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Research Article

EVALUATION OF IN VITRO AND IN VIVO ANTIOXIDANT POTENTIAL OF THE ETHYL ACETATE FRACTION OF *CYPERUS PANGOREI RHIZOME*

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ABSTRACT

Background and Aim: Antioxidants derived from natural sources, particularly those rich in flavonoids and phenolic compounds, have attracted considerable interest for their potential health benefits in combating oxidative stress-related diseases. This study aimed to evaluate the antioxidant potential of the ethyl acetate (EtAc) fraction obtained from *Cyperus pangorei* rhizome, focusing on both in vitro and in vivo assessments. **Methods:** The EtAc fraction was subjected to RP-HPLC analysis to identify and quantify phenolic compounds and flavonoids. Acute toxicity testing was performed to assess the safety profile of the EtAc fraction. In vitro antioxidant activity was determined using DPPH and ABTS+ radical scavenging assays. In vivo evaluation of antioxidant activity was conducted in Triton X-100-induced oxidative stress model in mice, measuring malondialdehyde (MDA) content, glutathione (GSH) levels, and superoxide dismutase (SOD) activity in liver tissues. **Results:** RP-HPLC analysis confirmed the presence of quercetin, luteolin, and apigenin in the EtAc fraction. The fraction exhibited significant dose-dependent antioxidant activity in both DPPH and ABTS+ assays, with IC50 values indicating potent scavenging effects. In the Triton X-100-induced stress model, administration of the EtAc fraction led to a reduction in MDA levels, restoration of GSH levels, and enhancement of SOD activity in mouse liver tissues. **Conclusion:** The findings suggest that the EtAc fraction from *C. pangorei* possesses promising antioxidant properties, as evidenced by its ability to mitigate oxidative stress-related damage both in vitro and in vivo.

INTRODUCTION

The human body relies on a sophisticated network of enzymatic and non-enzymatic antioxidants to counteract the detrimental impact of free radicals and oxidants [1]. These reactive species are implicated in various diseases, including cancer, cardiovascular issues, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease, alcohol-induced liver disease, ulcerative colitis, aging, and atherosclerosis [2]. In the realm of modern healthcare, natural antioxidants derived from plants, animals, microbes, marine organisms, and other natural sources play a pivotal role in promoting health and addressing critical disease conditions [3].

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Recent times have witnessed a growing interest in exploring the therapeutic potential of medicinal plants as antioxidants to mitigate tissue injuries induced by free radicals [4]. While synthetic antioxidants exist as a means of protection against oxidative stress, their use can be hampered by economic challenges and potential health risks, including increased toxicity and mutagenicity [5]. Consequently, stringent regulations have propelled the search for naturally occurring antioxidants, prompting extensive investigations into various plant species [5].

Cyperus pangorei, a member of the Cyperaceae family, is gaining recognition for its potential as a natural source of antioxidants. This plant, native to India and Southeast Asia, has a long history of traditional use in treating various ailments, including digestive issues, fever, and jaundice [6]. The plant has been subject to investigation for various pharmacological activities, including antidiabetic [7], antioxidant [8], anticancer [9], anti-inflammatory [10],

hepatoprotective [11], and analgesic [12] properties. Notably, *C. pangorei* has garnered attention due to its bioactive constituents, particularly flavonoids and phenolic compounds, distributed across various parts of the plant [13]. These compounds are well-known for their antioxidant potential, attributed to their capacity to scavenge free radicals, inhibit oxidative enzymes, and chelate metal ions [14]. Previous studies have established the antioxidant activity of *C. pangorei* rhizome extract in in vitro settings [8]. This present study pioneers a more comprehensive exploration into its antioxidant potential, focusing on evaluating the in vitro and in vivo antioxidant potentials of the standardized *C. pangorei* rhizome extract.

MATERIALS AND METHODS

Chemicals and Reagents

The phytochemical standards-quercetin (purity ≥95%), luteolin (purity ≥ 98%), and apigenin (purity ≥ 95%)-were obtained from Sigma-Aldrich (St. Louis, MO, USA). Reagents for the assays, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH, 96%), 2,2′-azinobis-3-ethyl benzothiazole-6-sulfonic acid (ABTS, 98%), potassium persulfate (99.5%), Vitamin C (99%), Triton X-100, 2-thiobarbituric acid (2-TBA), 1,1,3,3-tetraethoxypropane (TEP), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT), and ethylenediaminetetraacetic acid (EDTA), were sourced from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA) was procured from HiMedia laboratory, India. All other chemicals and reagents used in this study were of analytical grade.

