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Research Article

Assessment of Antioxidant and Hepatoprotective Effects of *Kappaphycus alvarezii* (Doty) Doty ex Silva Against Carbon Tetrachloride-Induced Liver Injury in Rats

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ABSTRACT

Kappaphycus alvarezii (Doty) Doty ex Silva, commonly known as red algae, holds economic significance as a primary source of κ-carrageenan, which exhibits promising medicinal and therapeutic properties. This study aims to assess the antioxidant potential as well as hepatoprotective activity of the ethanolic extract of *K. alvarezii* (EEKA). The assays utilised to determine the antioxidant properties of EEKA were total phenolic content (TPC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging. In addition, the ability of EEKA to ameliorate experimentally induced liver injury in Sprague-Dawley rats caused by carbon tetrachloride (CCl_4) was evaluated. The biochemical assays involved measuring liver marker enzymes (AST and ALT) in serum as well as determining the levels of reduced glutathione (GSH), lipid peroxidation (LPO) via malondialdehyde, catalase (CAT), and glutathione S-transferase (GST) in liver homogenates. The TPC and DPPH radical scavenging activity of EEKA demonstrated relatively low antioxidant properties compared to standard references. However, CCl_4-induced groups exhibited significantly increased levels of AST and ALT, along with depletion of antioxidant status (GSH, CAT, and GST) indicated by LPO. Pretreatment with EEKA resulted in slightly decreased liver marker enzyme activity and LPO, coupled with an increase in antioxidant status. These findings suggest that EEKA contains active principles capable of counteracting the hepatotoxic effects induced by CCl_4.

Keywords: *Kappaphycus alvarezii*; antioxidant; hepatoprotective; carbon tetrachloride; oxidative stress.

INTRODUCTION

Seaweed is a type of macroalgae that includes both cultivated and wild types. It is divided into three groups: brown algae (Phaeophyta), green algae (Chlorophyta), and red algae (Rhodophyta) (Araujo & Peteiro, 2021). Brown and red algae are frequently utilised as food sources, being rich in carbohydrates and low in fat, akin to vegetables like lettuce and celery, and serving as effective roughage agents (Peñalver et al., 2020). Aware et al. (2022) reported that 100 g of algae provides ample daily human vitamin requirements, including vitamins A, B2, and B12, and a significant portion of vitamin C. Natural seaweed compounds or extracts

offer milder effects compared to synthetic drugs like butylated hydroxyanisole and butylated hydroxytoluene. Around 500 species of seaweed have been utilised by humans for agricultural, culinary, and chemical purposes. Notably, red seaweeds such as *Kappaphycus*, *Eucheuma*, *Gracilaria*, as well as brown kelps such as *Laminaria* and *Undaria*, are cultivated in aquaculture for human consumption or gelling compound extraction (Leandro et al., 2020). Significantly, seaweed production saw a notable surge from 2000 onwards, reaching 174,100 tonnes in 2018, indicating an average annual growth from 16,100 tonnes in 2000 (FAO, 2020).

Kappaphycus, a unicellular red alga belonging to the genus Rhodophyta, is a marine macroalgae within the Rhodophyceae class. Specifically, *Kappaphycus alvarezii* (Doty) Doty ex Silva, previously referred to as *Eucheuma cottonii*, is categorised under the order Gigartinales, which comprises significant phycocolloid producers primarily found in the families Furcellariaceae, Gigartinaceae, and Solieriaceae (Rupert et al., 2022; Jalal et al., 2023). Notably, certain members of Gigartinales are highly sought after commercially due to their unique properties. *K. alvarezii* has been extensively employed due to its notable nutritional attributes. This extensive analysis focuses on the most recent publications concerning the pharmacological and phytochemical properties of *K. alvarezii*, which demonstrate its prospective utility in the nutraceutical, cosmeceutical, and pharmaceutical sectors. *K. alvarezii* is rich in various phytochemical constituents, such as lectins, phenolics, flavonoids, alkaloids, terpenoids, phytosterols, and the polysaccharide carrageenan, all of which potentially contribute to its pharmacological activity. *K. alvarezii* has been shown to possess antioxidant anti-inflammatory, antibacterial, antiviral, antifungal, anticancer, and antidiabetic properties in both in vivo and in vitro studies (Jalal et al., 2023). Because of its phytochemical and pharmacological attributes, *K. alvarezii* holds economic importance as a primary producer of κ-carrageenan (Bulboa Contador et al., 2020). Research has highlighted the potential of *K. alvarezii* in protecting against liver disease, attributed to its rich antioxidant content, particularly phenolics. These antioxidants have shown promise in shielding the liver from oxidative stress, a critical factor in liver cirrhosis (Sekar & Chandramohan, 2008; Shihab et al., 2023).

