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In Vitro Antioxidant and In Vivo Hepatoprotective Properties of *Wissadula periplocifolia* Extract

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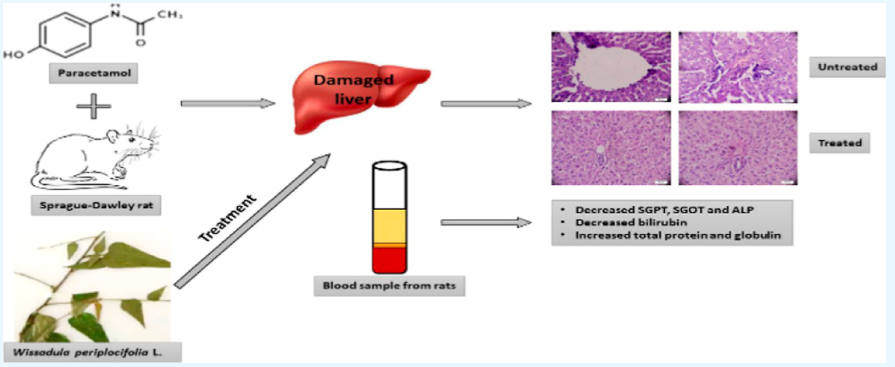

ABSTRACT: *Wissadula periplocifolia* (L.) Thwaites is a traditional medicinal plant belonging to the family Malvaceae, used in folk medicine for inflamed snake bites and bee stings. The current study was designed to investigate the in vitro antioxidant and in vivo anti-inflammatory and hepatoprotective activities of 80% ethanol extract of *W. periplocifolia* and its different fractions. The crude ethanolic extract (CEE) was then serially fractionated with petroleum ether fraction (PEF), chloroform fraction (CHF), and aqueous fraction (AQF). The antioxidant activity was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay, anti-inflammatory activity was determined in the xylene-induced ear edema model, and hepatoprotective activity was measured in the paracetamol-induced hepatic injury model. PEF showed a significant scavenging effect with an IC_{50} value of 33.5 μg/mL, followed by CEE (IC_{50} = 42.2 μg/mL), CHF (IC_{50} = 77 μg/mL), and AQF (IC_{50} = 80 μg/mL), compared to standard butylated hydroxytoluene (IC_{50} = 14.8 μg/mL). Both doses of CEE (250 and 500 mg/kg) could reduce ear edema by 41.3 and 50%, respectively, compared to standard diclofenac sodium (76.09%). Moreover, CEE significantly reduces the elevated liver enzymes (ALT, AST, and ALP), compared to control. Nevertheless, it elevated blood protein and reduced the blood bilirubin level ($p < 0.01$), compared to control. Histopathological studies also indicated significant protection of the liver from paracetamol-induced liver damage. In conclusion, *W. periplocifolia* could be a good source of antioxidant and hepatoprotective phytochemicals; meanwhile, toxicological and pharmacokinetic studies are recommended.

1. INTRODUCTION

Our ancestors were totally dependent on a wide variety of plants to treat themselves from disease until the introduction of synthetic medicines.[1−3] It is found that almost 2000 racial groups all over the world have their own unique medical understanding and experience.[4,5] This is because some vital components that possess antioxidant and anti-inflammatory properties are used in certain physiologic conditions.[6] There were 90 drugs that are commercially available or approved worldwide in the period of 1982 to 2002, and 79% of them were from natural sources.[7−9] Still a large portion of the population use medicinal plants as primary source to treat different diseases such as fever, pain, inflammation, diarrhea, and many other illnesses.[10,11]

Liver is a major site for extensive metabolism and excretion. It also has influence on almost every biochemical process such as physical development, immunity, nutrition, energy supply,

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and breeding.[12] Therefore, it has exposure to a wide range of xenobiotics, and some of them can create reactive oxygen species (ROS) that can induce oxidative tissue damage.[13,14] These ROS can react with the cell membrane, which ultimately leads to lipid per-oxidation and ultimately inflammation.[15,16] It has been established that these radicals can work as pathological mediators in a wide variety of complications such as heart disease, diabetes, gout, and cancer. Antioxidants can react with these free radicals and turn them into harmless compounds.[17] Although we have seen the enormous development of advanced medicine, no effective medications are available that can protect the damage of the liver cell; moreover, some of them cause severe side effects.[18] For this reason, some researchers recommended using a wide variety of medicinal plant-derived phytochemicals for the treatment of liver diseases.[19]

Wissadula periplocifolia (L.) from the family "Malvaceae", a medicinal plant, is distributed all over Bangladesh, but it has not been extensively investigated yet.[20] Previous studies have suggested that this species contains higher antioxidant properties than some of the other plants in the same family.[21] Selecting *W. periplocifolia* (WP) for investigation was based on the fact that the Malvaceae family has broad antioxidant properties and have folkloric uses in different liver diseases.[22] The paracetamol-induced hepatotoxicity model is widely used to investigate the hepatoprotective effects of drugs of plant origin.[23] However, no scientific data are available regarding the effect of this plant extract on paracetamol-induced intoxication in rats. Plant extracts have also been indicated to eradicate the development of liver damage induced by acetaminophen or paracetamol in the experimental model.[10] It works by enhancing the antioxidant enzyme activity and reducing the amount of lipid peroxidation. Paracetamol is an over-the-counter (OTC) drug widely used for its analgesic and antipyretic effects. It is primarily metabolized in the liver and eliminated by different biochemical reactions such as conjugation with sulfate and glucuronide and then excreted through kidneys. Higher doses of paracetamol showed significant hepatocellular damage or necrosis. So, it can be used as a good way for the screening of hepatoprotective activity in paracetamol-induced liver injury. Paracetamol-induced hepatotoxicity has been attributed to the formation of toxic metabolites as well as elevation of different biological markers such as alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin and reduction of serum protein, which are the results of the damage of the cellular integrity of the liver.[24−27] Toxic metabolites such as *N*-acetyl-*p*-benzoquineimine (NAPQI) can lead to extensive lipid peroxidation by oxidizing liver intracellular GHS rapidly together with irreversible modification of cellular protein, cell death and ultimately organ damage.[28,29] Reactive metabolites can exert initial cell stress through a wide range of mechanisms including depletion of glutathione (GSH) or binding to enzymes, lipids, nucleic acids, and other cell structures.[30] Therefore, the aim of the study has been focused on the hepatoprotective activities of the ethanolic extract of WP on paracetamol-induced hepatic toxicity in experimental rats. This study opens an area for scientists and drug researchers to perform phytochemical analysis of the extract and in silico studies on the target proteins to assess pharmacokinetics (absorption, distribution, metabolism, and elimination evaluations) and pharmacodynamic properties of the extract components.[31−36]