Experimental Animals

This study used adult Swiss albino mice (25–30 grams), randomly assigned to 5 groups for in vivo antioxidant investigation and 6 groups for acute oral toxicity assessment (n=6 per group). Mice were housed in standard conditions with a 12-hour light/dark cycle, temperature (25 ± 2°C), and humidity (55.6 ± 10%), with access to standard food and water ad libitum. The experimental protocols adhered to Animal Care and Use Committee guidelines and were approved by the Institutional Animal Ethics Committee of CMJ University, Meghalaya, India.

Plant Materials and Extract Preparation

Plant materials for this study were obtained from the rhizome of *C. pangorei*, collected from the Sabong region of the East Midnapore district in West Bengal, India, and authenticated at the Department of Microbiology, CMJ University in Meghalaya, India. The collected rhizome was air-dried and crushed into a fine powder consistency. Approximately 500 g of this powdered material was subjected to extraction with 70% ethanol using the cold maceration method over 15 days at 25°C with intermittent shaking. The resulting extract was filtered, evaporated under reduced pressure using a vacuum rotary evaporator at 45ºC, and then further dried using a lyophilizer, yielding 53.68 g of extract (10.74%, w/w). This extract underwent solvent-solvent partitioning, where the desiccated extract was reconstituted in water, heated to eliminate waxy constituents, and then partitioned successively with n-hexane, chloroform, and ethyl acetate. The remaining aqueous portion was dried to yield the aqueous fraction. Phytochemical analysis (total phenolics and flavonoids) and preliminary DPPH assay demonstrated that the ethyl acetate (EtAc) fraction displayed the most potent antioxidant activity, accompanied by the highest phenolic and flavonoid contents among the various fractions.

Chromatography (RP-HPLC) and Standardization

The RP-HPLC system (Waters, Milford, USA) used in this study featured a 600-controller pump, a UV-Vis detector with multiple-wavelength detection capabilities, a Rheodyne 7725i injector with a 20 µl loop, and integrated Empower2 integration software. Chromatographic separation employed a Luna C18 (2) 100 Å, 250 x 4.6 mm column packed with 5 mm particles (Phenomenex, Torrance, USA). The chromatographic conditions and standardization of the EtAc fraction followed the method described by Chen et al. [15]. The mobile phase comprised 0.1% formic acid, acetonitrile, and methanol, with a column temperature of 30°C, a flow rate of 1 mL/min, and a total run time of 45 min. Analysis was performed at a wavelength of 350 nm, with an injection volume of 20 µL. Quantification of luteolin, apigenin, and quercetin in the EtAc fraction relied on their respective calibration curves, and identification was based on retention time (RT) comparison between standards and the EtAc fraction.

In Vitro Antioxidant Study

DPPH assay

The in vitro antioxidant potential of the EtAc fraction was assessed through the DPPH assay following the method of Brand-Williams et al. [16]. Various concentrations (5, 10, 20, 40, and 80µg/mL) of the EtAc fraction and vitamin C were prepared in analytical-grade methanol. Each concentration (0.5mL) was mixed with 3mL of methanol and 0.3mL of a 0.5mM DPPH solution in methanol. Five concentrations (5, 10, 20, 40, and 80µg/mL) of the EtAc fraction and vitamin C were prepared in analytical grade methanol. In separate test tubes, 0.5mL of each concentration of the EtAc fraction and vitamin C was mixed with 3mL of methanol and 0.3mL of a 0.5mM DPPH solution in methanol. After thorough mixing, the reaction mixtures were incubated in the dark at room temperature for 30 minutes. The mixture of methanol (3.3mL) and sample (0.5mL) was served as blank. The control solution was prepared by mixing methanol (3.5mL) and DPPH radical solution (0.3mL). The experiments were conducted in triplicate. Absorbance

values were measured at 517nm using a spectrophotometer, and the DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ DPPH radical scavenging activity} = \frac{Ac - As}{Ac} \times 100$$

Where, As represents the absorbance of the sample, while Ac signifies the absorbance of the control.