Despite this, there is still a scarcity of studies examining the antioxidant activity of *K. alvarezii* concerning oxidative stress and antioxidant enzymes in hepatic tissues. Bridging the gap between acknowledging antioxidant activity and demonstrating its therapeutic impact, particularly in liver health, necessitates further investigation. Thus, the aim of this study is to assess the antioxidant properties of the ethanolic extract of *K. alvarezii* (EEKA) and their potential impact on liver disease, particularly through experimentation involving carbon tetrachloride (CCl_4)-induced liver damage in rats. The current study seeks to address the knowledge deficit by conducting a thorough investigation into the potential therapeutic advantages of EEKA in mitigating oxidative stress-induced liver disease induced by CCl_4. The objective of this undertaking is for a scholarly contribution towards the realm of enhanced liver disease treatment modalities.

MATERIALS AND METHODS

Laboratory supplies

Ethanol, ascorbic acid, gallic acid, sodium carbonate (Na_2CO_3), Folin-Ciocalteu reagent, CCl_4, 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, 2,4-dinitrophenylhydrazine (DNPH), α-ketoglutarate, L-aspartate, L-alanine, and other biochemical assays were obtained from Sigma-

Aldrich (Burlington, MO, USA). The chemicals and reagents that were used have been selected for their exceptional purity.

The equipment used in this study were obtained from the Biotechnology Research Institute, Universiti Malaysia Sabah, Sabah, Malaysia. It included a freezer (Thermo Fisher Scientific, Waltham, MA, USA), rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland), Soxhlet extractor (Bionics Scientific, Delhi, India), freeze dryer (Labconco, Kansas City, MO, USA), spectrophotometer (PerkinElmer, Waltham, MA, USA), refrigerated centrifuge (Beckman Coulter, Brea, CA, USA), and homogeniser (Kinematica AG, Malters, Switzerland).

Sample preparation

Whole *K. alvarezii* samples (Figure 1) were collected in July 2012 from the Mini Estate Sejahtera of Sebatik Island in Semporna, Sabah, Malaysia. Their identification and authentication were done by Assoc. Prof. Dr. Wilson Thau Lym Yong from the Biotechnology Research Institute, Universiti Malaysia Sabah. The samples (voucher no.: GS004) were placed in the plant tissue culture laboratory at the same institute. Upon collection, the samples underwent washing with tap water to eliminate salt, epiphytes, and debris (Sivasankari et al., 2006). Subsequently, they were rinsed in distilled water. Following the cleaning process, the raw seaweeds were shade-dried for 72 h.

Figure 1: Sample of *K. alvarezii* obtained from the Mini Estate Sejahtera on Sebatik Island.

Sample extraction

Extraction followed the traditional method outlined by Jinoni et al. (2024) with slight alteration. The extraction process involved the use of a Soxhlet extractor, followed by the removal of ethanol solvent using a rotary evaporator, and freeze-drying to eliminate water content and to obtain the crude extract. Prior to extraction, the samples were homogenised into fine powder using a conventional blender and stored at –20 °C. Approximately 60 g of powdered samples were weighed and transferred into a beaker. Soxhlet ethanolic extraction was performed using 20 g of the sample in 100 mL of 95% ethanol at 78 °C. The rotary evaporator was employed to concentrate the resulting extract. Subsequently, the extracts were transferred into containers, securely sealed, and preserved in a freezer at –80 °C for a duration of 48 h. To facilitate lyophilisation, the tubes were sealed with parafilm, punctured with small holes using a toothpick, and subjected to freeze-drying at –80 °C for 48 h. The resulting powdered outcome was stored in room temperature until further analysis.

Total phenolic content

The determination of total phenolic content (TPC) followed a protocol similar to that described by Awang et al. (2023) with some modifications. Gallic acid concentrations (10–200 µg/mL) were prepared from a 1 mg/mL stock. Folin-Ciocalteu reagent (1.5 mL) was added, followed by incubation at room temperature for 5 min. Na_2CO_3 solution (1.5 mL) was then added, and the mixture was left in the dark for 90 min. Absorbances were measured at 720 nm, and results were expressed as mg GAE/g dry extract.

DPPH assay

The DPPH method, as outlined by Jinoni et al. (2024) with slight modifications, was used to assess the free radical scavenging activity of EEKA. Stock solutions of the sample (5 mg/mL) were prepared, from which eight concentrations (10–2400 µg/mL) were obtained. Each tube contained 2.7 mL of DPPH solution. After vortexing and incubating for 60 min at room temperature, absorbances at 517 nm were measured using a spectrophotometer. Ascorbic acid served as the positive control. The DPPH scavenging capacity of the extract was determined using Equation (1).

$$\text{DPPH free radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

where $A_{control}$ represents the absorbance of the solution without the extract, and A_{sample} represents the absorbance of the extract mixed with the DPPH solution. The results were converted to IC_{50} values (representing the sample concentration necessary to impede 50% of DPPH radicals) through the process of extrapolating the regression analysis.

