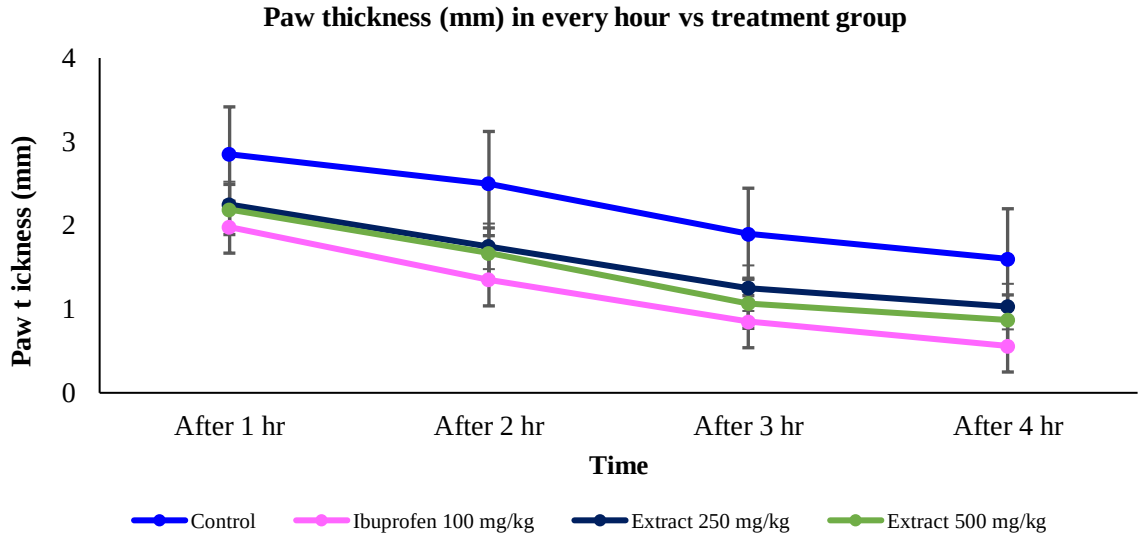

Figure 6. Paw thickness (mm) in every hour for negative control, positive control and *H. longifolia* extract.

3.9. Neuropharmacological Activity

3.9.1. Forced Swimming Test

H. longifolia extract significantly increased immobility times when given orally at dosages of 50, 100, and 200 mg/kg in comparison to the control group ($p < 0.05$). This finding indicates that the extract may have a major effect on immobility at the dosages of 50, 100, and 200 mg/kg it showed the immobility time significantly 132.4, 112.2 and 126.2 s respectively while standard showed 180.2 s. Further investigation is required to determine any possible therapeutic advantages. Similarly, as expected, a significant increase in immobility durations ($p < 0.05$) was also observed with the usual medication diazepam (1 mg/kg, intraperitoneal). These findings highlight how crucial it is to carry out more studies and create efficient remedies for this illness (Figure 7).

The tail suspension test is one of the widely used tests for appraisal of the antidepressant-like effect in medicine [31] noting that based on the duration of immobility time, this model has CNS depression-like evidence which is manifested as an extended duration of immobility while a short duration of immobility in this model has antidepressant effect. Nevertheless, in the forced swimming test, CNS depressive effect was observed. The experiment was also generalized to know whether this tail suspension test could be employed to provide a fast and sensitive method for establishment of the psychotropic nature of the substance. Consequently, the energy provided by mice to escape from suspension forms the measuring principle in total. During this test, the mice's movements were observed in concern with the energy and certain power that they built gradually. It significantly increased the extent of the mice's motor rest period, which has indicated CNS depressive effect of the extract.

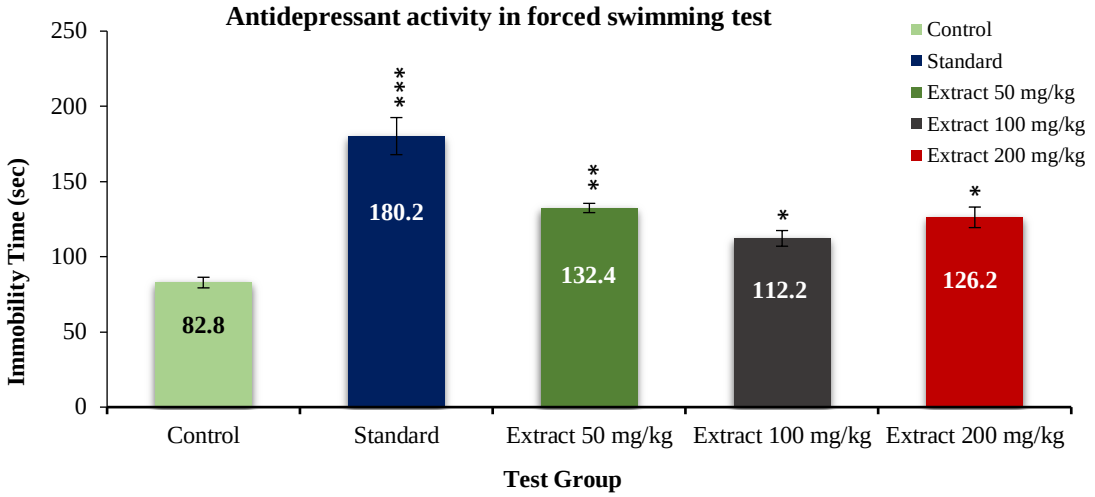

Figure 7. Effects of extract administration on the immobility time in the Forced Swim Test *H. longifolia* extract and Diazepam. Significance: $^{**}p < 0.01$, $^{***}p < 0.001$.

