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EVALUATION OF THE AMELIORATIVE EFFECT OF THE PHILIPPINE NATIVE BLUEBERRY *Vaccinium myrtoides* (Blume) Miq. FRUIT EXTRACT AGAINST CYCLOPHOSPHAMIDE-INDUCED HEPATOTOXICITY IN MICE

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ABSTRACT – The Philippines is home to several plant species, some of which have economic and medicinal values. Exploring foods rich in polyphenols, such as members of the plant genus *Vaccinium* (e.g, blueberries), have antioxidant activity with protective effects against hepatotoxicity. This study examined the possible hepatoprotective effect of Philippine native blueberry *Vaccinium myrtoides* (Blume) Miq. crude ethanolic fruit extract (BBE) against cyclophosphamide-induced liver toxicity in mice. Two concentrations of BBE were tested. Adult male ICR mice were randomly sorted into four (4) groups, namely: 1. Control Phosphate Buffered Saline [(PBS), 1% body weight (BW)]; 2. Cyclophosphamide [(CP), 150 mg/kg BW]; 3. Low dose of crude blueberry extract [(BBE1), 100 mg/kg BW] + CP; and 4. High dose of crude blueberry extract [(BBE2), 400 mg/kg BW)] + CP. Results revealed that CP and BBE1+CP treatments had a significant increase in liver weight on day 14 versus day 7. This could be due to the damage from CP exposure. Histopathological scoring showed that administration of BBE treatments showed a visible reduction of necrosis and steatosis compared to those given only CP, even if recorded liver lesion scores were statistically equal due to the limited number of animals used. Present findings suggest the potential of *V. myrtoides* crude extract in ameliorating the hepatotoxic effects of cyclophosphamide. Hence, this pioneering study on the antioxidative activity of a Philippine native blueberry can serve as a springboard for further examination of its hepatoprotective property.

Keywords: antioxidant, blueberry, hepatoprotection, Philippine native plants, ROS scavenging activity, Vaccinium myrtoides

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INTRODUCTION

Harnessing native or local plant species as food sources will help encourage both the diversification of food production and improved adaptation of local landscapes to cater to the demands of an ever-changing environment (Provenza, 2008). The native Philippine blueberry, *Vaccinium myrtoides* (Blume) Miq., is understudied and underutilized. It is used as food and medicine (Magciano, 2010; Coronel, 2011; Magdalita et al., 2014). Galvez (2015) noted that the decoction of *V. myrtoides* stem serves as wash or antiseptic during fever. Ethnobotanical studies from some local communities in Ifugao (Balangcod and Balangcod, 2009) and Benguet (Chua-Barcelo, 2014; Balangcod and Balangcod, 2018) mentioned that the fruit of *V. myrtoides* serves as food or processed as candies, jams, jellies, juices, and wine. It is also used as an ink or dye, as a traditional medicine to improve eyesight and for diabetes, cancer, flu, and as an antioxidant.

Blueberries, which belong to the genus *Vaccinium*, are widely popular due to their taste and numerous health benefits. Whether consumed daily as fruit, juice, or processed products, its high antioxidant content brought about by polyphenolic compounds, such as flavonoids and anthocyanins, effectively act as antioxidants (Olas, 2018). Antioxidants are associated with managing increased oxidative stress that gives rise to chronic illnesses stemming from various conditions such as stress, poor diet, excessive alcohol consumption, drugs, and disease (Ma et al., 2018; Kalt et al., 2020; Rashidinejad, 2020).

The *Vaccinium* genus has been well-studied in temperate areas. Its health benefits stem from its phenolic, flavonoid, and anthocyanin contents. Blueberries have been recorded to be cardioprotective (Ahmet et al., 2009), antioxidant (Senevirathne et al., 2010; Huang et al., 2016), antimicrobial (Burdulis et al., 2009; Pervin et al., 2013; Silva et al., 2013; Oprea et al., 2014; Silva et al., 2015), tumor-reducing (Seeram et al., 2006; Calò and Marabini, 2014; Sun et al., 2015), anti-inflammatory and anti-nociceptive (Torri et al., 2007; Samad et al., 2014; Nardi et al., 2016), and neuroprotective (Vyas et al., 2013 using fruit and leaf).