Experimental procedure

The animal experiment was carried out in adherence to the ethical guidelines of the university and federal laws governing animal experimentation. The Animal Ethics Committee granted approval for the study under protocol number UMS/IP7.5/M3/4-2012. For the investigation, male Sprague-Dawley rats aged 7 to 8 weeks, with a body weight ranging from 150 to 250 g, were chosen based on their good health. Before commencing the experiments, each animal was subjected to a period of acclimation to laboratory conditions. The animals were accommodated in a regulated setting within the animal housing facility of Biotechnology Research Institute, Universiti Malaysia Sabah. Throughout the study, the rats were provided with access to tap water and rodent chows to ensure their well-being and nutritional needs were met.

The experimental animals were categorised into four groups (n = 6), as indicated in Table 1. In Group I, animals were administered normal saline for a period of 14 days. Group II received normal saline for 14 days, followed by the administration of CCl_4 in corn oil (1:1) on the 13th and 14th days. Group III and Group IV were administered EEKA at doses of 150 mg/kg b.wt. and 300 mg/kg b.wt., respectively, for 14 consecutive days, followed by the administration of CCl_4 in corn oil (1:1) on the 13th and 14th days. All administrations were carried out orally using oral gavage via force feeding.

Table 1: Summary of experimental procedure.

Group	Treatment	Period
I	Normal saline, p.o., normal group	14 days
II	Normal saline, p.o. + CCl_4 (1.0 mg/kg b.wt.), p.o., control group	14 days
III	EEKA (150 mg/kg b.wt.), p.o. + CCl_4 (1.0 mg/kg b.wt.), p.o., pretreatment	14 days
IV	EEKA (300 mg/kg b.wt.), p.o. + CCl_4 (1.0 mg/kg b.wt.), p.o., pretreatment	14 days

Each animal that had been treated was sacrificed 24 h after the final administration of EEKA or hepatotoxin inducers. Following the induction of anaesthesia with mild ether, the subject was decapitated. Plasma samples were isolated from blood samples extracted via cardiac puncture into tubes coated with lithium heparin. Following the excision, connective tissue was removed from the liver, and it was cleansed with saline to eliminate any potential blood contamination. Subsequently, the liver tissue was stored in a freezer at –80°C for biochemical analyses to assess the function of hepatic antioxidant enzymes.

Estimation of serum ALT and AST

Blood serum, collected through centrifugation for 15 min at 1,500 rpm and kept at –20 °C for ALT and AST analysis, underwent separate enzymatic assays. For ALT, 0.5 mL of 200 mM L-alanine and 2 mM α-ketoglutarate were combined, incubated at 37 °C for 10 min. Afterward, 0.1 mL of serum was introduced, volume adjusted using sodium phosphate buffer to 1.0 mL, and left to incubate for 30 min. For AST, a similar process was followed using 200 mM L-aspartate and 2 mM α-ketoglutarate with a 60-min incubation. Afterward, 1 mM DNPH was added, incubated for 20 min, and the resulting colour change was measured after 30 min at 510 nm (Reitman & Frankel, 1957).

Preparation of post-mitochondrial supernatant

The tissue fractionations utilised in this study were prepared in accordance with standard procedure, which was modified from the approach outlined by Mohandas et al. (1984) and as described by Iqbal et al. (1999). About 1.17% KCl (w/v) was added to a chilled phosphate buffer (0.1 M, pH 7.4) in which the liver tissue was pulverised with a homogeniser. After centrifugation in a refrigerated centrifuge at 2,000 rpm at 4 °C for 10 min, the remnants of the cell nucleus were separated from the liver homogenate. In order to obtain the post-mitochondrial supernatant (PMS), a 30-minute centrifugation at 10,000 rpm and 4 °C was performed. The reduced glutathione (GSH), lipid peroxidation (LPO), catalase (CAT), and glutathione S-transferase (GST) activities were all determined using this PMS.

