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Evaluation of Antioxidant Enzyme Activity and Antibacterial Potential of *Eichhornia crassipes* Leaf Extracts

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Abstract

Eichhornia crassipes (water hyacinth) is an invasive species of aquatic plant that was assayed for its antioxidant enzyme activity and any antibacterial properties using water and alcohol extracts of leaves and flowers harvested from the Tigris River (Baghdad, Iraq). The water extracts were assayed for glutathione S-transferase (GST) and peroxidase (POX) activities and the presence of heavy metals (iron, zinc, copper) while the alcohol extracts were assayed for secondary metabolites and antibacterial properties against one Gram-positive bacterium (*Staphylococcus aureus* and *Bacillus* spp.) and two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The results showed that GST (28.123 U/L) and POX (167.323 U/L) activities were significantly higher in the leaves than in the flowers (GST: 8.31 U/L and POX: 91.267 U/L), and this corresponded with the heavy metal accumulation in leaves. For both the samples of alcoholic extract, as well as the two species of bacteria tested, the activity was considerable against the Gram-positive microorganisms at concentrations of 60 and 80 mg/mL, producing inhibition zones of 14-32 mm, but showed no activity against the Gram-negative bacteria. Various secondary metabolites were present, including alkaloids, terpenoids, saponins, tannins, and flavonoids, which provided the study with its medicinal value. This research on *E. crassipes* contributes to evidencing its bioremediation potential and application to produce bioactive compounds.

Keywords: Antioxidant activity, antibacterial potential, *Eichhornia crassipes* Leaf Extracts, glutathione S-transferase (GST) activity, peroxidase (POX) activity

1. Introduction

Eichhornia crassipes, commonly referred to as the water hyacinth, is a free-floating aquatic plant that is infamous for its fast growth and invasive characteristics in freshwater systems (Gopal, 1987). Water hyacinths have received a considerable amount of attention recently thanks to their ability to uptake heavy metals and pollutants. Hence, the potential of *E. crassipes* as a bioremediation option in contaminated waterways is of growing interest (Mishra & Tripathi, 2008). In Iraq, specifically the Tigris River in Baghdad, the river was inundated with industrial effluent, domestic sewage, and agricultural runoff. These activities have contributed to an accumulation of heavy metals (e.g., iron, copper, and zinc) found in the Tigris River (Al-Ani et al., 2019), which are detrimental to aquatic ecosystems and humans and require sustainable remediation (Hussein & Alwan, 2018).

The ability of *E. crassipes* to survive in contaminated environments has been attributed to its excitable antioxidant defense system (mainly through enzymes, particularly glutathione S-transferase (GST), and peroxidase (POX)). These enzymes help combat oxidative stress associated with the toxicity of heavy metals, allowing *E. crassipes* the capacity to hyperaccumulate pollutants (Jing et al., 2007; Foyer & Noctor, 2005). *E. crassipes* is also capable of producing secondary metabolites such as flavonoids, alkaloids, and tannins, which possess antioxidant and antimicrobial capabilities and have potential pharmacological activity (Harborne, 1998; Cowan, 1999). Basol et al. (2014) objective was to explore the anti-activity of *E. crassipes* to germs, and they claimed they discovered the effectiveness of these secondary metabolites against bacteria, particularly the Gram-positive bacteria (Lalitha & Jayanthi, 2014).

This study aims to evaluate in a detailed manner the antioxidant enzyme activity (GST and POX), accumulation of heavy metals for aqueous extracts of *E. crassipes* leaves and flowers, identification of secondary metabolites, and antibacterial activity of the alcoholic extracts of the samples. Specifically, this study aims to quantitatively measure enzyme activity and heavy metal concentration, identify the bioactive compounds, and measure the antibacterial efficacy against selected bacterial strains to assess the natural capacity of the plant to perform the twin purposes of environmental remediation and pharmaceutical development.