Cyclophosphamide (CP), a widely used anticancer drug, is also known as a powerful immunosuppressant used especially for autoimmune disorders and organ transplant operations to avoid organ rejection (Bhat et al., 2018). However, it produces toxic side effects that damage different organs; examples include the heart, lungs, kidney, spleen, and reproductive organs (El-Kholy et al., 2017; Al-Salih et al., 2020; Kim and You, 2021), among others. One notable organ that it affects is the liver since it is the site of metabolism of this drug by hepatic microsomal cytochrome P450 mixed function oxidase system into phosphoramide mustard, which is responsible for its anti-neoplastic effect, and acrolein, which elicits the damages through increasing oxidative stress.

Several studies have used different temperate *Vaccinium* species for hepatoprotection for various cases (Bao et al., 2008; Wang et al., 2010a; Wang et al., 2010b; Wang et al., 2013; Gong et al., 2014; Shi et al., 2014; Ravan et al., 2017; Muceniece et al., 2019; Yan et al., 2019) since blueberries have been noted for its antioxidant effects from its phytochemical content. However, tropical *Vaccinium* species like *V. myrtoides* have not yet been examined. This study aims to investigate the possible hepatoprotective property of the crude freeze-dried ethanolic *V. myrtoides* fruit extract on CP-induced liver injury in mice. The findings of this study could help highlight the health benefits of this native blueberry species and increase its visibility and market presence.

MATERIALS AND METHODS

Fruit Sample Preparation and Extraction

Fresh fruits of *V. myrtoides* were obtained from a local farm in Atok, Benguet. The identity of the blueberries was verified through an MNH-UPLB report of identification in 2016 by the UPLB Museum of Natural History (CAHUP). Firm, dark, purple fruits were considered mature and were the ones harvested through random sampling. These were brought to the laboratory immediately while stored in an ice cooler and eventually were washed with running water, pat-dried, weighed, and stored in resealable bags at -80°C (Operon® Ultra-Low Temperature Freezer) until further use. Small portions (100 g) were freeze-dried by a lyophilizer (Labconco® FreeZone 2.5 Liter Benchtop Freeze Dry System). Samples were then subsequently crushed by a Wiley® Mini-Mill grinder (sieve 20) twice to produce a coarse, granule-like consistency. Ground samples were then kept refrigerated (4°C) in amber bottles until use.

The modified extraction procedure was based on Grace et al. (2014). The powdered sample (2.5 g) was extracted with 80 % ethanol (250 mL) via mortar and pestle, then subjected to suction filtration to remove residue, resulting in a clear, homogenous, and debris-free extract. Maceration with 80 % ethanol and filtration was carried out until the filtrate was colorless. Extractions were done thrice. Filtrates were pooled and concentrated through rotary evaporation (Büchi Rotavapor® R-114/S) at 40 °C.

Animal-Based Assays

Male 8–10-week-old ICR mice (weighing 30-40 g), were obtained from the Laboratory Animal Facility, Research Institute for Tropical Medicine, Muntinlupa City, Philippines, and were acclimatized for one week. Mice were housed individually in polycarbonate cages with stainless steel top (Techniplast®, Italy). Commercial mice pellets and drinking water were provided *ad libitum*.

These were then randomly assigned into four (4) treatment groups:
(1) control phosphate-buffered saline [(PBS), 1X, (1% body weight (BW)] as the negative control (n=7);
(2) cyclophosphamide (150 mg/kg BW, Xyclomed®, Korea) as the positive control (n=7);
(3) crude blueberry extract 100 mg/kg BW (BBE1) + CP as the low dose (n=6); and
(4) crude blueberry extract 400 mg/kg BW (BBE2) + CP as the high dose (n=6).

The BBE extract was reconstituted in 1X PBS in a 10-mL volumetric flask and then sterilized with 25-nm nylon wielded syringe filter (0.22 μm, LabFil®, China) under a biosafety cabinet. Previous mice-based assays used concentrations of *Vaccinium* extracts from 50 to 200 mg/kg (Hiroyuki et al., 2009; Sun et al., 2015; Nardi et al., 2016; Pervin et al., 2016). CP was also reconstituted with 1X PBS. The dosages in our study were based on Huyan et al. (2011) and Xu and Zhang (2015).