ABTS+ radical scavenging assay

The antioxidant property of the EtAc fraction obtained from *C. pangorei* was evaluated using the ABTS+ radical cation scavenging assay. The assay was performed as described by Zheleva-Dimitrova et al. [17]. The ABTS+ radical cation was generated by mixing 7mM of ABTS with 2.45mM of potassium persulfate ($K_2S_2O_8$). The mixture was incubated in darkness at room temperature. To assess the scavenging ability of the EtAc fraction towards the ABTS radical cation, 3mL of the ABTS+ solution was mixed with 0.2mL of different concentrations (5, 10, 20, 40, and 80µg/mL) of the EtAc fraction. Ascorbic acid was used as a reference. The reaction mixture was left at room temperature for 6 minutes. The absorbance of the cuvettes was measured at 734 nm. Methanol was used as the blank, and the ABTS+ solution was used as the control. The percentage inhibition was determined using the following formula:

$$\% \text{ Inhibition } = \frac{(\text{OD of control} - \text{OD of sample})}{\text{OD of control}} \times 100$$

Where, OD is the optical density.

Acute toxicity study

An acute oral toxicity study adhering to OECD guidelines 423 [18] was conducted on Swiss albino mice. Various doses (50, 100, 200, 400, 800, and 16000 mg/kg body weight) of the EtAc fraction dissolved in aqueous solution were administered to mice following an overnight fast. Each group comprised 6 animals. Immediate signs of toxicity were closely monitored during the first 3 hours, followed by an additional 24 hours to detect any mortality. The animals were observed for an additional 14-day period to evaluate the presence of delayed toxicity.

***In Vivo* Antioxidant Activity**

Experimental protocol and doses

The experimental doses for the EtAc fraction were established through an acute toxicity study. To assess the in vivo antioxidant activity, a mouse model of triton X-100 induced oxidative stress, as outlined by Ghule et al. [19], was utilized. EtAc fraction concentrations were prepared using a 0.9% NaCl normal saline solution and administered over a 7-day period as follows:

- Group I (normal control): Received 0.2 mL distilled water (p.o) 30 minutes before intraperitoneal (i.p.) injection of normal saline (2.5 mL/kg body weight).
- Group II: Given 0.2 mL distilled water (p.o) 30 minutes before i.p. injection of Triton X-100.
- Group III: Administered 50 mg/kg/day of EtAc fraction (p.o) 30 minutes before i.p. injection of Triton X-100.
- Group IV: Administered 100 mg/kg/day of EtAc fraction (p.o) 30 minutes before i.p. injection of Triton X-100.
- Group V: Administered 200 mg/kg/day of EtAc fraction (p.o) 30 minutes before i.p. injection of Triton X-100.
- Group VI: Given 27.47 mg/kg/day of vitamin C (p.o), equivalent to a human dose of 250 mg/kg, 30 minutes before i.p. injection of Triton X-100.

Preparation of Tissue Homogenates

Liver samples were dissected and promptly washed with ice-cold saline to eliminate excess blood. Liver homogenates (5% w/v) were prepared in cold 50 mmol/L potassium phosphate buffer (pH 7.4) using a tissue homogenizer. The cell debris was removed by centrifugation at 5000 r/min for 15 min at 4 °C using refrigerated centrifuge [20]. The supernatant was used for the estimation of malondialdehyde (MDA), reduced glutathione (GSH) levels and superoxide dismutase (SOD) activity.

Measurement of MDA

The thiobarbituric acid reactive substance (TBARS) assay is a well-established method used to assess lipid peroxidation, a crucial indicator of oxidative stress. This assay quantifies TBARS produced during lipid peroxidation. In this study, the quantification of MDA content followed a spectrophotometric method elucidated by Colado et al. [21]. The assay involved the preparation of a composite mixture comprising 500 µL of tissue homogenate supernatant blended with phosphate buffer (pH 7.4), along with 300 µL of 30% w/v TCA, 150 µL of 5M HCl, and 300 µL of 2-TBA (2%, w/v). This mixture was incubated for 15 minutes at 90°C, followed by centrifugation at 12,000 x g for 10 minutes. The resulting pink-colored supernatant was analyzed spectrophotometrically at 532 nm to determine MDA concentration, using a standard curve generated with 1,1,3,3-TEP.