3.9.2. Tail-Suspension Test

Comparing the results of the test sessions with the control group the extract at the doses of 50, 100 and 200 mg/kg increased the immobility times significantly ($p < 0.05$). The result of the extract on behavior in this test is presented in the (Figure 8) below. This finding indicates that the extract has a major effect on immobility at the dosages of 50, 100, and 200 mg/kg it showed the immobility time significantly 113.2, 128.4 and 145.8 s respectively while standard showed 152.6 s. In the present study similar to that of the control group after diazepam (1 mg/kg, i.p), the duration of immobility time was significantly increased ($p < 0.05$).

In order to evaluate whether animal models exhibit antidepressant-like behavior, the forced swimming test is frequently employed. In this concept, a reduction in immobility time signifies antidepressant action, whereas a longer immobility time represents a CNS depression-like impact [31]. However, in the forced swimming test, a CNS depressive effect was noted. The experiment was also expanded to see if the tail suspension test might be used as a quick and accurate way to determine whether a substance has psychotropic qualities. In essence, the energy generated by mice attempting to break free of their suspension serves as the basis for the measuring principle. The mice's motions throughout this test were examined in terms of the energy and power that they gradually developed. The extract dramatically lengthened the mice's immobility period, suggesting that it had CNS depressive effects. In a mouse model, the common medication diazepam also showed CNS depressive effects. It has been suggested that the tail suspension test has higher pharmacological sensitivity and is less taxing than the force swimming test [32,33].

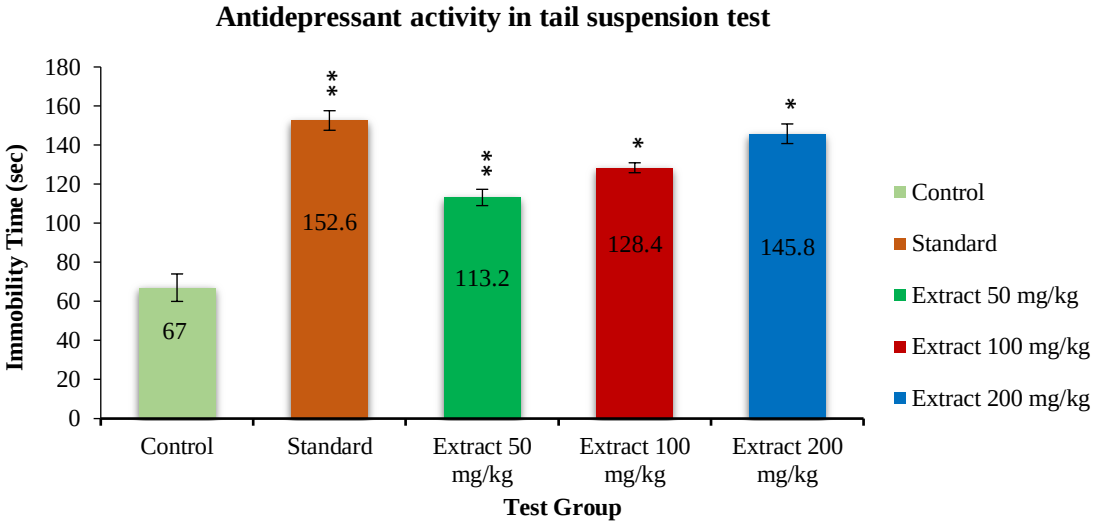

Figure 8. Effects of ethanolic leaves extract administration on the immobility time in the Tail suspension Test. *H. longifolia* Extract and Diazepam. Significance: $^{**}\ p < 0.01$, $^{***}\ p < 0.001$.

4. Conclusions

The therapeutic value of plant extracts is directly related to phytochemical compositions they contain. Several bioactive components present in *H. longifolia* include flavonoids, glycosides, alkaloids, terpenoids, acidic compounds, tannins etc. The extract proved more efficient in DPPH and FRAP scavenging than ascorbic acid because its IC_{50} value. The bioactive substances present in the plant form a strong basis for examining the pharmacological prospects of *H. longifolia* especially in its antimicrobial properties. In the antimicrobial assessment *E. coli* demonstrated maximum susceptibility to the antibacterial effect and it displayed the highest susceptibility to the microorganism while, *B. megaterium* demonstrated minimal inhibitory capacity. The thrombolytic test revealed that extract has ability to clot lysis. The extract exhibited no toxicity and behavioral changes in addition to no recorded fatalities. Among the treatment groups the inhibitory effect on inflammation reached 54.22% with 250 mg/kg and 60.27% with 500 mg/kg at the four-hour mark. The tail suspension and forced swimming tests demonstrated that the extract showed psychotropic features in addition CNS depressive properties. Future research will employ modern technology to identify and analyze the bio-compounds responsible for the evaluation of drug mechanisms and performance through in silico methodology will establish their potential use in medical drug development to address multiple healthcare conditions.

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