Estimation of hepatic enzyme activities

The procedure utilised to measure GSH was that of Jollow et al. (1974). At 412 nm, the absorbance was ascertained utilising a spectrophotometer. The outcomes were quantified and expressed in µmol reduced GSH/g tissue. The procedure for determining the malondialdehyde (MDA) content, which serves as an indicator of LPO, was delineated by Iqbal et al. (1999): the utilisation of thiobarbituric acid reacting substances (TBARS). At 535 nm, a spectrophotometer was utilised to determine the optical density of the supernatant, which directly correlated with the amount of MDA generated in each sample. The findings were measured as nmol MDA formed/g tissue. The CAT activity was measured in accordance with the procedure outlined by Thanebal et al. (2021) and Claiborne (1985). At 240 nm, the changes in absorbance of the solution in the reaction mixture were detected with a spectrophotometer, and the activity was computed as nmol H_2O_2 consumed/min/mg protein. The GST activity was determined utilising the methodology outlined by Habig et al. (1974) and Athar & Iqbal (1998). The absorbance

was quantified utilising a spectrophotometer at 340 nm, and the catalytic activity was determined as nmol CDNB conjugate formed/min/mg protein.

Statistical analysis

The results were presented using mean ± standard deviation (SD). To facilitate comparisons, a one-way analysis of variance (ANOVA) was employed, followed by the application of Tukey's Honestly Significant Difference (HSD) test. Data analysis was conducted using IBM SPSS Statistics v. 17.0. Establishing a significance level of p-value < 0.05 was performed.

RESULTS

Effect of EEKA on TPC and DPPH radical scavenging activity

The TPC in EEKA was found to be 18.92 ± 0.31 mg GAE/g dry extract, indicating relatively low concentrations of TPC. Additionally, the DPPH assay of EEKA was evaluated at concentrations ranging from 150 to 5000 µg/mL. The IC_{50} values for DPPH radical scavenging activity were determined as follows: ascorbic acid (75.00 ± 0.84 µg/mL) and EEKA (4150.00 ± 0.41 µg/mL), indicating lower antioxidant properties.

Effects of EEKA on serum ALT and AST

The assessment of liver structural integrity involves determining the activities of aminotransferases (ALT and AST). In comparison to the normal group, the serum levels of AST and ALT increased significantly ($p < 0.05$) by 53% and 68%, respectively, in the control group, as shown in Figure 2. For 150 mg/kg b.wt., no significant differences ($p > 0.05$) occurred compared to CCl_4, with only a 7% decrease for AST and a 4% decrease for ALT, respectively. However, EEKA pretreatment at 300 mg/kg b.wt. significantly ($p < 0.05$) inhibited increase in serum ALT and AST induced by CCl_4 by 14% and 25%, respectively.

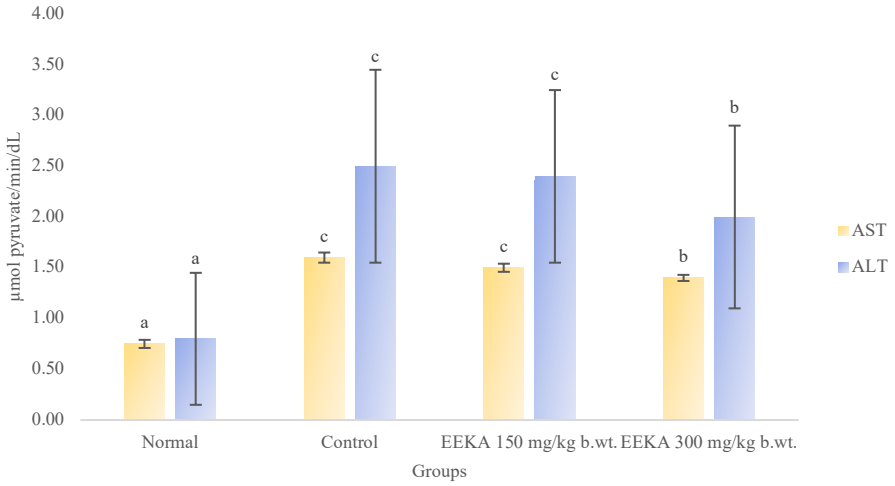

Figure 2: Effects of EEKA on serum ALT and AST levels. Each bar depicts the mean ± SD (n = 6). Distinct letters (within a bar) signify statistically significant variations (one-way ANOVA, Tukey's HSD test, $p < 0.05$).

Effects of EEKA on GSH activity

The GSH content in liver tissue was significantly ($p < 0.05$) reduced by 56% in the control group, as illustrated in Figure 3. This indicates the presence of oxidative stress in comparison

to the normal group. On the contrary, administering CCl_4 followed by pretreatment with EEKA at doses of 150 and 300 mg/kg b.wt. provided significantly ($p < 0.05$) protection against GSH depletion, with reductions of 50% and 69%, respectively.