Measurement of GSH

To quantify the levels of GSH, it was reacted with 5,5'-dithiobis (2-nitrobenzoic acid) (commonly known as Ellman's reagent), resulting in the formation of a yellow chromophore. The absorbance of this chromophore was then measured using a spectrophotometer, following the method outlined by Ellman [22]. For the assay procedure, equal volumes of liver homogenate and a 10% TCA solution were combined and subjected to centrifugation at 2000 x g for 10 minutes at 4°C. The assay sample comprised 0.1

mL of the resulting supernatant, 2 mL of phosphate buffer at pH 8.4, 0.5 mL of DTNB, and 0.4 mL of double-distilled water. After thorough mixing using a vortex mixer, the absorbance of the assay mixture was measured at 412nm within 15 minutes. GSH concentration was calculated using a standard curve of known GSH concentrations.

Measurement of SOD activity

The SOD activity in the liver homogenate was assessed following the method described by Okado-Matsumoto and Fridovich[23]. This method utilizes spectrophotometric analysis to measure the inhibition of NBT reduction within the nonenzymatic phenazine methosulfate-nicotinamide adenine dinucleotide system. A reaction mixture was prepared, consisting of 0.1 M phosphate buffer (pH 7.8), 0.41mM NBT, 0.33 mM EDTA, 0.01 mM PMS (phenazine methosulfate), and 0.8mM NADH. The reaction was initiated by adding a specific quantity of liver homogenate to this mixture. Optical density at 540 nm was measured using a spectrophotometer to determine the initial extinction. After 5 min, the optical density was recorded again to ascertain the extinction after 5 min. SOD activity was expressed as U/mg protein, where U represents a relative unit of activity defined as the amount of SOD required to inhibit NBT reduction by 50%, normalized to a 1 mg protein sample.

Statistical Analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests using Graph Pad Prism 5.0 statistical software. Data are expressed as means ± SEM, and a significance threshold of $P < 0.05$ was applied to determine statistical significance.

RESULTS

RP-HPLC Analysis

RP-HPLC chromatogram of EtAc fraction showed sharp peaks for the presence of quercetin, luteolin, and apigenin at retention times (RT) of 17.33 ± 0.15, 18.97 ± 0.17, and 32.13 ± 0.13 min, respectively (Figure 1). From the calibration curves, the contents of quercetin, luteolin, and apigenin in the EtAc fraction were found to 2.89 ± 0.51%, 1.89 ± 0.17%, and 3.85 ± 0.19%, respectively.

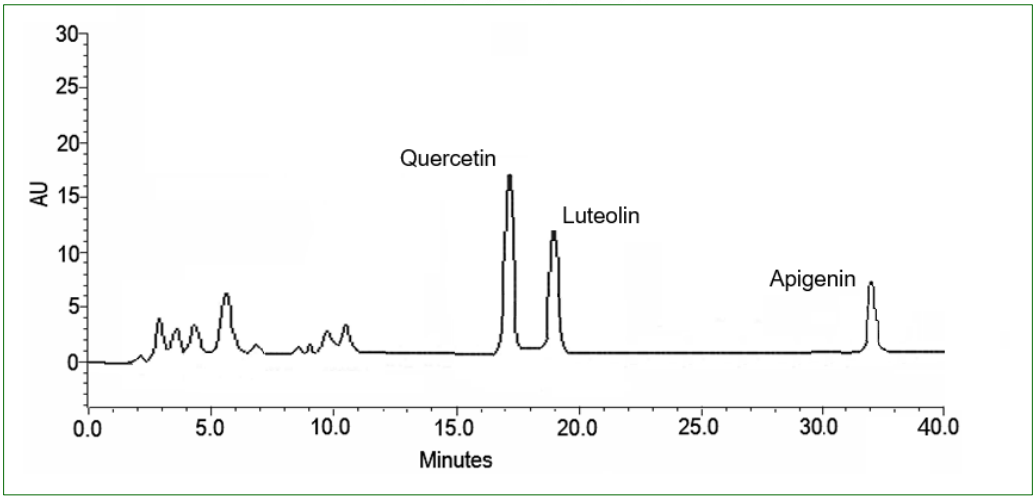

Figure 1: RP-HPLC chromatogram of EtAc fraction of *C. pangorei* rhizome

Acute Toxicity

No mortality or signs of toxicity were observed in mice orally administered with the EtAc fraction at doses up to 1600 mg/kg body weight during the 14-day study period. However, additional dosing was not conducted to determine the LD_{50} value.