2. Materials and Methods

2.1.Sample Collection

In 2024, leaves and flowers of *E. crassipes* were collected from the Tigris River, Al-Jadiriyah area of Baghdad, Iraq, where the pollution is substantial (Al-Ani et al., 2019). Samples were collected early in the morning in order to reduce environmental deviations with the washing of the leaves and flowers with distilled water to remove debris and surface contaminants, followed by air drying for 48 hours at 25°C. The samples were kept in the dark in airtight containers until they were processed for study in order to avoid damage (Sofowora, 1993).

2.2. Preparation of Aqueous Extracts

Aqueous extracts of *E. crassipes* leaves and flowers samples were made using a blender and by homogenizing 10 g of fresh leaves or flowers with 100 mL sterile distilled water. The homogenate was filtered through Whatman No. 1 filter paper, and the resulting filtrate was centrifuged at 5000 rpm for 10 minutes to remove debris. The supernatant was analyzed for enzyme activity and heavy metals after being stored for 24 hours at 4°C. Immediately after preparation, it was best to analyze any putative enzymes for activity, if practical (Trease & Evans, 2002).

2.3. Preparation of the Alcoholic Extract

Alcoholic extracts were prepared by finely grinding 10 g of air-dried leaves and flowers (both) into a fine powder using a mortar and pestle. The powder was extracted using 100 mL of a 1:1 methanol: water mixture using constant agitation for 24 hours at room temperature. The mixture was then filtered through Whatman No. 1 filter paper, and the combined filtrate was allowed to evaporate to dryness using a rotary evaporator at 40ºC under reduced pressure. The dried extract (1 g) was dissolved in 10 mL of sterile distilled water to create a stock solution. Serial dilutions of 20, 40, 60, and 80 mg/mL were made and stored at room temperature in the dark until use (Harborne, 1998).

2.4.Analysis of antioxidant enzyme activity

2.4.1. Glutathione S-Transferase (GST) activity

GST activity was measured in a spectrophotometer by measuring the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB), measured at 340 nm using a UV-Vis spectrophotometer (Habig et al., 1974). The total volume of the reaction mixture contained 50 µL of aqueous extract, 900 µL of 100 mM phosphate buffer (pH 6.5), 1 mM GSH, and 1 mM CDNB, and the absorbance was measured after one minute and three min. The activity was determined using the following formula:

$$\textbf{GST activity (U/L)} = 10 \times (\Delta A / \Delta T),$$

where ΔA is the change in absorbance, and ΔT is the change in time (minutes). The GST activity as one unit is defined as the amount of enzyme that catalyses the formation of 1 µmol of GS-CDNB conjugate per min, per liter (Mannervik, 1985).

2.4.2. Peroxidase (POX) Activity

Peroxidase activity (POX) was determined with guaiacol as the substrate, measuring the formation of tetraguaiacol at 470 nm (Chance & Maehly, 1955). The reaction mixture was 1,800 µL of 50 mM phosphate buffer (pH 7.0), 50 µL of aqueous extract, 20 mM guaiacol, and 10 mM hydrogen peroxide. Absorbance was measured after 1 min. POX activity was calculated using the following:

$$\textbf{POX activity (U/L)} = (\Delta A \times Vt) / (\Delta T \times Vs \times \varepsilon),$$

where ΔA is the change in absorbance, Vt is the total volume (1,800 µL), Vs is the volume of sample (50 µL), ΔT is the reaction time (1 min), and ε-molar extinction coefficient of tetraguaiacol (7100 L mol^{-1} cm^{-1}). One unit of POX activity is defined as the amount of enzyme that produces 1 µmol of tetraguaiacol per minute per liter (Pütter, 1974).

2.4.3. Heavy Metal Analysis

Iron, copper, and zinc concentrations were determined by atomic absorption spectroscopy (AAS) using a Perkin Elmer instrument with standard solutions of each metal for calibration (limit of detection: 0.01 mg/L). Aqueous extracts were acid-digested with 5% nitric acid, filtered through 0.45 µm syringe filters, and then analyzed (APHA, 2017). Results were expressed as mg/L.