The experiment lasted for 15 days. The frequency and duration of the cyclophosphamide treatment were adapted from Catap et al. (2018). The control, BBE1, and BBE2 treatments were given orally daily using a gauge 22 stainless-steel gavage, while CP was administered intraperitoneally every three days (days 1, 4, 7, 10, and 13). Both BBE1 and BBE2 received CP an hour after administration of the blueberry extracts.

Mice sampling was done on the 8th and 15th days to assess the effects of 7- and 14-day treatments on induced liver damage and immunosuppression, apart from innate and adaptive immunity (Catap et al., 2018). Studies also concerned with the hepatoprotective effects of extracts against cyclophosphamide-induced hepatotoxicity examined their mice at the end of the experiment, which ranged from 7 (El-Naggar

et al., 2013; Shokrzadeh et al., 2014; Habibi et al., 2015) up to 20 days (El-Kholy et al., 2017; Jiang et al, 2020). Three mice were randomly selected from each group (n=12) on day 7 and the remaining mice (n=14) on day 14 were euthanized by cervical dislocation. Livers were pat-dried then weighed before these were prepared for histopathology. All experiments conducted in mice were approved by the UPLB Institutional Animal Care and Use Committee with approval number AR-2018-258.

Histopathology of the Liver

The collected livers were preserved in 10 % formalin, processed using standard paraffin technique, sectioned at 4μm, and stained with Hematoxylin and Eosin for histopathological examination. The stained liver sections were examined under the microscope. Scoring was based on Liang et al. (2014), where liver lesions (inflammation, fatty liver or steatosis, fibrosis, and necrosis) were scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (marked). Values of mean liver lesions range from 0.00-0.99 (changes absent), 1.00-1.99 (mild changes), 2.00-2.99 (moderate changes), and 3.0 (marked changes). Photographs were taken at different magnifications using a research microscope with camera attachment (Olympus® USA). All sections were evaluated blindly by the researcher and veterinary pathologist.

Statistical Analysis

Liver weights were recorded as mean ± SD. Statistical significance of liver weights of treatments within and between treatment days was evaluated with repeated measures ANOVA. The interaction of liver weight in corresponding treatment groups between day 7 and day 14 was examined with Independent Samples T-test using the statistical software Jamovi (version 1.6). Liver lesion scores were evaluated with Fisher's Exact Test using Rstudio (version 1.4.1106). A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Liver Weight and Microscopic Lesions

Results showed that CP treatment for 14 days demonstrated the highest increase of liver weight across all groups and was significantly heavier (M = 2.46 g, SD = 0.17) compared to the mice that received the same treatment for 7 days (M = 1.85 g, SD = 0.17, $t(5) = 4.76$, $p = 0.005$) . Likewise, mice treated with BBE1 (100 mg/kg BW)+CP for 14 days (M = 2.39 g, SD = 0.15) also had significantly heavier liver weights than the mice that received this treatment for 7 days (M = 2.02g, SD = 0.09, $t(4)$, $p = 0.019$) (Figure 1). For the control and BBE2+CP treatment groups, their mean liver weights for both observation days were not significantly different. Comparison of mean liver weights of mice from different treatment groups for day 7 and day 14 was also not significantly different ($p > 0.05$). Mice given BBE1+CP treatment had slightly heavier weights than those given BBE2+CP.