DPPH Radical Scavenging

The Figure 2a illustrates the DPPH free radical scavenging activity of the EtAc fraction. The EtAc fraction exhibited a remarkable dose-dependent DPPH scavenging effect, with an IC_{50} value of 4.21 μg/mL (Figure 2b). Particularly, at a higher concentration (80 µg/mL), the extract demonstrated the highest percentage of DPPH radical scavenging activity (62.46 ± 1.27%, $P \leq 0.001$). In this study, vitamin C, employed as a reference antioxidant, also exhibited a dose-dependent radical scavenging capacity with an IC_{50} value of 2.35 μg/mL, consistently demonstating superior DPPH radical scavenging activity across all dosage levels.

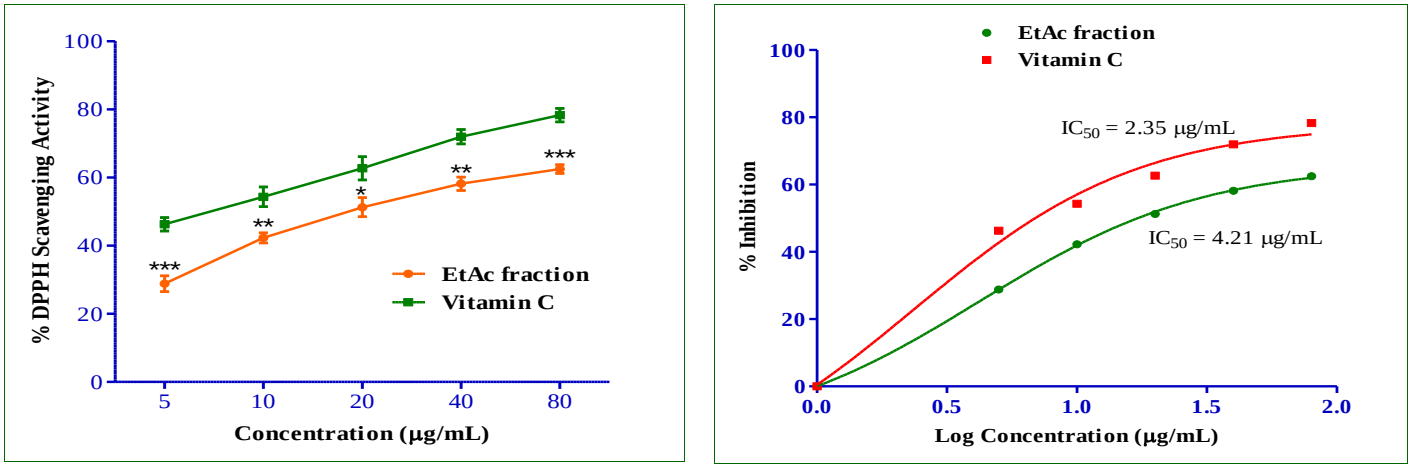

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Figure 2: (a) DPPH radical scavenging activity, and (b) the IC_{50} values of EtAc fraction and vitamin C. The presented data represents the mean ± SD from three independent experiments. Statistical comparison: Vitamin C vs. EtAc fraction (*P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05).**

ABTS+ Radical Scavenging

The results of the ABTS scavenging activity for both the EtAc fraction and vitamin C is depicted in Figure 3a. Overall, both the EtAc fraction and the reference standard (vitamin C) displayed substantial and dose-dependent radical scavenging activities. The IC_{50} values for the ABTS free radical scavenging capacity were determined to be 2.14 µg/mL for the EtAc fraction and 1.79 µg/mL for vitamin C, as shown in Figure 3b. At a higher concentration (80 µg/mL), the EtAc fraction exhibited an ABTS free radical scavenging capacity of 59.21 ± 2.97%, while vitamin C showed a capacity of 66.39 ± 4.97%.