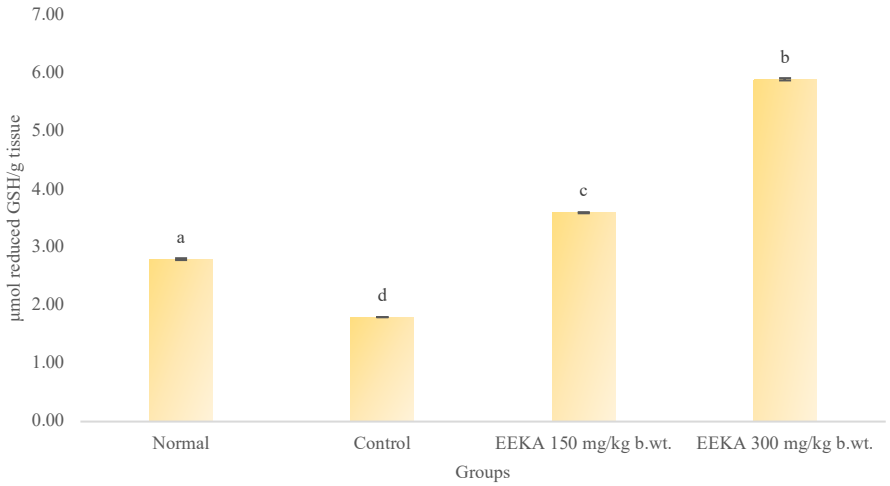

Figure 3: Effects of EEKA on GSH levels. Each bar depicts the mean ± SD (n = 6). Distinct letters (within a bar) signify statistically significant variations (one-way ANOVA, Tukey's HSD test, $p < 0.05$).

Effects of EEKA on LPO activity

Utilising MDA and TBARS reactivity with TBA, which produced a pink chromophore, the TBARS level in liver tissue was determined. The levels of TBARS (expressed as MDA) in the liver homogenate of rats subjected to CCl_4 treatment exhibited a substantial ($p < 0.05$) increase of 4% compared to the normal group, as detailed in Figure 4. However, EEKA at doses of 150 and 300 mg/kg b.wt. significantly ($p < 0.05$) decreased TBARS levels in comparison to the control group by 25% and 43%, respectively.

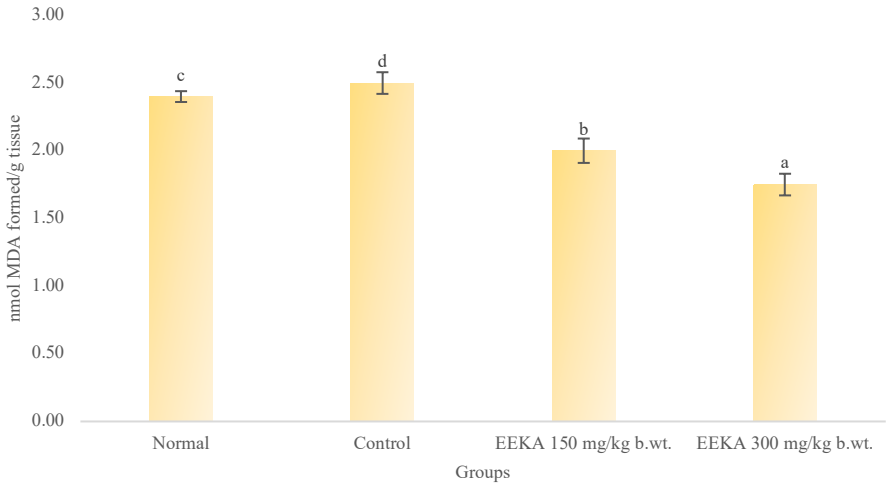

Figure 4: Effect of EEKA on LPO levels. Each bar depicts the mean ± SD (n = 6). Distinct letters (within a bar) signify statistically significant variations (one-way ANOVA, Tukey's HSD test, $p < 0.05$).

Effects of EEKA on CAT activity

Figure 5 illustrates the CAT activity in control and experimental groups of rats. The CAT enzyme activity showed a substantial ($p < 0.05$) seven-fold reduction after CCl_4 administration compared to the normal group. In contrast, pretreatment of rats with EEKA (at doses of 150 and 300 mg/kg b.wt.) significantly ($p < 0.05$) increased CAT activity by 58% and 77%, respectively, in comparison to rats treated solely with CCl_4. This indicates that EEKA pretreatment of rats could potentially restore the antioxidant capacity depleted by CCl_4.