2. RESULTS

2.1. Determination of Total Phenolics. The phenolics contents of crude ethanolic extract (CEE) and its three fractions, such as petroleum ether fraction (PEF), chloroform fraction (CHF) and aqueous fraction (AQF), were 378.94, 339.92, 202.44, and 266.81 mg of gallic acid equivalent (GAE)/g of dried extracts, respectively (Figure 1). Comparing the phenolics content of CEE with different fractions, it was observed that PEF contained the highest number of phenolic compounds.

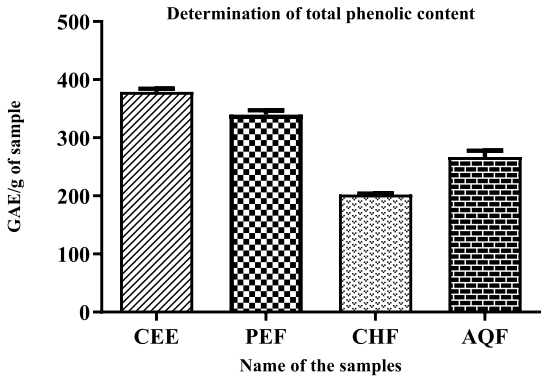

Figure 1. Determination of total phenolics content (mg GAE/g of dried extract) of CEE and its three fractions. CEE: crude ethanolic extract, PEF: petroleum ether fraction, CHF: chloroform fraction, AQF: aqueous fraction, GA: gallic acid. Values are the mean of triplicate experiments and represented as mean ± STD.

2.2. Reducing Power Capacity Assay. The ferric reducing power of the different fractions of WP was determined by using ascorbic acid (AA) as standard. The reductive capabilities of different extractives and AA are shown in Figures 2 and 3. The CEE showed less activity compared to AA.

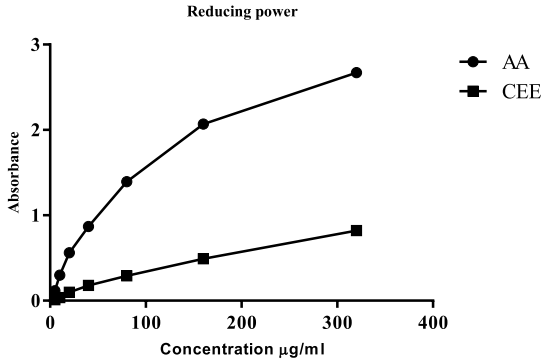

Figure 2. Determination of the reducing power capacity of CEE against standard AA.

Among the fractions, the PEF (absorbance 1.03) had the highest reducing activity and compared to standard AA (absorbance 2.67) in terms of iron-reducing activity. Other fractions, such as CHF (absorbance 0.48) and AQF (absorbance 0.79), had moderate antioxidant activity compared to AA at 320 μg/mL. These results demonstrated that PEF had significant iron reducing capacity. However, the AQF and CEE fractions showed moderate activity. The iron

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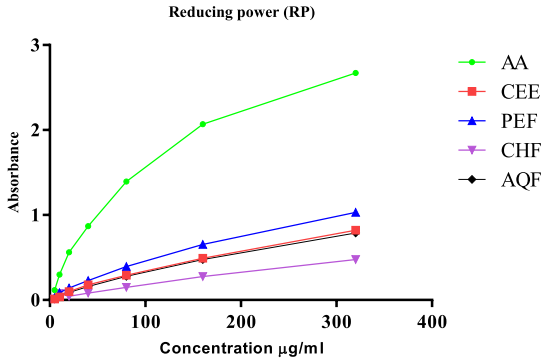

Figure 3. Determination of the reducing power capacity of CEE and its three fractions. CEE: Crude ethanolic extract, AQF: aqueous fraction, CHF: chloroform fraction, PEF: petroleum ether fraction, AA: ascorbic acid (standard).

reducing power of different extractives and AA showed the following order: AA > PEF > CEE > AQF > CHF.

2.3. 1,1-Diphenyl-2-picrylhydrazyl Radical Scavenging Assay. The antioxidant activity of the extractives of WP was determined by a widely used and most reliable DPPH radical scavenging assay method. The ability of the extractives to scavenge the stable DPPH radical that contains an odd electron depends on the antioxidant assay. This radical gives absorbance at 517 nm and decolorizes after neutralization by the antioxidants. The activity is proportional to the concentration of the extractives.

The DPPH radical scavenging assay by PEF, CEE, CHF, AQF, and standard butylated hydroxytoluene (BHT) are given in Table 1 and Figures 4 and 5. The IC_{50} values of BHT and CEE were 14.8 and 42.2 μg/mL, respectively.

Table 1. DPPH Free Radical Scavenging Activity of CEE and Its Three Fractions at Different Concentrations

extract/fraction	CEE	PEF	CHF	AQF	BHT
IC_{50} (μg/mL)	42.2	33.5	77	80	14.8

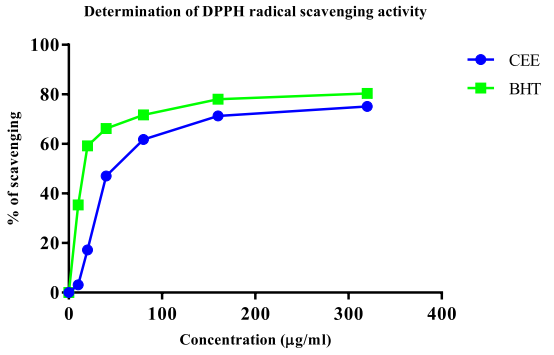

Figure 4. Determination of the DPPH radical scavenging activity of CEE against BHT (Standard).