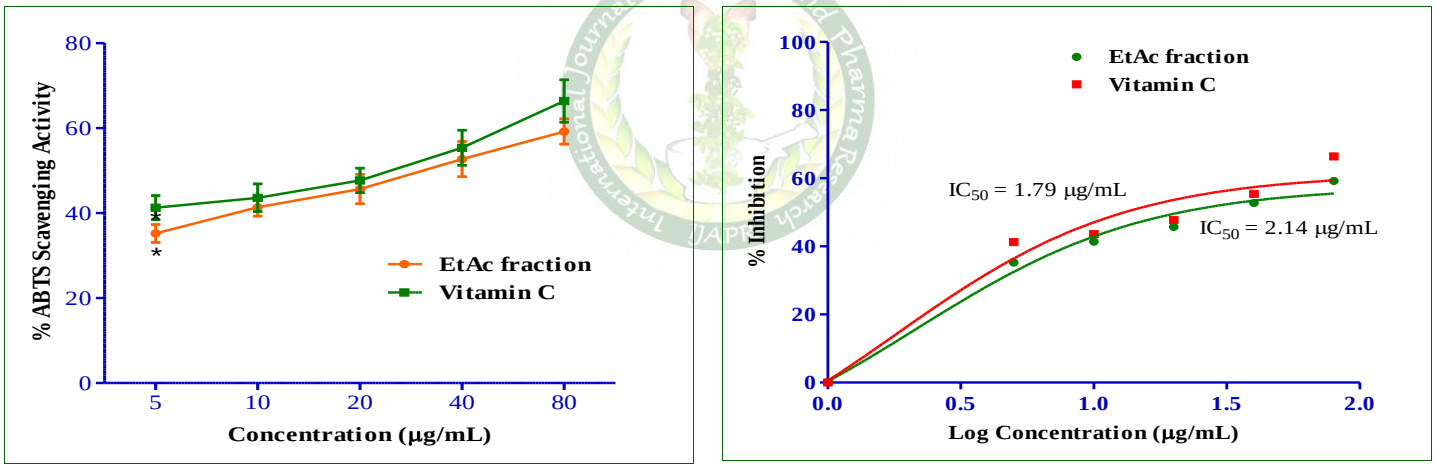

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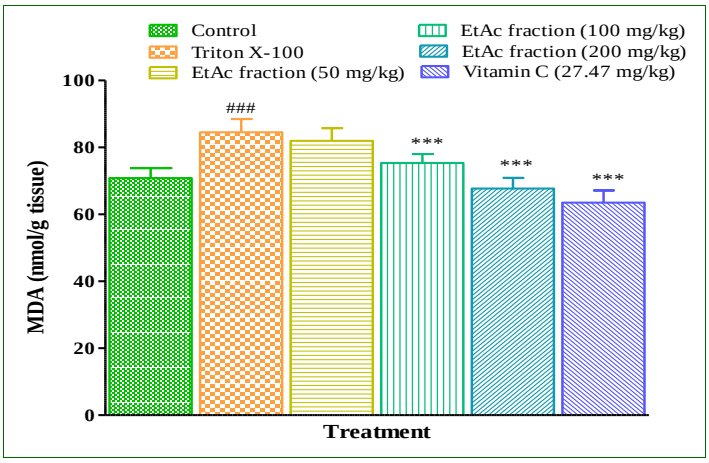
Figure 3: (a) ABST radical scavenging activity, and (b) IC_{50} values of EtAc fraction and vitamin C. The presented data represents the mean ± SD from three independent experiments. Statistical comparison: Vitamin C vs. EtAc fraction (*P ≤ 0.05).