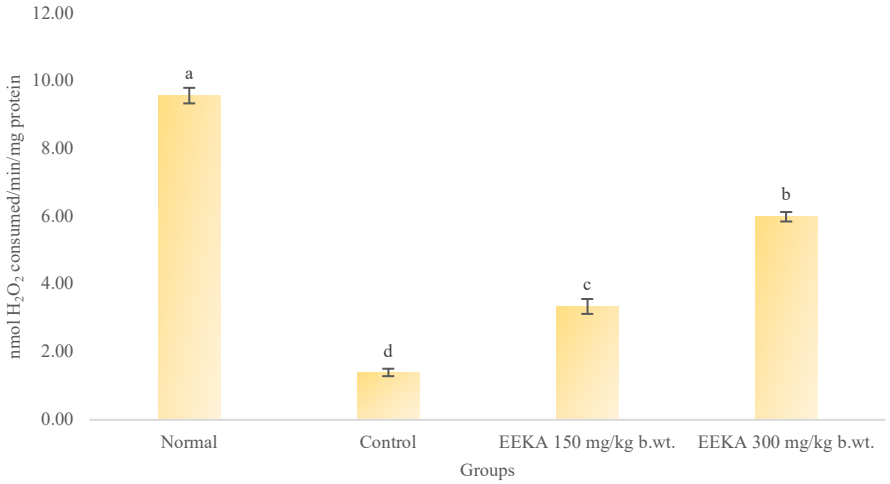

Figure 5: Effect of EEKA on CAT levels. Each bar depicts the mean ± SD (n = 6). Distinct letters (within a bar) signify statistically significant variations (one-way ANOVA, Tukey's HSD test, $p < 0.05$).

Effects of EEKA on GST activity

As shown in Figure 6, the GST activity of liver tissue homogenates from all experimental groups was quantified. The GST activity demonstrated a substantial ($p < 0.05$) decrease of 54% in the control group in comparison to the normal group. This signifies the presence of oxidative stress in hepatic tissues and the depletion of antioxidant reserves. Conversely, in the pretreatment groups (150 and 300 mg/kg b.wt.) with EEKA prior to CCl_4 intoxication, GST activity was significantly increased ($p < 0.05$) by 17% and 42%, respectively, compared to the control group.

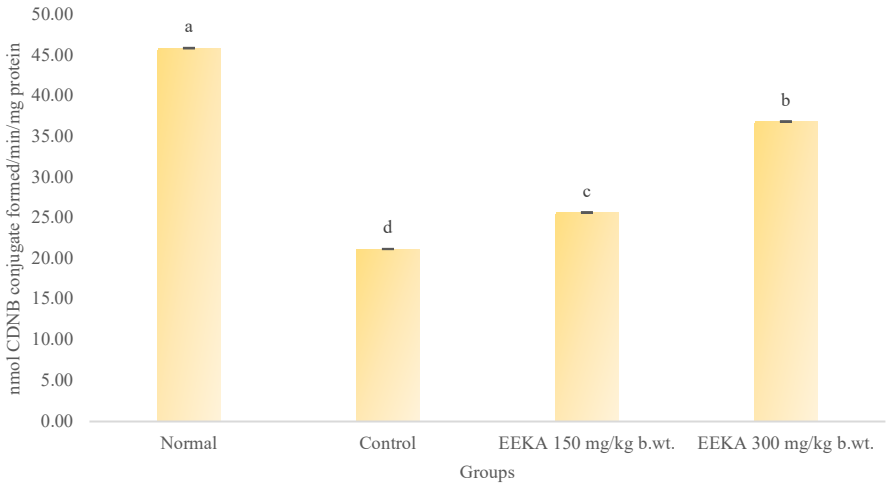

Figure 6: Effect of EEKA on GST levels. Each bar depicts the mean ± SD (n = 6). Distinct letters (within a bar) signify statistically significant variations (one-way ANOVA, Tukey's HSD test, $p < 0.05$).

DISCUSSION

K. alvarezii is a red tropical seaweed that holds significant economic value due to high demand for its cell wall polysaccharides, specifically κ-carrageenan (Lim et al., 2017; Rupert et al., 2022). Carrageenan is composed of sulphate half-esters affixed to the sugar unit of linear polysaccharide chains, which impart water solubility, the ability to form a highly viscous solution, and stability over a broad pH range. Each of its three general forms (κ, λ, and ι) possesses distinct gelling characteristics. Ethanol, with a low boiling point of 69 °C, is commonly used as a solvent in extraction processes because it helps prevent damage or overheating of the chemical compounds (Miazek et al., 2017). The selection of an extraction technique is substantially influenced by the nature of the plant and its components (Lefebvre et al., 2021). The Soxhlet extractor, a laboratory apparatus designed for compound extraction from solid materials, is utilised in the extraction process. Subsequently, the ethanol solvent was delicately extracted from the sample via rotary evaporation using a rotary evaporator, yielding the unrefined extract. Consequently, the ethanol extraction method using a Soxhlet apparatus is preferred over water extraction due to its effectiveness.