2.4. Acute Toxicity Study. The oral acute toxicity study of ethanolic extract of WP was determined following Organization for Economic Co-operation and Development (OECD) guideline 423 with simple modification and the limit dose was 6000 mg/kg. None of the experimental animals showed any toxic symptoms or mortality after administration of CEE of WP at different concentrations such as 2000, 4000, and 6000 mg/kg body weight. General behavior such as

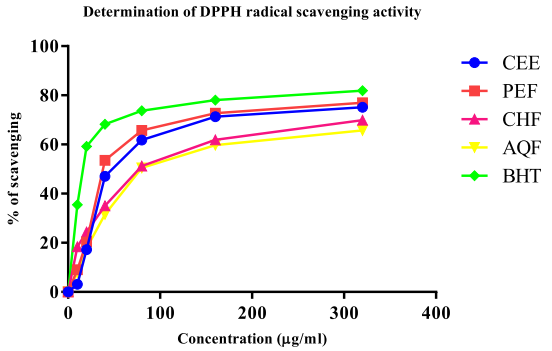

Figure 5. Determination of DPPH free radical scavenging activity of CEE and its three fractions against BHT (standard) at various concentrations. CEE: Crude ethanolic extract, AQF: aqueous fraction, CHF: chloroform fraction, PEF: petroleum ether fraction, BHT: butylated hydroxytoluene (standard).

behavior, eating pattern, skin color, and temperature of the treatment groups did not change while compared with the control group within a short period of time (4 h), followed by a long period (72 h). Therefore, it can be concluded that a dose of 6000 mg/kg body weight remains safe and the LD_{50} should be more than 6000 mg/kg body weight. However, some signs of lethargy and drowsiness were observed for the first 2 h after the administration of 4000 mg/kg and 6000 mg/kg body weight compared to the control group.

2.5. Xylene-Induced Ear Edema Test. The results from the experiment indicated that the group that was treated with standard diclofenac sodium showed significant ($p < 0.001$) 76.09% inhibition of edema compared to control group (Table 2 and Figure 6). Other two groups that were treated with CEE

Table 2. Effect of CEE Extract of *W. periplocifolia* (WP) in Xylene-Induced Ear Edema Test[a]

group	dosage (mg/kg)	ear weight difference mg	inhibition (%)
control		9.20 ± 0.86	
diclofenac sodium	100	2.20 ± 0.37***	76.09
WP	250	5.40 ± 0.93**	41.30
	500	4.60 ± 1.12*	50.00

[a]The values are presented in mean ± SEM ($n = 5$). An independent sample t-test was carried out to analyze this relationship. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared with the control group.

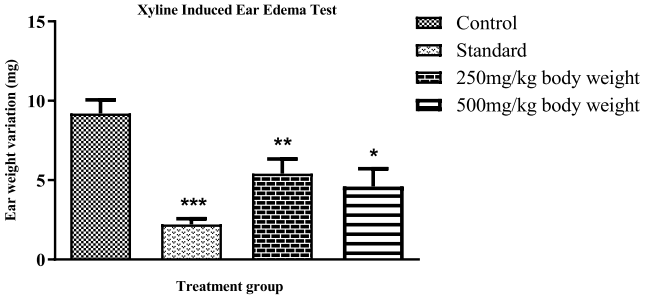

Figure 6. Effect of CEE extract of WP in a xylene-induced ear edema test. The values are presented in mean ± SEM ($n = 5$). Independent sample t-test was carried out to analyze this relationship. For *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared with the control group.

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Table 3. Effects of the CEE of *W. periplocifolia* on Hepatocellular Biomarkers[a]

treatment group	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
normal control (regular food and water)	32.2 ± 1.497	23.2 ± 2.035	156.2 ± 3.426
negative control (only paracetamol)	54.8 ± 5.643^{z}	49.2 ± 1.934^{z}	197.2 ± 10.837^{y}
positive control (Paracetamol + Silymarin)	42.4 ± 1.691	28.2 ± 2.728^{c}	132.8 ± 6.240^{c}
D250 (Paracetamol + 250 mg/kg CEE)	46.4 ± 1.720	32.2 ± 3.382^{c}	172.0 ± 6.473
D500 (Paracetamol + 500 mg/kg CEE)	38.4 ± 2.135^{b}	20.2 ± 1.0198^{c}	114.6 ± 4.445^{c}

[a]The values are presented in mean ± SEM (n = 5). One-way ANOVA was performed, followed by Tukey and Dunnett tests, to analyze this relationship. $^{x}P < 0.05$, $^{y}P < 0.01$, and $^{z}P < 0.001$ when compared against control. On the other hand, $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ when compared against negative control.

of *W. periplocifolia* at a dose of 250 mg/kg and 500 mg/kg showed 41.3% ($p < 0.01$) and 50% ($p < 0.05$) inhibition of edema, respectively, compared to the control group.

2.6. Hepatoprotective Study. *2.6.1. Assessment of Liver Enzymes.* Elevated serum levels of biochemical markers such as ALT, AST, and ALP indicate liver damage. When the negative control group was treated with only paracetamol, a significant serum level of ALT, AST, and ALP was observed. The standard group that was treated with silymarin showed a significantly lower level of these biochemical markers when compared with the negative control group (Table 3 and Figure 7). The other two groups that were treated with 250 and 500 mg/kg of CEE of *W. periplocifolia* also significantly lowered these biochemical markers when compared with the negative control group. The 500 mg/kg body weight was shown to be more protective than the 250 mg/kg body weight.