2.4.4. Qualitative Analysis of Secondary Metabolites

Alcoholic extracts were subjected to phytochemical screening procedures for the presence of alkaloids, terpenoids, saponins, tannins, and flavonoids using standard procedures (Harborne, 1998; Evans, 2009). An equal number of reagents (A and B) was supplied for each test, and results were based on changes in color or precipitate formation. Alkaloids were tested in a similar manner using Wagner's (A) and Dragendorff's (B) reagents. Terpenoids were tested using Salkowski's (A) and Liebermann-Burchard (B) reagents. Saponins were identified by phenol foam formation with distilled water (test A) and sodium bicarbonate (test B). Tannins were tested using ferric chloride (A) and lead acetate (B). Lastly, flavonoids were tested using sodium hydroxide (A) and sulfuric acid (B) (Sofowora, 1993).

2.5. Antibacterial Activity Assay

Bacterial strains (*E. coli*, *P. aeruginosa*, *S. aureus*, and *Bacillus* spp.) were obtained from the Microbiology Laboratory, Department of Life Sciences, College of Sciences for Women, Baghdad. The agar well diffusion method was used for the assessment of antibacterial activity (Hammer et al., 2003). Muller-Hinton agar plates were inoculated with 10^5 CFU/mL bacterial suspensions (held with a sterile cotton swab), and five wells were made at equal distances using a cork borer (5 mm diameter). Each well was filled with 0.2 mL of extract at 20, 40, 60, or 80 mg/mL concentrations, and a control well was filled with sterile distilled water. Following incubation at 37°C for 24-48 hours, inhibition zones were measured in mm with a digital caliper (Bauer et al., 1966).

2.6.Statistical Analysis

Paired t-tests were used to compare enzyme activities and heavy metal concentrations of flowers and leaves. Antibacterial inhibition zones were analyzed using one-way ANOVA and Tukey's post-hoc test to differentiate concentrations and bacterial strains. The criteria for statistical significance were set at $p < 0.05$. All analyses were conducted with SPSS version 25, and data are reported as means ± standard deviation (n = 3) (Zar, 1999).

3. Results

3.1.Antioxidant Enzyme Activity

Aqueous extracts of *E. crassipes* leaves produced significantly higher GST activity (28.123 ± 1.02 U/L) than flowers (8.31 ± 0.45 U/L), which is a 29.55% increase ($p < 0.01$). For the leaves, $\Delta A = 0.027$ (0.312 - 0.285), $\Delta T = 1$ minute, which generated 28.123 U/L. For the poise flowers, $\Delta A = 0.016$ (0.130 - 0.114), $\Delta T = 2$ minutes, generating 8.31 U/L. The POX activity for leaves occurred at a rate of 167.323 ± 3.56 U/L, whereas for the flowers it occurred at a rate of 91.267 ± 2.13 U/L, where $p < 0.01$. For the poise flowers, $\Delta A = 0.033$, whereas for the poise flowers, $\Delta A = 0.081$, $Vt = 1800$ µL, $Vs = 50$ µL, $\Delta T = 1$ minute, $\varepsilon = 7100$ L mol^{-1} cm^{-1} (see Table 1). It is reasonable to infer that the leaves were exposed to far more environmental conditions, stimulating a greater abundance of antioxidant enzymes.

Table 1. Antioxidant enzyme activity in aqueous extracts of *Eichhornia crassipes*.

Extract	GST Activity (U/L)	POX Activity (U/L)
Flower	8.31 ± 0.45	91.267 ± 2.13
Leaf	28.123 ± 1.02	167.323 ± 3.56

Note: Values are means ± standard deviation (n = 3). Differences are statistically significant ($p < 0.01$).

3.2.Heavy Metal Concentrations

Leaves accumulated significantly higher concentrations of heavy metals compared with the flowers (see Table 2). Iron was existing at concentrations of 29.46 ± 1.23 mg/L for the leaves, and 2.2 ± 0.15 mg/L for the flowers ($p < 0.001$). Copper existed at 1.15 ± 0.06 mg/L for the leaves and at 0.81 ± 0.04 mg/L for the flowers ($p < 0.05$). Zinc existed at 26.5 ± 1.45 mg/L for leaves and at 10 ± 0.62 mg/L for flowers ($p < 0.05$). The greater concentration of heavy metal accumulation is attributed to the increased surface area of the leaves, meaning greater physical contact with polluted water and increased extractive uptake of metal.