The EEKA, evaluated for its TPC and antioxidant properties using the DPPH radical scavenging assay, shows potential for commercialisation in human health consumption. The Folin-Ciocalteu method was used to determine the TPC in this investigation. The method involves the reduction of metal oxides by phenolic acids, resulting in a blue solution with maximal absorption at 765 nm (Blainski et al., 2013; Pérez et al., 2023). Phenolic compounds, known for their antioxidant activities and potent chain-breaking antioxidant properties (Losada-Barreiro et al., 2022), exhibit physiological functions and hepatoprotective effects. These effects are attributed to their redox characteristics (Al Mamari, 2021; Shi et al., 2022), essential for neutralising free radicals, decomposing peroxides, and suppressing singlet and triplet oxygen. Similarly, DPPH is a valuable reagent for assessing the scavenging ability of compounds against free radicals. In alcoholic solutions, it undergoes reduction to its non-radical form DPPH-H, in the presence of hydrogen-donating antioxidants. Characterised by maximal absorbance at 517 nm, DPPH is widely used for evaluating the radical scavenging capacity of various compounds. The formation of the non-radical form of DPPH-H is indicated

by a visual change from purple to yellow during the reduction of alcoholic DPPH solution (Gulcin, 2020).

The investigation revealed that EEKA exhibits varying degrees of scavenging ability against DPPH radicals, depending on its concentration. The DPPH radical scavenging activity of EEKA was confirmed by its IC_{50} value, which represents the concentration of an antioxidant required to achieve a 50% reduction in the initial concentration of DPPH radicals (Gulcin, 2020). A lesser IC_{50} value signifies a more potent capacity of sample to function as a DPPH scavenger. In this study, an IC_{50} value of 4150.00 ± 0.41 µg/mL was determined, indicating weaker antioxidant activity compared to the positive control (75.00 ± 0.84 µg/mL). While phenolic compounds constitute a significant group, other antioxidant secondary metabolites such as flavonoids and tannins also play a crucial role in combating oxidative stress and safeguarding liver health (Yogi & Mishra, 2017; Vun-Sang & Iqbal, 2023).

CCl_4 has long been recognised as a hepatotoxin and is known to cause liver damage (Thanebal et al., 2021). Its main route of entry into water is through industrial waste, stemming from its use in chlorofluorocarbons synthesis (Unsal et al., 2020). The accumulation of adipose tissue in the liver and centrilobular necrosis are both associated with CCl_4. Numerous studies have shown that hepatotoxicity induced by CCl_4 is catalysed by cytochrome P-450 in the endoplasmic reticulum of hepatocytes, making it a common model for hepatotoxicity examination (Li et al., 2015). CCl_4 undergoes biotransformation at the endoplasmic reticulum-based cytochrome P-450 system, resulting in the production of the trichloromethyl free radical (CCl_3•). When cellular lipids and proteins react with CCl_3• in the presence of oxygen, trichloromethyl peroxyl radical is generated. Consequently, cell mortality occurs as a result of trichloromethyl peroxyl radicals initiating LPO and disrupting Ca^{2+} homeostasis (Unsal et al., 2020).

Free radicals are produced under specific environmental conditions and as part of normal cellular processes in the body. These molecules lack an electron, making them electrically charged. To stabilise this charge, free radicals seek to either acquire or donate an electron from or to neighbouring molecules. Antioxidants counteract these molecules by neutralising them before they initiate the chain reaction leading to oxidative damage (Gulcin, 2020; Chaudhary et al., 2023; Vun-Sang et al., 2023). To ascertain the capacity of EEKA to alleviate oxidative stress and damage caused by free radicals, its proposed antioxidant properties were evaluated using a rat model of CCl_4-induced hepatotoxicity in an experimental setup. Thus, the evaluation of hepatic health can be accomplished by estimating the activities of serum AST and ALT, enzymes predominantly localised in the cytoplasm and present significantly in the cytosol of hepatocytes (Li et al., 2015). EEKA found in seaweed may have acted to protect the plasma cellular membrane of hepatocytes from being breached by reactive metabolites generated by CCl_4 exposure. This prevention action could have reduced hepatocyte injury and consequently decreased AST and ALT leakage due to cell destruction. The diminished expression of these transaminases in rats treated with EEKA following toxin exposure may explain this phenomenon (Wang et al., 2017).