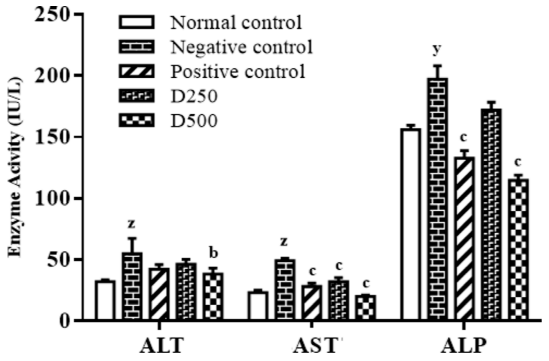

Figure 7. Effects of CEE of WP on ALT, AST, and ALP levels of normal and paracetamol-treated rats.

2.6.2. Determination of Albumin and Globulins. When the liver function is impaired, the level of protein is significantly decreased in the negative control group compared with the normal control. The other two groups which were treated with 250 mg/kg and 500 mg/kg body weight showed an improved serum level of protein while compared with the negative control group, as shown in Table 4 and Figure 8.

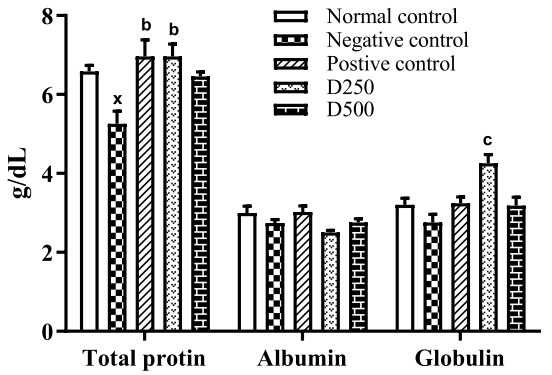

Figure 8. Effects of CEE of WP on total protein, albumin, and globulin levels of normal and paracetamol treated rats.

2.6.3. Determination of the Bilirubin Level. When the old red blood cells break down, the bilirubin comes out to the circulation. It is neutralized by the healthy liver and is a normal physiological process. However, when the function of the liver was altered, it could not neutralize the free bilirubin properly, which ultimately led to an increase in the level of bilirubin to an unhealthy level. The results from the experiment showed that the negative control group produced a significant ($p < 0.01$) amount of bilirubin when compared with the normal control group (Table 5 and Figure 9). The other three groups such as standard, 250 mg/kg, and 500 mg/kg group of CEE of *W. periplocifolia* showed a significantly ($p < 0.01$) lowered level of bilirubin from the serum.

2.6.4. Liver Histopathology. The histopathological results provide strong support for biochemical analysis. The liver section of the normal control group showed normal liver cells along with apparent cytoplasm, nucleus, and nucleolus along with undamaged central vain and hepatic portal vain in it. The

Table 4. Effects of CEE of *W. periplocifolia* on Total Protein, Albumin, and Globulin Levels of Normal and Paracetamol-Treated Rats[a]

treatment group	total protein (g/dL)	albumin (g/dL)	blobulin (g/dL)
normal control (regular food and water)	6.58 ± 0.159	3.00 ± 0.170	3.20 ± 0.170
negative control (only paracetamol)	5.26 ± 0.319^{x}	2.74 ± 0.093	2.76 ± 0.206
positive control (Paracetamol + Silymarin)	6.96 ± 0.420^{b}	3.02 ± 0.156	3.24 ± 0.163
D250 (Paracetamol + 250 mg/kg CEE)	6.96 ± 0.319^{b}	2.50 ± 0.055	4.26 ± 0.218 ^{c}
D500 (Paracetamol + 500 mg/kg CEE)	6.46 ± 0.108	2.76 ± 0.093	3.18 ± 0.215

[a]The values are presented in mean ± SEM (n = 5). One-way ANOVA was performed, followed by Tukey and Dunnett tests, to analyze this relationship. $^{x}p < 0.05$, $^{y}p < 0.01$, and $^{z}p < 0.001$ when compared against control. On the other hand, $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$ when compared against negative control.

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Table 5. Effects of CEE of *W. periplocifolia* on the Total Bilirubin Level of Normal and Paracetamol-Treated Rats[a]

treatment group	total bilirubin (mg/dL)
normal control (regular food and water)	0.64 ± 0.051
negative control (only paracetamol)	1.28 ± 0.218y
positive control (Paracetamol + Silymarin)	0.66 ± 0.051b
D250 (Paracetamol + 250 mg/kg CEE)	0.68 ± 0.037[b]
D250 (Paracetamol + 500 mg/kg CEE)	0.58 ± 0.037b

[a]The values are presented in mean ± SEM (n = 5). One-way ANOVA was performed, followed by Tukey and Dunnett tests, to analyze this relationship. [x]P < 0.05, [y]P < 0.01, and [z]P < 0.001 when compared against control. On the other hand, [a]P < 0.05, [b]P < 0.01, and [c]P < 0.001 when compared against negative control.

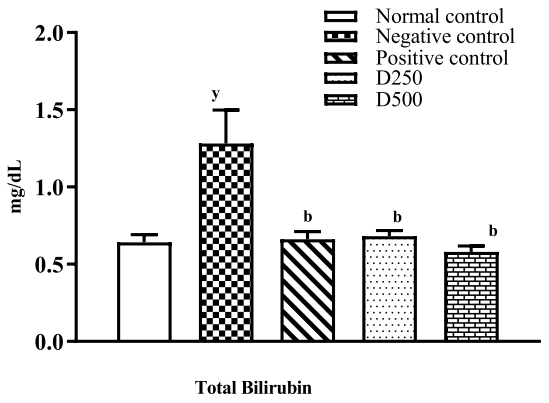

Figure 9. Effects of CEE of WP on total bilirubin levels of normal and paracetamol-treated rats.

paracetamol-intoxicated negative control group exhibits loss of hepatic architecture such as cell necrosis, Kuppfer cell hyperplasia, and blocking and rupture of central and hepatic portal vain. The animal groups treated with CEE of WP at doses of 250 mg/kg and 500 mg/kg showed significant protection from liver injury caused by paracetamol, while compared to the negative control group. Figures 10A and 11A show the normal structure of central vein, hepatic portal vein, and surrounding hepatocytes. Figures 10 and 11B show focal centrolobular necrosis (red arrow) around both the central and portal vein. Figures 10C and 11C show a large decrease in the necrotic area around central and portal vein with diffuse Kuppfer cells (green arrow) than the only paracetamol treated group. Figures 10 and 11C,D show almost the same structure with no damage around central and portal vein with diffuse Kuppfer cells (green arrow).