MDA Content

The study assessed the impact of the EtAc fraction on MDA content in the livers of Triton X-100-induced stressed mice (Figure 4a). Triton X-100 induced a significant increase in MDA levels compared to the control group (84.54 ± 3.96 nmol/g tissue vs. 70.87 ± 2.94 nmol/g tissue, respectively; $P < 0.001$). However, treatment with EtAc fraction at doses of 100 mg/kg (75.335 ± 2.71 nmol/g tissue) and 200 mg/kg (67.74 ± 3.14 nmol/g tissue), b.w., as well as Vitamin C at 27.47 mg/kg (63.53 ± 3.63 nmol/g tissue), b.w., demonstrated a significant reduction in MDA levels compared to the Triton X-100 group ($P < 0.001$ for all comparisons). These results highlight the potential antioxidative effect of EtAc fraction and Vitamin C in mitigating oxidative stress induced by Triton X-100 in mouse liver tissues, suggesting their promising role as therapeutic agents against oxidative stress-related liver damage.



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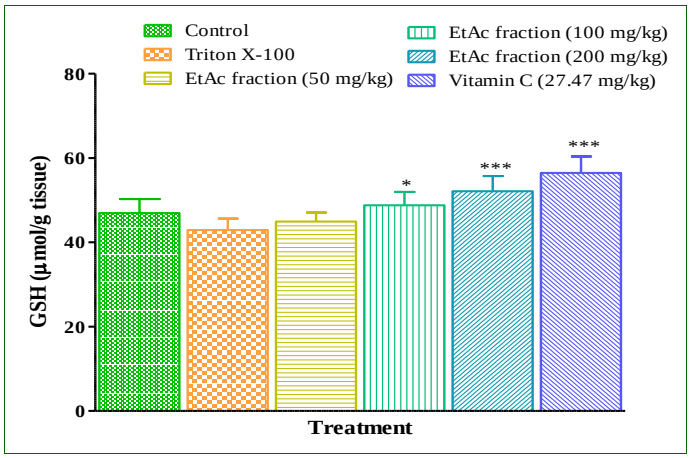

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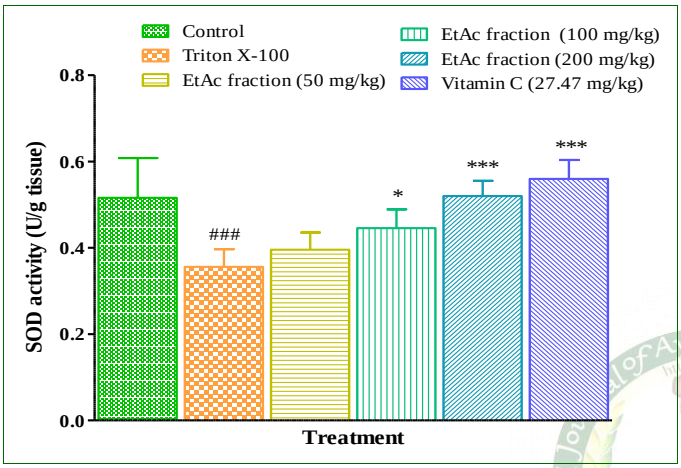

c

Figure 4: Effects of EtAc fraction on (a) MDA content, (b) GSH content and (c) SOD activity in the liver of mice treated with Triton X-100. Each bar represents mean ± SEM (n = 6). Statistical analysis was done through One-way ANOVA followed by Dunnett's multiple comparison test. Statistically significant differences compared as: Control Vs Triton X-100 (###$P < 0.001$); Triton X-100 Vs different EtAc fraction concentration and Vitamin C treated groups (*$P < 0.05$ and ***$P < 0.001$).

GSH Content

The study investigated the impact of EtAc fraction on GSH levels in the livers of Triton X-100-treated mice. The negative control, Triton X-100, exhibited a significant reduction in GSH content (42.915 ± 2.71 µmol/g tissue, $P < 0.001$) compared to the control group (46.95 ± 3.32 µmol/g tissue). Administration of EtAc fraction at doses of 100 mg/kg and 200 mg/kg, as well as Vitamin C, increased GSH levels significantly, indicating potential protection against Triton X-100-induced oxidative stress (Figure 4b). However, the lower EtAc fraction dose (50 mg/kg) did not significantly alter GSH content compared to Triton X-100-treated mice. These results suggest a dose-dependent response of EtAc fraction, with higher concentrations exhibiting stronger antioxidant effects against Triton X-100-induced decrease in liver GSH levels.