This is the first study examining the protective effect of crude *V. myrtoides* fruit extract on mice livers exposed to CP. The mean liver weights of each treatment group within days 7 and 14 were statistically equal. Comparison of the day 7 and day 14 treatment pairs showed that the CP and the BBE1+CP had significantly heavier liver weights on day 14 than their day 7 records, while the control and BBE2+CP did not have any significant changes (Figure 1). Existing research has observed both significant and non-significant changes in liver weight when administered with CP. Oyagbemi et al. (2016) noted that there was a significant weight gain in the liver of CP-treated rats compared to the control, but posttreatment of gallic acid resulted in a significant reduction compared to CP-treated rats. A similar case was observed by Shi et al. (2014), wherein rats given CP had a higher liver/body weight ratio compared to the control. Following

treatment with *V. corymbosum* anthocyanin extract, it was significantly lowered compared to the CP group. Other studies did not observe significant changes when administered with CP alone (Al-Salih et. al, 2020; Patwa et al., 2019; Zhang et al., 2021), while Kanno et al. (2009) noted a significant decrease in liver weight in mice administered with 150-250 mg/kg doses of CP.

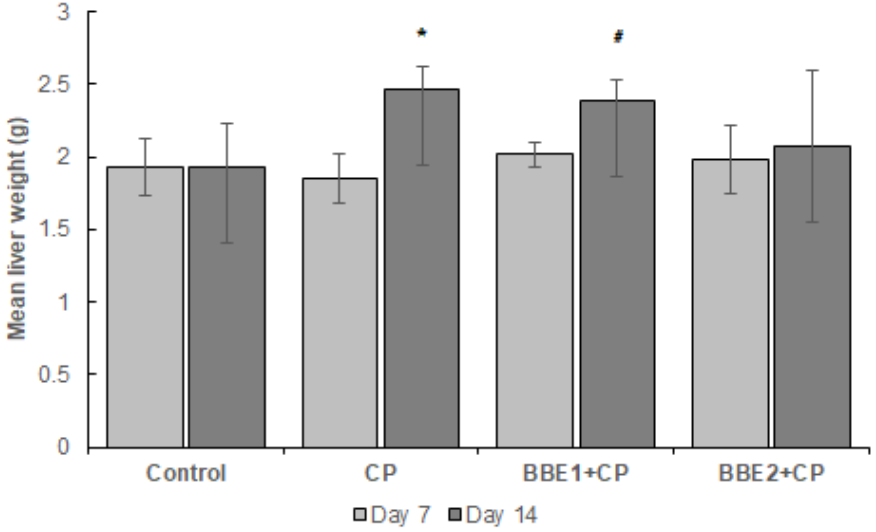

Figure 1. Liver weights of the treatment groups on days 7 and 14. CP: cyclophosphamide, BBE1: crude blueberry extract (100 mg/kg BW), BBE2: crude blueberry extract (400 mg/kg).
$^{*}p < 0.05$ CP at Day 14 compared with CP at Day 7. $^{\#}p < 0.05$ BBE1+CP at Day 14 compared with BBE1+CP at Day 7.

Greaves et al. (2012) noted that an increase in liver weight could be due to several factors, such as an increase in lipid or glycogen content that could be due to cell damage, congestion, hepatocellular hypertrophy, or circulatory factors. The development of steatosis in hepatocytes is associated with the cell being unable to extrude lipids or from cellular injury and toxicity (Greaves et al., 2012). Increased liver weight in CP-treated rats or mice was also detected in various studies (Shi et al., 2014; Oyagbemi et al., 2016; Jiang et al., 2020) that recorded disrupted hepatic architecture and cellular damage. Similar to what is observed in our study, the possible cause of the increase could be the cellular damage from exposure to CP and the steatosis observed in the microscopic anatomy.

The control group generally exhibited typical liver microanatomy, such as the normal histological structure of hepatocytes, sinusoidal spaces, and central vein, and did not have any other significant findings (Figure 2A). However, compared to the control, mice solely given CP showed distortion of hepatic architecture as well as dilatation and congestion of hepatic sinusoidal spaces (Figure 2B). Mild to moderate steatosis around the central vein, along with swollen hepatocytes, were also noted. Although the BBE1+CP (Figure 2C) and BBE2+CP (Figure 2D) groups were also given CP, lesser liver damage was observed in

both compared to the lone CP-treated group. The BBE1+CP group, compared to the lone CP-treated group, showed a marked decrease of vacuolar degeneration and necrosis of most hepatocytes with minimized dysplastic changes, less dilatation of central and portal veins, and reduction of sinusoids congestion. It showed a relatively normal hepatic histological structure despite CP-induced oxidative damage. The BBE2+CP group also exhibited a moderate decrease of vacuolar degeneration and necrosis of most hepatocytes compared to the CP-treated group. Less dilatation of central and portal veins was also observed, along with the reduction of sinusoidal congestion. However, the lower dose of BBE seemed to encourage better recovery from CP exposure, as it recorded a lesser degree of steatosis and necrosis on days 7 and 14 compared to the BBE2+CP (Table 1).