3. DISCUSSION

Phenolic compounds are known to have potential antioxidant properties. More than 8000 familiar compounds are constituted in this class of plant metabolites such as phenols and tannins. The main dietary antioxidants are phenolics, and they exert higher antioxidant capacity even better than various essential vitamins.[37] Various research showed that phenolic compounds extracted from hydroalcoholic fraction contain certain potent natural antioxidants such as catechin, quercetin, and isorhemnetin.[15] The present study indicates that WP has a high total phenolic content. The total phenolic contents of different extractives such as CEE and its three fractions PEF, CHF, and AQF were found to be 378.94, 339.92, 202.44, and 266.81 mg of GAE/g, respectively. Among the three fractions, the PEF had the highest reducing capacity, while the CHF had the lowest reducing capacity at a concentration of 320 μg/mL (Figure 3). Taken together, the results demonstrated that WP has a very significant antioxidant activity. The antioxidant activity, which is associated with phenolic compounds, is the ability to scavenge free radicals that were formed.[38] Our investigation revealed that the PEF showed significant scavenging with an IC_{50} of 33.5 μg/mL, followed by CEE (IC_{50} = 42.2 μg/mL), CHF (IC_{50} = 77 μg/mL), and AQF (IC_{50} = 80 μg/mL), while the standard BHT showed the highest radical scavenging activity (IC_{50} = 14.8 μg/mL). In iron reducing capacity, the CEE and its three fractions showed the following order: AA > PEF > CEE > AQF > CHF. These compounds can sometimes bind with various minerals, which is beneficial for the body, for example, hydroxyl radicals can be generated by iron and copper by Fenton and Haber−Weiss reactions.[39] Different phenolic antioxidant compounds were isolated from *W. periplocifolia* such as 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, (*E*)-cinnamic acid, tamgermanetin, and 7,4′-di-*O*-methylisoscutellarein.[20]

Regarding the acute toxicity study, a dose of 6000 mg/kg body weight (the highest tested dose) did not show any signs of toxicity like change in behavior, skin effects, or breathing. There was no impairment in food intake and water consumption, suggesting that the plant extract is safe even at

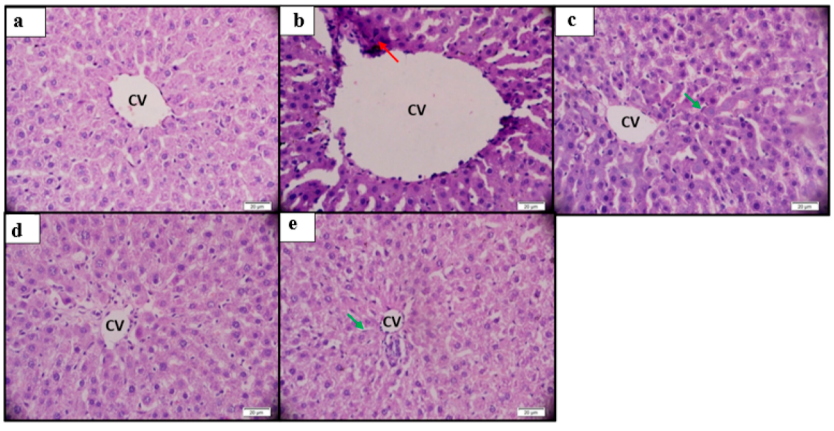

Figure 10. Histopathology of liver tissues showing the hepatic central vein in the normal group (A), negative control (paracetamol only; B), positive control (paracetamol + silymarin; C), D250 (250 mg CEE + paracetamol; D), and D500 (500 mg CEE + paracetamol; D).

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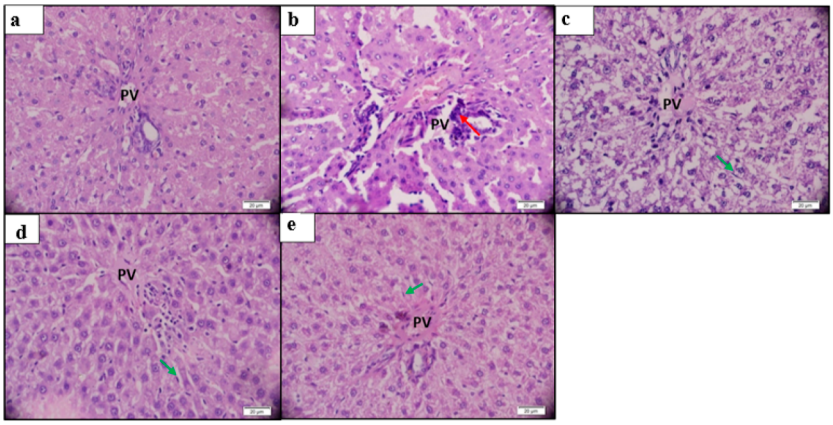

Figure 11. Histopathology of liver tissues showing the hepatic portal vein in the normal group (A), negative control (paracetamol only; B), positive control (paracetamol + silymarin; C), D250 (250 mg CEE + paracetamol; D), and D500 (500 mg CEE + paracetamol; D).

high tested dose (6000 mg/kg). Thus, LD50 should be more than 6000 mg/kg body weight.