Table 2. Heavy metal concentrations in aqueous extracts of *Eichhornia crassipes*.

Extract	Iron (mg/L)	Copper (mg/L)	Zinc (mg/L)
Flower	2.2 ± 0.15	0.81 ± 0.04	10 ± 0.62
Leaf	29.46 ± 1.23	1.15 ± 0.06	26.5 ± 1.45

Note: Values are means ± standard deviation (n = 3). Differences are statistically significant ($p < 0.05$ for copper and zinc, $p < 0.001$ for iron).

3.3.Qualitative Analysis of Secondary Metabolites

The alcoholic extract was confirmed to be positive for alkaloids, terpenoids, saponins, tannins, and flavonoids (Table 3). Both alkaloids produced a white precipitate with Reagent A and a brown precipitate with Reagent B. Terpenoids showed a reddish-brown color with Reagent A and a brown precipitate with Reagent B. Saponins were characterized by foam, and both reagents produced a white precipitate. Tannins produced a blue-green color with Reagent A and a dry yellow precipitate with Reagent B. Flavonoids produced an orange-red color with Reagent A and red with Reagent B. The data presented confirms that the plant contains bioactive compounds characterized by potential antioxidant and antimicrobial properties.

Table 3. Qualitative analysis of the secondary metabolites in the alcoholic extract.

Metabolite	Reagent A Result	Reagent B Result
Alkaloids	+White precipitate	+Brown precipitate
Terpenoids	+Reddish-brown	+Brown precipitate
Saponins	+Dense foam	+White precipitate
Tannins	+Blue-green	+Yellow precipitate
Flavonoids	+Orange-red	+Red

Note: Positive readings (+) indicate the presence of the metabolite.

3.4.Antibacterial Activity

The alcoholic extract showed strong antibacterial activity against Gram-positive bacteria (*S. aureus* and *Bacillus* spp.) at concentrations of 60 and 80 mg/mL with inhibition zones of 14 to 32 mm (Table 4, Figure 1). *Bacillus* spp. had inhibition zones of 25 ± 1.2 mm at 60 mg/mL and 32 ± 1.5 mm at 80 mg/mL ($p < 0.05$). S. aureus had inhibition zones of 8 ± 0.5 mm at 40 mg/mL, 14 ± 0.8 mm at 60 mg/mL, and 15 ± 0.9 mm at 80 mg/mL ($p < 0.05$). No inhibition was observed against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) at any concentration (Figure 2). The control (sterile distilled water) showed no inhibition across all strains.

Table 4. Antibacterial Activity of Alcoholic Extract (Inhibition Zone in mm).

Bacteria	20 mg/mL	40 mg/mL	60 mg/mL	80 mg/mL	Control
E. coli	-	-	-	-	-
P. aeruginosa	-	-	-	-	-
Bacillus spp.	-	-	25 ± 1.2	32 ± 1.5	-
S. aureus	-	8 ± 0.5	14 ± 0.8	15 ± 0.9	-

Note: Values are means ± standard deviation (n = 3). Inhibition zones for Bacillus spp. and S. aureus at 60 and 80 mg/mL were statistically significant ($p < 0.05$).

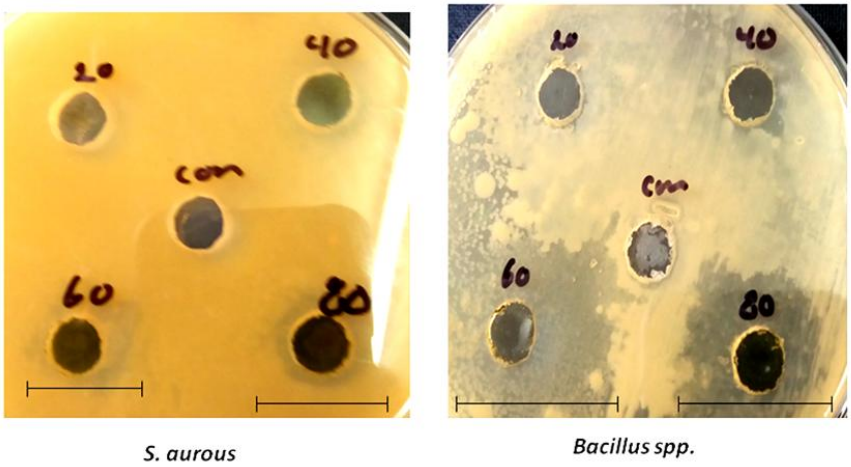


Figure 1. Antibacterial activity of alcoholic extract against gram-positive bacteria.