Pathological oxidative stress can lead to cellular deformity (Gulcin, 2020). The level of GSH significantly influences tissue vulnerability to oxidative injury and hepatic depletion. Tripeptides, prevalent in the liver, act as non-enzymatic biological antioxidants with properties on both intracellular and extracellular surfaces (Iqbal et al., 2020; Vun-Sang et al., 2022). GSH, a significant nonprotein sulphydryl compound, performs various biological functions, including preserving the reduced form of membrane protein-sulphydryl groups. GSH levels

were substantially lower in rats exposed to CCl_4 compared to those induced with CCl_4 and treated with EEKA. This disparity suggests that EEKA treatment of CCl_4-induced rats may increase susceptibility to CCl_4. One primary hypothesis suggests that the free radical derivatives of CCl_4 contribute to the formation of lipid peroxidases, leading to hepatopathy. Therefore, effective protection against CCl_4-induced toxicity requires significant antioxidant activity or prevention of free radical generation (Vun-Sang et al., 2022). The current investigation revealed a notable increase in MDA level in the group exclusively treated with CCl_4, indicating elevated levels of LPO causing tissue damage and compromising the ability of antioxidant defence systems to counter excessive free radical production (Iqbal et al., 2020; Thanebal et al., 2021; Vun-Sang et al., 2022; Iqbal et al., 2023; Shah et al., 2023). EEKA treatment considerably inhibited LPO, indicating its antioxidant activity.

Within the body, an effective defence mechanism exists to prevent and neutralise damage caused by free radicals. Oxidative enzymes, including CAT, play a crucial role in this defence mechanism. CAT, classified as a phase 2 enzyme, serves as a non-enzymatic antioxidant and acts as the initial line of defence against reactive oxygen species. It is widely distributed across all animal tissues, with the liver demonstrating the highest activity levels (Iqbal et al., 2020; Thanebal et al., 2021). The primary function of CAT is to convert active oxygen molecules into non-toxic compounds. Reduced CAT activities in the liver were observed in the CCl_4-treated groups, which can lead to various detrimental consequences due to the accumulation of superoxide radicals and hydrogen peroxide (H_2O_2). (Vun-Sang et al., 2022; Iqbal et al., 2023; Shah et al., 2023) CAT primarily protects tissues from highly reactive hydroxyl radicals and decomposes H_2O_2. Interestingly, in the EEKA pretreated group, hepatic CAT activity in CCl_4-intoxicated rats significantly increased. Phase 2 enzymes play a crucial role in detoxifying xenobiotics, providing the body with defence mechanisms against potential environmental hazards (Iqbal et al., 2020; Vun-Sang et al., 2022; Iqbal et al., 2023; Shah et al., 2023). GST, an innate antioxidant defence mechanism found in living tissues, is a key player in this process. A reduction in GST activities is associated with an accumulation of highly reactive radicals, leading to detrimental consequences such as cellular membrane impairment and malfunction (Iqbal et al., 2023; Shah et al., 2023). CCl_4 administration results in the production of peroxyl radicals, leading to the inactivation of GST enzymes and a significant decrease in GST activity in exposed rats. Based on the results, EEKA demonstrates the potential to maintain or enhance phase 2 enzymes, including GST.

Prior research has conclusively established that antioxidative enzymes serve as the principal defence mechanism against ROS and other free radicals. This research aims to assess the antioxidant potential of EEKA against ROS, using a rat hepatotoxicity model induced by CCl_4 intoxication. The investigation evaluates the levels of ALT and AST in serum, as well as GSH, LPO, CAT, and GST in hepatic tissues. This study enhances understanding of how *K. alvarezii* administration may reduce oxidative damage in the liver. Additionally, this research suggests that EEKA could serve as a viable nutritional alternative to current pharmacological methods for reducing hepatotoxicity.

Relying solely on TPC and DPPH assays may limit the comprehensive assessment of antioxidant potential in EEKA, as these assays provide valuable but incomplete insights into antioxidant activity. They may not identify the specific compounds that contribute significantly to antioxidant activity, crucial for understanding the oxidative stress mechanism. Therefore, additional assays such as ferric reducing antioxidant power and oxygen radical absorbance capacity should be emphasised for EEKA to further delineate its ability to mitigate oxidative stress, while high-performance liquid chromatography can elucidate the contribution of other

compound groups to its antioxidant properties. Additionally, the absence of mandatory histopathological analysis represents a significant limitation. Such analysis offers insights into hepatic tissue integrity and complements biochemical analyses, which are essential in animal protocols for evaluating mechanisms or evidence of tissue inflammation induced by substances such as CCl_4. Future recommendations would encompass a broader range of antioxidant assays and histopathological examinations to provide a more comprehensive understanding hepatoprotective potential of EEKA and its impact on hepatic health.

CONCLUSIONS

In summary, the findings suggest that EEKA has the potential to augment or sustain the functionality of hepatic antioxidant enzymes, offering protection against liver injury induced by CCl_4. This protective effect of EEKA can be ascribed to its capacity to function as a scavenger of free radicals, intercepting those involved in CCl_4 metabolism via microsomal enzymes. Incorporating antioxidant-rich marine resources such as *K. alvarezii* into a regular diet may help restore balance, given their multifaceted effects. Hence, based on the results, EEKA exhibits potential as a hepatoprotective agent, likely due to its antioxidant compounds.

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