Our current study showed that characteristic anti-inflammatory response in mice paw-licking after administration of formalin which were then treated with CEE of WP at 250 body and 500 mg/kg body weight produced significant analgesic effects. The CEE showed significant 60.96 and 71.1% ($P < 0.001$) inhibition at 250 and 500 mg/kg body weight, respectively, compared to 78.31% inhibition 100 mg/kg body weight of standard Diclofenac sodium in formalin induced paw-licking. In this study, it was found that treatment of mice with CEE of WP at 250 and 500 mg/kg body weight resulted in a significant anti-inflammatory effect on the formalin-induced paw-licking. The CEE also showed significant 60.96 and 71.1% ($p < 0.001$) inhibition at 250 and 500 mg/kg body weight compared to 78.31% inhibition at 100 mg/kg body weight of standard diclofenac sodium in formalin-induced paw licking test. So, it can be inferred that CEE of WP is effective in reducing inflammatory pain at a dose of 250 and 500 mg/kg body weight. Xylene-induced ear edema test in mice also showed significant inhibition of inflammation, which is 41.3% ($P < 0.01$) and 50% ($P < 0.05$) at 250 mg/kg body weight and 500 mg/kg body weight, respectively, compared to standard 76.09% for diclofenac sodium. It has been proven that ROS are associated with inflammatory conditions.[40] There is a profound relationship between antioxidants with respect to hunting ROS and anti-inflammatory effects and therefore play an influential role in the treatment of inflammatory diseases.[41] The evidence of both antioxidant and anti-inflammatory properties by the CEE may confirm this correlation. Therefore, these properties may describe the traditional use of the plant in the treatment of inflammatory conditions caused by insect bites. A previous study isolated flavone, namely, 7,4′-di-*O*-methylisoscutellarein from *W. periplocifolia* aerial parts revealed its anti-inflammatory activity via inhibition of neutrophils' recruitment and reduction of IL-1β and TNF-α.[20] In another anti-inflammatory study in brazil, it was found that *W. periplocifolia* (L.) C. Presl (25 mg/kg) could reduce the paw carrageenan-induced edema.[42] Another species, namely, *Wissadula amplissima,* exhibited a significant anti-inflammatory activity, as represented by remarkable inhibition on lipoxygenase and xanthine oxidase.[43]

The most common diseases of the liver are hepatitis, alcoholism, drug induced liver disease, hepatocellular carcinoma, and so on. Liver may also be susceptible to reactive oxygen species (ROS).[44] The primary target of oxidative stress is the parenchymal cells, which lead to liver damage. Mitochondrion and other cellular ingredients such as microsomes and peroxisomes can produce ROS that can damage the liver cell. Without proper elimination of those radicals, this ROS can trigger lipid peroxidation, causing the proliferation of cancer, heart disease, and neurodegeneration as well as inflammation.[45−47] An inherited and sophisticated antioxidant system has already been developed in mammals to minimize the effects of ROS. The enormous functional reserve of the liver masks the clinical impact of mild liver damage, but with the progression of repeated derange, it may become life-threatening.[48]

Different biochemical markers such as ALT, AST, ALP, serum bilirubin level, and total proteins are widely used to examine if there is any liver injury.[49,50] The coadministrations of CEE of WP have showed limiting the level of those marker enzymes, and this is a commonly accepted agreement that the serum level of these marker enzymes ALT, AST, ALP, and serum bilirubin returns to a normal level, indicating the healing of hepatic parenchymal cells and regeneration of hepatic cells. CEE of WP significantly reduce blood ALT, AST, and ALP ($p < 0.001$) while comparing with the negative control group. WP exhibited excellent hepatoprotective properties by keeping the most common serum biochemical marker at a normal level. Different scientific reports suggested that polyphenolics like flavonoids have protective effects on liver due to their antioxidant properties.[51] Phytochemically, WP also found to contain triterpenoids, tannins, steroids, and alkaloids might play a role in hepatoprotective activity.[20,21] The increased serum ALT, AST, and ALP level and decreased serum protein level in paracetamol-treated rats found in our study were probably due to inflammation or hepatocellular damage as in the histopathological data showed severe necrosis in paracetamol-treated rats. This could be due to the formation of ROS, which is caused by the oxidative threat posed by paracetamol. This necrosis causes an uncontrolled release of inflammatory cellular contents. All the changes, which were visualized via histopathological studies, were very much reduced by treating the rats with CEE of *WP*.

It is well known that paracetamol is an antipyretic and analgesic drug that is usually safe in therapeutic doses, but high doses can cause fatal hepatic damage in experimental animals and humans and are employed as an experimental hepatotoxic agent.[52] It is metabolized in liver by glucuronidation and sulfation enzymes, and when high doses of paracetamol is

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administered, these metabolizing enzymes became exhausted, and the excess paracetamol converts by CYP_{450} oxidase system into *N*-acetyl-*p*-benzoquinone imine (NAPBQI), which is a highly reactive metabolite and causes ROS overproduction and lipid peroxidation.[53] This active metabolite then reacts with the sulfhydryl group of glutathione (GSH) by covalent bonding, resulting in GSH depletion, and an alteration in homeostasis of liver cells leads to change in the permeability of the cell membrane with a subsequent cellular swelling, karyolysis, hepatocyte vacuolization, and elevation of liver enzymes.[54] So, antioxidants can play a vital role protecting the liver in paracetamol-induced liver injury.[55] The phenolics contained in CEE as plant-derived compounds are stated as antioxidants that decrease different destructive processes in human beings owing to their capability to scavenge ROS and degrade its production or potentiate the production of antioxidant enzymes and their protective action.[56]

The current study showed that complementary antioxidant, anti-inflammatory, and hepatoprotective properties of *W. periplocifolia* may be interrelated and mediated through its anti-inflammatory and antioxidant activities.