Inhibition zones for S. aureus (left) and Bacillus spp. (right) at 60 and 80 mg/mL concentrations, shown with black lines around wells.

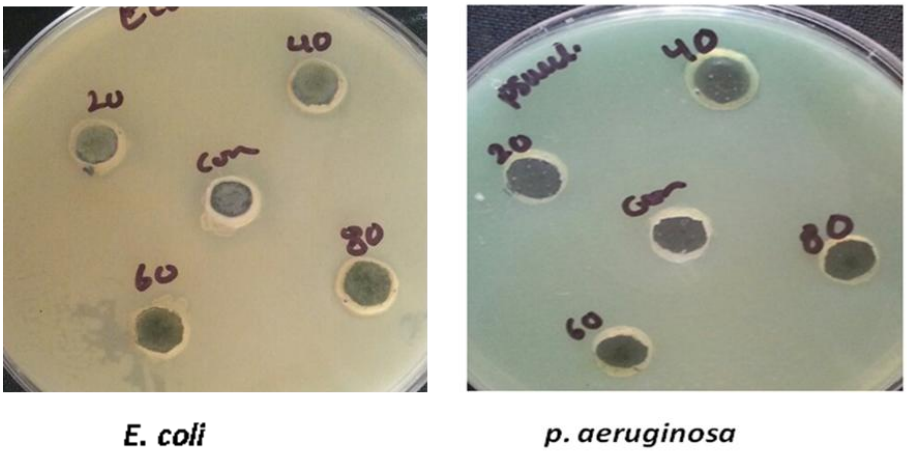


Figure 2. No antibacterial activity against Gram-negative bacteria.

No inhibition zones for E. coli and P. aeruginosa at any concentration.

4. Discussion

4.1. Antioxidant Enzyme Activity

The GST and POX activity for leaves of *E. crassipes* was significantly higher than for flowers, which is consistent with the involvement of the leaves being the first contact point with polluted water. Leaves have a greater surface area and are in direct contact with much higher concentrations of heavy metals, and likely other environmental factors that can stress these biological systems and induce the expression of antioxidant enzymes (Jing et al., 2007; Foyer & Noctor, 2005). GST detoxifies xenobiotics by facilitating their binding to glutathione, and POX detoxifies reactive oxygen species (ROS) generated during oxidative stress (Mishra & Tripathi, 2008). The elevated GST activity of 29.55% and POX activity of 83.3% in the leaves suggests a significant defense mechanism to protect against pollution and accept the very high pollution levels in the Tigris River, where physical, chemical, and biological contaminants accumulate and exceed WHO limits (Al-Ani et al., 2019; WHO, 2011).

4.2.Heavy Metal Accumulation

Higher concentrations of iron, copper, and zinc indicate their uptake will occur in leaves, the endpoint of heavy metal uptake. The Tigris River receives 680,000 m^3 of sewage, untreated, every day in Baghdad, which would account for the abundant heavy metals (Al-Ani et al., 2019; Hussein & Alwan, 2018). Iron deposits were significantly higher in leaves and assumed to be better retained than transported to other plant tissues, with an increase of about 13 times, 29.46 mg/L compared to 2.2 mg/L in the flowers, and Marschner (1995) suggested that iron does not usually translocate in higher organisms.

Copper is an essential micronutrient element that is important for respiration and photosynthesis, but at high concentrations, it may become toxic. The usefulness of copper is regulated by metallochaperones that bind free copper to guard against cellular toxic levels of free copper (Jing et al., 2007; Yruela, 2005). Zinc is essential for many enzymes, including polyphenol oxidase and cytochrome oxidase, and is present in a soluble form (Zn^{2+}). It can be sequestered in high quantities in leaves (Broadley et al., 2007). This suggests that *E. crassipes* could be a hyperaccumulator of Zn and could therefore be employed for bioremediation in contaminated water bodies (Raskin et al., 1997).