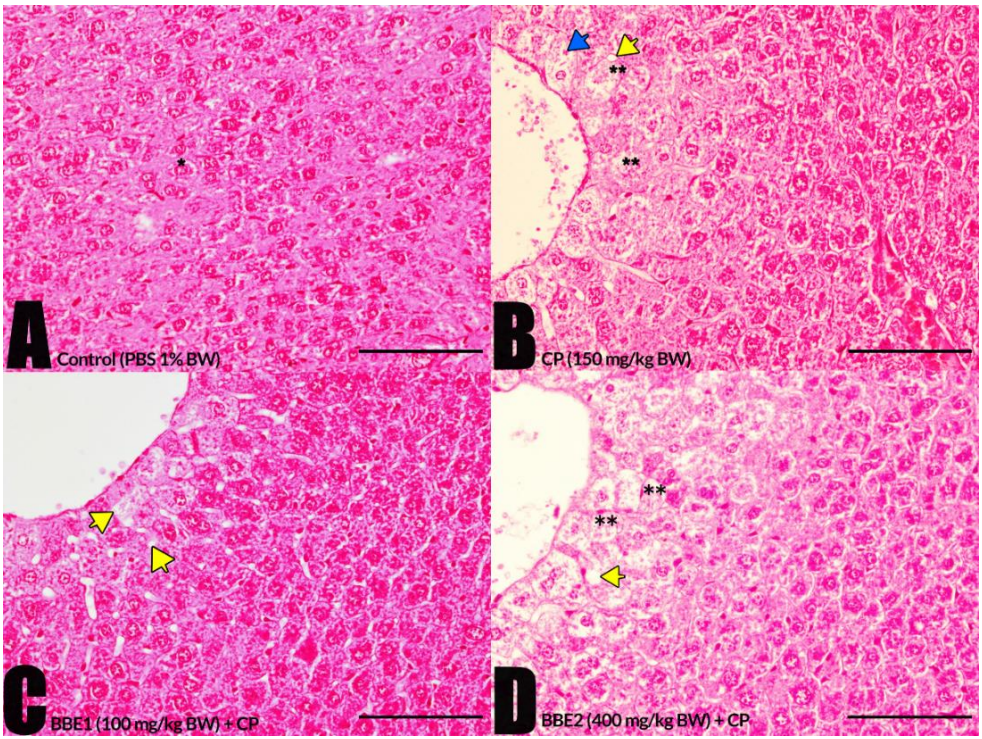

Figure 2. Liver microscopic anatomy of the different experimental groups after day 7. Normal hepatocytes (*) were observed in the Control group. Hallmarks of cyclophosphamide (CP) toxicity, such as necrotic (**) and swollen hepatocytes (blue arrowhead) and mild steatosis (yellow arrowhead) were seen on both days 7 and 14 (B). Low dose (100 mg/kg) of BBE (C) alleviated the hepatic injuries such as necrosis and enlarged hepatocytes. High dose (400 mg/kg) also dampened some of the CP-induced damage, such as swollen hepatocytes (BBE = crude blueberry extract). Bar = 100 µm.

Despite some visible differences that were numerically favored, there were no statistically significant differences ($p > 0.05$) among the treatments and between days 7 and 14 (Table 1). Mild to

moderate steatosis and necrosis were observed in all treatments on both days 7 and 14 but did not reach significant values for both observation days. Only the CP group attained both mild and moderate steatosis compared to other treatment groups. Compared to the CP group, the BBE1+CP recorded a lesser degree of necrosis and steatosis on days 7 and 14. The BBE2+CP group only had mild steatosis at the end of day 14.

Table 1. Mean scores obtained in the evaluation of the histopathological lesions in the livers of mice treated with Control, CP, and BBE for 14 days.