4. MATERIALS AND METHODS

4.1. Chemicals and Reagents. AA (Merck, Germany), acetic acid (Merck, Germany), butylated hydroxy toluene (BHT) (Sigma chemical company, USA), barium sulfate (SIGMA), DPPH (Sigma chemical company, USA), diclofenac sodium (Beximco Pharmaceuticals Ltd.), paracetamol (Beximco Pharmaceuticals Ltd.), silymarin (Beximco Pharmaceuticals Ltd.), ethanol (Merck, Germany), ferric chloride (Sigma chemical company, USA), trichloro acetic acid (Merck, Germany), formalin (Merck, Germany), xylene (Merck, India), gallic acid (Sigma-Aldrich, USA), ketamine (Gonoshasthaya Pharma Ltd.), potassium ferricyanide (Merck, Germany), phosphate buffer (Sigma-Aldrich, USA), and sodium carbonate (Sigma chemical company, USA) were used.

4.2. Preparation of Plant Materials. Whole green parts of WP were collected from Boyra, Khulna, in May 2017 in fresh condition. The plant was identified by an expert taxonomist from the Bangladesh National Herbarium, Mirpur, Dhaka (DACB accession number 45994). The parts of the herb were cleaned, shade-dried for 5 days, grounded by using a grinding machine at Department of Pharmacy, BSMRSTU, and stored at room temperature.

About 500 g of grounded plant materials was taken into a well cleaned amber colored bottle and immersed in the materials with 1500 mL of ethanol (80%). The bottle was sealed for 7 days along with its contents, with occasional shaking. After 7 days, the content was filtered through a cotton filter and then with Whatman no. 1 filter paper. The filtrate was then concentrated at a temperature below 50 °C with a rotary evaporator under reduced pressure to get a brownish mass (60 g) crude ethanolic extract. About 30 g of the ethanolic extract was fractionated using the method as described by Kupchan with simple modification,[57] and ultimately, PEF (petroleum ether fraction) 4.2 g, CHF (chloroform fraction) 8.2 g, and AQF (aqueous fraction) 13 g were obtained to be used for further study. A schematic flowchart illustrating the fractionation process is shown in Figure 12.

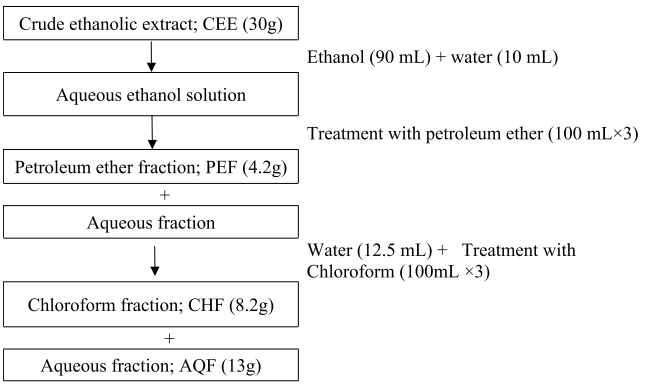

Figure 12. Schematic flowchart of the fractionation process.

4.3. Determination of Total Phenolics. The extent of total phenolics of the different extractives was determined with simple modification in which the Folin−Ciocâlteu reagent (FCR) was used as the oxidizing agent and gallic acid (GA) was used as the standard.[58] An aliquot of different extracts or standard with different concentrations was mixed with 2 mL of FCR (10 times diluted with distilled water), and to each of the test tubes, 2 mL of sodium carbonate was added. The test tubes were then vortexed for about 15 s and then kept in an incubator at 25 °C for 20 min to complete the reaction. The absorbance was then measured against blank at 760 nm using a spectrophotometer. The content of total phenolic compounds was demonstrated in terms of gallic acid equivalents, GAE.

4.4. Reducing Power Capacity Assay. The reducing power capacity of different extractives was evaluated by previously described method[59] with simple modification. 0.25 mL extractives/standard of various concentration, 0.625 mL of potassium buffer (0.2 M), and 0.625 mL of potassium ferricyanide [$K_3Fe(CN)_6$], 1%solution were added into each of the test tubes. The test tubes were then placed for 20 min at 50 °C in an incubator to complete the reaction. 0.625 mL of solution of 10% trichloroacetic acid (TCA) was added into each of the test tubes. The mixtures were centrifuged for 10 min at 3000 rpm, and 1.8 mL of the supernatant was collected and mixed with 1.8 mL of distilled water and 0.36 mL solution of 0.1% ferric chloride ($FeCl_3$). The absorbance was then measured against blank at 700 nm using a spectrophotometer. Increased absorbance of the reaction mixture indicated an increased reducing power.

4.5. 1,1-Diphenyl-2-picrylhydrazyl Radical Scavenging Assay. To evaluate the free radical scavenging capacity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used with different extractives, which was previously suggested by[60] with simple modification. 1 mL ethanol solutions of extractives or standard (BHT) at different concentrations such as (5, 10, 20, 40, 80, 160, 320) μg/mL and 2.4 mL of ethanol solution of DPPH were added to each tube. The test tubes were then placed for 30 min at 25 °C in an incubator to complete the reaction. The absorbance was then measured against blank at 517 nm using a spectrophotometer. The following equation was used to calculate the percentage (%) of inhibition (I)

$$\% I = \{(A_0 - A_1)/A_0\} \times 100$$

Here, A_0 = the absorbance of the control and A_1 = the absorbance of the extract/standard. The % of inhibition vs concentration graph provided the IC_{50} value.

IC_{50} is an inhibitory concentration of an antioxidant, where it can scavenge 50% of the DPPH free radicals.

% Scavenging for each concentration was calculated, and a linear regression ($Y = mX + C$) was carried out. Then, % of

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inhibition was plotted against concentration and IC_{50} was calculated from the graph.

Here, m = slope, C = constant, Y = 50 (for IC_{50}), and X = inhibition concentration (μg/mL).

4.6. Experimental Animals. Swiss albino mice 4 weeks old, weighing between 20 and 30 g of both sexes, were propagated and reared in the animal house facility of the department of Pharmacy, Jahangirnagar University, maintaining room temperature of 23 ± 2 °C and at a humidity between 40 and 70%. For maintaining a constant hydration rate, water and food supply were terminated 12 h before the experimentation. All of the experimental animals were treated with proper ethics. The control group consisted of the same number of mice or rats as every treatment group. They are treated in the same ambient environment as the treatment groups with a sufficient supply of the same food and water.