SOD Activity

This study also investigated the effect of EtAc fraction on SOD enzyme activity in Triton X-100-induced oxidative stress in mice liver. Compared to the control group, the negative control group subjected to stress showed a significant reduction in SOD activity (0.355 ± 0.041 U/mg protein vs. 0.515 ± 0.093 U/mg protein, $p < 0.001$). Treatment with EtAc fraction at 50 mg/kg did not significantly improve SOD activity. However, at higher doses of 100 mg/kg ($p < 0.05$) and 200 mg/kg ($p < 0.0001$), EtAc fraction significantly restored SOD activity (0.445 ± 0.043 U/mg protein and 0.52 ± 0.035 U/mg protein, respectively), suggesting a potential dose-dependent protective effect against oxidative stress (Figure 4c). Moreover, the positive impact of EtAc fraction at 100 mg/kg and 200 mg/kg was comparable to that of Vitamin C at 27.47 mg/kg (0.56 ± 0.044 U/mg protein). These findings indicate that EtAc fraction may possess antioxidant properties, particularly at higher concentrations.

DISCUSSION

In recent years, antioxidants, particularly those abundant in flavonoids and phenolic compounds, have become focal points of attention owing to their potential health benefits and preventive applications against diseases [14]. Flavonoids and phenolic compounds, commonly occurring in plants, are recognized for their proficiency in counteracting oxidative stress-an outcome of the imbalance between the production of reactive oxygen species (ROS) or free radicals and the body's capacity to neutralize them [14]. Oxidative stress is implicated in cellular damage, DNA mutations, and the onset of various chronic diseases, including cardiovascular disorders, neurodegenerative conditions (such as Alzheimer's and Parkinson's

disease), and cancer [24]. Medicinal plants, being rich reservoirs of essential metabolites, hold promising potential for activities such as antioxidant, antimicrobial, anti-inflammatory, and anticancer effects [25]. Investigating antioxidants from natural sources, particularly plants, thus presents a significant avenue for exploration. Phytochemical constituents, including flavonoids and phenolic compounds found in diverse plant species, have undergone extensive scrutiny for their efficacy in combatting oxidative stress and associated disorders.

This study delves into the antioxidant potential of the standardized EtAc fraction from *C. pangorei* rhizome. Previous research has highlighted the presence of bioactive constituents, notably flavonoids and phenolic compounds, in various parts of this plant [26]. In our analysis, RP-HPLC confirmed the presence of quercetin, luteolin, and apigenin in the EtAc fraction, with subsequent quantification revealing their respective concentrations. Past studies have attributed these compounds to their ability to scavenge free radicals, inhibit oxidative enzymes, and chelate metal ions. Notably, quercetin has shown radical-scavenging activity and metal-chelating abilities, contributing to its antioxidative effects [27]. Luteolin has demonstrated anti-inflammatory and antioxidant activities, suggesting its potential in alleviating oxidative stress-induced damage [28]. Apigenin, another flavonoid, has been associated with antioxidant and anti-inflammatory effects, implying its role in shielding cells against oxidative damage [29].

The current investigation primarily focuses on evaluating the antioxidant potential of the EtAc fraction from *C. pangorei*, emphasizing both *in vitro* and *in vivo* assessments. *In vitro* assays, including the DPPH and ABTS+ radical scavenging assays, provided valuable insights into the dose-dependent antioxidant activities of the fraction, highlighted by notable IC_{50} values. Subsequently, the study extended its scope to an *in vivo* model, utilizing a Triton X-100-induced stress paradigm in mice. The EtAc fraction demonstrated significant antioxidant effects in the *in vivo* study, evident through the reduction in MDA levels, restoration of GSH levels, and enhancement of SOD activity in stressed mouse liver tissues. These findings underline the potential therapeutic efficacy of the EtAc fraction in mitigating oxidative stress-related damage at the organ level. The acute toxicity study indicated a favourable safety profile for the EtAc fraction, paving the way for future exploration of its clinical applications.

CONCLUSION

In conclusion, the EtAc fraction from *C. pangorei* shows promise as a natural antioxidant, demonstrating significant potential for therapeutic interventions against oxidative stress-related conditions. The comprehensive in vitro and in vivo assessments, along with the absence of acute toxicity, establish a strong foundation for further exploration and development of the EtAc fraction as a potential candidate for clinical applications in antioxidant-based therapies.

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