Treatment groups	Mean liver lesion scores			
	Steatosis		Necrosis	
	Day 7^{ns}	Day 14^{ns}	Day 7^{ns}	Day 14^{ns}
Control (PBS 1% BW)	0.33 ± 0.58	0.75 ± 0.96	0.00 ± 0.00	0.00 ± 0.00
CP (150 mg/kg BW)	2.33 ± 0.58	1.25 ± 1.50	1.00 ± 0.00	0.50 ± 0.58
BBE1 (100 mg/kg BW) + CP	0.67 ± 1.15	0.67 ± 1.15	0.00 ± 0.00	0.33 ± 0.58
BBE2 (400 mg/kg BW) + CP	0.67 ± 1.15	1.67 ± 1.53	0.67 ± 0.58	0.67 ± 0.58

^{ns}not significant at $p = 0.05$. CP: cyclophosphamide, BBE1: crude blueberry extract (100 mg/kg BW), BBE2: crude blueberry extract (400 mg/kg).

Compared to the control group (Figure 2A), those that were exposed to CP had visible damages and lesions typical of CP hepatotoxicity, such as enlargement of the hepatocytes, mild steatosis, and necrosis (Figure 2B). Several studies have also observed this even with different dosages and frequency of administration. The group of mice or rats were given only a single intraperitoneal dose of CP (50-200 mg/kg BW) either at the start (El-Naggar et al., 2013; Shokrzadeh et al., 2014; Habibi et al., 2015; Oyagbemi et al., 2016), in the middle (Shi et al., 2014; El-Kholy et al., 2017; Sherif, 2018) or towards the end (Germoush et al., 2016; Temel et al., 2020; Doustimotlagh et al., 2020) of the experiments had livers that manifested severe inflammation, necrosis, and lymphocytic infiltrations upon histopathological examination. Other studies that have administered lower dosages of CP continuously, such as 30 mg/kg for 10 days (Patwa et al., 2019) or 80 mg/kg for five days (Jiang et al., 2020), resulted in livers with similar lesions. It manifests the same damages as CCl_4 in the liver, which is the traditional model to induce liver injury (Wang et al., 2010a; Chen et al., 2012). Increased concentrations of acrolein in the liver contribute to the excessive oxidative stress that eventually overwhelms the innate antioxidant defense system, leading to lipid peroxidation, imbalanced antioxidant enzyme defense system, and destruction of its structure and biochemistry. The excess ROS also affects the pro-inflammatory and anti-inflammatory homeostasis that is also reflected in the disturbed hepatic condition (El-Naggar et al., 2014; Habibi et al., 2014; Shokrzadeh et al., 2014; Li et al., 2015; Al-Salih et al., 2020; Jiang et al., 2020). In all of the aforementioned studies, administration of the extract with high antioxidant activity to the mice or rats improved their liver condition, its associated enzyme and other markers, and histopathology.

In this study, the liver histopathology showed a reduction in CP-induced steatosis and necrosis in the BBE1+CP and BBE2+CP groups for both days 7 and 14 (Table 1). Compared to the control, those mice which were solely given CP had a seven-fold increase in steatosis during day 7 while both blueberry treatments only had twice the number of lesions. The BBE1+CP group also scored lower than the CP-treated group in terms of necrosis for days 7 and 14. The same set of scores was recorded for necrosis in the BBE2+CP group, which may mean a plateau of its effect. It could suggest that the BBE2 was able to prevent the progression of necrosis. However, it should be noted that the differences in the liver lesion scores were not

statistically significant. It may not have restored the liver condition at par with the control, but has reduced the damages compared to those given CP alone. This suggests that the antioxidant components of the blueberry extracts, such as its anthocyanins and flavonoids, and other phenolic groups (Barcelo et al., 2015; Villaverde et al., 2020), could have lessened the oxidative damage either by interrupting the propagation or by protecting the substrates from the previously generated ROS (Olas, 2018). The numerous C=C bonds and ring resonance structures can delocalize the unpaired electron, while the hydroxyl groups can donate hydrogen ions or electrons to the free radicals, terminating the progression of lipid peroxidation (Dai and Mumper, 2010; Skrovankova et al., 2015). Reduction of the effects of CP-induced oxidative injury through administration of biological compounds with high antioxidant capacity has been recorded. Gallic acid, a phenolic compound present in red wines, green teas, grapes, and different berries, was able to increase the antioxidant defense system in rat livers damaged by CP (Oyagbemi et al., 2016). Quercetin, a flavonoid found in berries, onions, and apples, also showed hepatoprotective effects through the management of oxidative stress and upregulation of the antioxidant enzymes (Sherif, 2018; Doustimotlagh et al., 2020).