4.7. Acute Toxicity Study. In this study, a limit dose of CEE of 6000 mg/kg was used. Under standard laboratory conditions, the experimental animals received overnight free access to water, except for any kind of food. About 25 Swiss albino mice (25−45 g) were divided into 5 groups and each of the groups consisted of 5 animals. 2 g of extract was dissolved in 10 mL of water and the following doses were administered according to the mice body weight. The first mice group was considered as the control, while the second, third, and fourth groups were considered as tested groups receiving oral extract at doses of 2000, 4000, and 6000 mg/kg, respectively. Before administration, the body weight of the experimental animals was measured to calculate the dose and the animals were observed for 4 h to check for any toxic effects. The animals were then observed further for 3 days to detect any toxic effects such as behavioral changes and other physiological parameters such as body weight, urinations, food intake, water intake, respiration, convulsion, tremor, temperature, constipations, changes in eye and skin colors, and so on. The oral acute toxicity study of the ethanolic extract of WP was executed following OECD (Organization for Economic Co-operation and Development) guideline 423 with simple modification.[61] High dosages of the plant extract were administered to different mice groups to observe the effects of the plant extract on their physiology. The acute toxicity study is a well known method to determine the LD_{50} value of the plant extract.

4.8. Xylene-Induced Ear Edema Test. In this study, a method described by ref 62 was used with some modification for the determination of inflammation activity. Four groups of mice having five mice in each group; the control group was treated with normal water (placebo; 10 mL/kg), standard group (diclofenac sodium; 100 mg/kg), and WP ethanol extract groups at dose 250 mg/kg body weight and 500 mg/kg body weight, respectively. After 1 h, 20 μL of xylene was injected on the anterior and posterior surface of each ear lobe. Every mouse received the same treatment, and the left ear lobe was used as the control. After 1 h of xylene application, the mice were sacrificed and a 3 mm circular section of ear were collected using Cork borer and weighted. Using the weight of the same portion of left ear (without xylene), the percentage of edema (inflammation) was calculated.

Percent inhibition can be calculated using the following formula

$$\%\ \text{inhibition} = \left(1 - \frac{\text{weight of edema (drug/standard)}}{\text{weight of edema (control)}}\right) \times 100$$

4.9. Assessment of Hepatoprotective Activity. *4.9.1. Experimental Animals and Procedure.* In this present experiment, Sprague−Dawley male rats aged 16−24 weeks (250−300 g) were kept at 25 ± 2 °C under a cycle of dark and light for 12 h with a supply of standard food and water. All rats were maintained by following the Institutional Guidelines for the Care and Use of Animals for Scientific purposes as well as the recommendation from the Helsinki Declaration. All the rats were maintained for 4 weeks using doses for the WP that were selected by following slight modification based on those reported in the acute toxicity study. After 1 week of acclimatization period, a total of 25 rats were divided into five groups, and each of the groups consisted of five rats. The grouping of rats was maintained in the following manner.

Group I: Considered as normal control group; only received water (10 mL/kg, per orally (*p.o.*)) once daily for 10 days, Group II: Considered as negative control group; received water (10 mL/kg *p.o.*) and paracetamol (700 mg/kg body weight *p.o.*) daily for 10 days, Group III: Considered as positive control group; received water (10 mL/kg *p.o.*), paracetamol (700 mg/kg body weight *p.o.*) and standard drug silymarin (100 mg/kg body weight *p.o.*), Group IV and V: considered as test group; received paracetamol (700 mg/kg body weight *p.o.*) and WP plant extract at a dose of 250 mg/kg and 500 mg/kg body weight *p.o.*, respectively, for 10 days.

4.9.2. Assessment of Liver Biomarkers. The current study was conducted to measure the hepatoprotective activity of the WP extract biochemically and histopathologically. The animals were sacrificed using ketamine after 7 days of treatment. Blood samples from each of the rats were collected from abdominal blood vessels and stored in a previously labeled centrifuge tube. For the clot formation, these tubes were allowed at a static condition for 30 min at room temperature. The blood samples were then centrifuged at 3000 rpm for 15 min, and serum samples were separated by a micropipette and collected into predefined Eppendorf tubes. Different biochemical markers such as alanine aminotransferase (ALT/SGPT), aspartate aminotransferase (AST/SGOT), albumin, globulin, total protein, and bilirubin were determined from the serum samples by a semiautomatic chemical analyzer named Erba Chem 5 V3, Germany. In the case of histopathological study, the liver was collected and preserved into 10% formalin and then treated with propanol, acetone, xylene, and paraffin to prepare paraffin blocks by the standard microtechnique defined by ref 63. A section of liver, about 5 μm cut with rotary microtome, pigmented with hematoxylin and eosin yellow was then observed under a microscope.

5. CONCLUSIONS

Based on our findings, *W. periplocifolia* exerts significant antioxidant and anti-inflammatory properties along with a significant hepatoprotective effect against paracetamol-induced hepatotoxicity. Furthermore, the present study supports the folk use of *W. periplocifolia* in the treatment of inflammatory conditions and adjuvant therapy for patients suffering from hepatic disorders. The hepatoprotective property of CEE could be related to the in vitro antioxidant activity and bioactive compound content. Even if the positive effect found in this

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study is attributed to polyphenolics contained in CEE, it would be interesting to isolate the individual compounds and explore their contribution to the hepatoprotective activity and underlying mechanism of action. Further toxicological and pharmacokinetic studies are recommended to fully characterize and figure out the extract as a complementary antioxidant, anti-inflammatory, and hepatoprotective drug upon chronic administration before implication of clinical trials.

■ ASSOCIATED CONTENT

Data Availability Statement

The data presented in this study are available in the article

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Notes

The authors declare no competing financial interest.

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