4.3.Secondary Metabolites

E. crassipes has been shown to contain complex phytochemicals such as alkaloids, terpenoids, saponins, tannins, and flavonoids in the alcoholic extract. Flavonoids and tannins are known antioxidants, acting as free radical scavengers and chelators of metal ions, and may mediate the resistance of *E. crassipes* to metals and pollutants (Harborne, 1998; Rice-Evans et al., 1997). Alkaloids and terpenoids have well-documented antimicrobial activities, whereas saponins can have hemolytic and antimicrobial activity (Sparg et al., 2004; Wink, 1993). Based on the identified phytochemicals, *E. crassipes* may be a source of bioactive compounds for pharmaceuticals, particularly natural antioxidants and antimicrobials (Cowan, 1999).

4.4.Antibacterial Activity

The differential antibacterial activity against Gram-positive bacteria (*S. aureus*, *Bacillus* spp.) exhibited by the alcoholic extract of *E. crassipes* can likely be explained by the putative differences seen in the cell wall structure of the two types of bacteria. Gram-positive bacteria have a thick peptidoglycan layer and lack an outer membrane, making them susceptible to phytochemicals found in plants such as flavonoids and tannins (Nikaido, 2003; Cowan, 1999). Conversely, Gram-negative bacteria (*E. coli* and *P. aeruginosa*) also possess an outer membrane, which decreases the potential efficacy of the extract as a barrier (Vaara, 1992). The larger zone of inhibition of *Bacillus* spp. when compared to *S. aureus* (32 mm compared to 15 mm at 80 mg/mL) indicates a sensitivity difference between the two bacteria. This may arise from differences in the peptidoglycan or differences in the permeability of the membrane (Lalitha & Jayanthi, 2014). The results from this study support findings from previous work, which reported the antibacterial effectiveness of *E. crassipes* extracts against Gram-positive pathogens (Jayanthi & Lalitha, 2011). The discovery of a lack of activity against Gram-negative bacteria demonstrates the ability to optimize extraction formulation in order to improve its spectrum of activity (Bauer et al., 1966).

4.5.Environmental and Pharmacological Implications

In the presence of heavy metals, the observed high accumulation and antioxidant enzyme activity in *E. crassipes* leaves suggest its potential as an inexpensive bioremediator of degraded aquatic systems, such as the Tigris River. *E. crassipes* would eliminate metals via absorption, thereby lessening the bioavailability of metals in the environment and water through bioremediation, and lessening health risk (Mishra & Tripathi, 2008; Raskin et al., 1997). However, because of its invasive nature, it requires some form of propagation/cultivation to avoid disrupting the ecology of aquatic habitats (Gopal, 1987). The observed antibacterial activity in the alcoholic extract may also point towards its promise as a natural preservative/antimicrobial agent and pursue further investigation as it pertains to unique infections caused by Gram-positive bacteria (Cowan 1999). The existence of secondary metabolites further supports the continuum for the development of pharmaceuticals from *E. crassipes* (Harborne, 1998); future studies should be directed towards isolation and characterization of active compounds, optimizing extraction processes, and in vivo assessment of their application (Newman & Cragg, 2012).

5. Conclusion

Eichhornia crassipes has immediate potential to function as a bioremediator and biological source of bioactive compounds. Higher GST and POX activities in leaves and higher accumulated heavy metals imply a capability of the leaves to aid in detoxifying water that has been contaminated. Secondary metabolites, especially flavonoids and tannins, demonstrate the pharmacological nature of the plant. The selective antibacterial action of *E. crassipes* against Gram-positive bacteria shows a rationale for the development of natural antimicrobials. In agreement with other studies, the findings of this paper highlight the use of *E. crassipes* in association

with sustainable bioremediation projects and the extraction of phytochemicals derived from *E. crassipes* for pharmaceutical purposes. Future studies should attempt larger-scale trials for bioremediation, identify specific bioactive compounds, and assess any clinical manifestations.

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