Previous research has documented the protective ability of blueberry antioxidants by ameliorating the effects of oxidative damage through its polyphenols. Wang and colleagues (2013) observed reduced hepatic fibrosis in CCl_4-damaged mice livers given "Rabbiteye" blueberry juice since it prevented liver inflammation and lipid peroxidation. Anthocyanins extracted from *V. corymbosum* fruit ameliorated CP-induced liver injury in rats by reducing inflammation and acting as antioxidants (Shi et al., 2014). Using *V. arctostaphylos* extract, Ravan et al. (2017) also reported an improvement of rat hepatic markers from CCl_4 exposure. Yan and colleagues (2019) reported a similar trend since blueberries were able to reduce liver fibrosis in rats and at the same time aid in maintaining their normal gut microbiome. In a recent study by Yang and colleagues (2021), it was found that purified anthocyanins and polyphenols had higher antioxidant activity, while the crude anthocyanin and polyphenol extracts were better at tumor suppression and improved overall immune function possibly attributed to the synergistic effect of its components.

Similar to other members of the *Vaccinium* genus, the fruits of *V. myrtoides* also contain polyphenolic compounds that highly suggest its antioxidant activity, which has reduced the oxidative damage caused by cyclophosphamide injury. Barcelo et al. (2015) noted that fresh *V. myrtoides* fruits contain phenolics (59.12 mg gallic acid equivalent (GAE)/100 mg fresh weight) and its subgroup, flavonoids (186.44 mg quercetin equivalents (QE)/100 mg fresh weight). It also exhibited antioxidant activity, as indicated by its 80.02% DPPH radical scavenging activity. A related study done showed that the 1:10 ethanolic freeze-dried extract of the fruit of *V. myrtoides* contained phenolics (184.29 mg GAE/100 mg fresh weight) and flavonoids (124.60 mg QE/100 mg fresh weight) and albeit lower, also exhibited 47.70% DPPH radical scavenging activity (Villaverde et al., 2020). Polyphenolic compounds in the fruit extract could have assisted in the amelioration of the hepatic lesions brought about by cyclophosphamide administration. Still, additional parameters should be explored to verify its hepatoprotective action further.

CONCLUSION AND RECOMMENDATIONS

The present study determined the hepatoprotective property of *V. myrtoides* fruit extract (BBE) on cyclophosphamide (CP)-induced liver injury in adult male ICR mice. A significant increase in liver weight was observed in the CP and BBE1+CP groups on day 14 compared to day 7, which could have been caused by the injury from increased oxidative stress from CP exposure. Examination of the liver microanatomy showed a reduction of steatosis and necrosis in mice treated with BBE compared to the CP-alone treated mice even if the recorded liver lesion scores were statistically equal for all groups possibly due to the limited

number of animals used. These preliminary findings show improvement in some of the CP-induced hepatic injuries with BBE treatment. It is further recommended that follow-up studies use a higher number of mice and inclusion of serum markers and other transcription factors involved in inflammation to better grasp the blueberry antioxidant components specific action/s on the hepatic damage brought about by cyclophosphamide.

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STATEMENT OF AUTHORSHIP

The first author conceptualized the study, collected the specimens, performed the experiments, gathered and analyzed the data, and made the drafts of the paper. The second author assessed the quality of the fruit extracts. The third author handled the histopathological assessment of the murine livers. The fourth author supervised the conceptualization of the study and plant extraction. The fifth author supervised the conceptualization of the study and the lab animal handling and protocols. All were involved in writing and reviewing the manuscript.

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