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Research Article

Aqueous Extract of *Malus domestica* Inhibits Human Platelet Aggregation Induced by Multiple Agonists

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Abstract

Cardiovascular diseases such as hypertension, myocardial infarction, and atherosclerosis are the major cause of morbidity and mortality in humankind and not only afflict industrialized nations but also affect developing countries such as Pakistan. We attempted to screen one of the most common fruits, *Malus domestica* (MD or apple), used in Pakistan for its potential cardio-protective effects. The crude aqueous extract (MD-Aq) was prepared and investigated for inhibitory activity against several important cardiovascular drug targets. Activities of antioxidant enzymes such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) were studied, as well as total antioxidant status (TAS). Calcium channel blocking activity was measured using guinea pig ileum in an isolated tissue bath setup. Platelet aggregation studies were carried out using a dual-channel aggregometer. MD-Aq showed dose-dependent improvement in the GPX activity. The highest SOD levels were observed with 10 mg/ml and were 185 U/L compared to 174 U/L for saline, which means no significant effect by MD-Aq. TAS levels were highest at 10 mg/ml of MD-Aq, reaching 1.75 mmol/L of plasma. At 10 mg/ml, close to 90% inhibition of KCl-induced opening of calcium channels was observed, compared with the effect of 1mM Verapamil. At each dose of 1, 5, and 10 mg/ml, a significantly higher arachidonic acid-induced inhibition of platelet aggregation was observed as compared to the saline effect. MD-Aq effects on platelet-activating factor-induced platelet aggregation were less potent. We concluded that MD-Aq could elevate GPX and TAS levels, is inactive at SOD, and is excellent as a calcium channel blocker, a more potent blocker of arachidonic acid-induced aggregation than platelet-activating factor-induced aggregation. These activities can help treat various cardiovascular diseases.

Keywords: *Malus domestica*, calcium channels, platelet aggregation, glutathione peroxidase, superoxide dismutase

1. Introduction

It has been observed that *Malus domestica* (MD or apple) fruit and its peel possess potent antioxidant properties that can significantly mitigate the growth of colon and liver cancer cells (Escarpa and Gonzalez 1998). This is because the apple consists of a unique collection of phytocompounds that diminish the growth of cancer cells (Escarpa and Gonzalez 1998). Apples boast the highest antiproliferative activity compared to 11 other commonly used fruits. It is believed that the apple's peel imparts most of the antiproliferative effect as removing the apple peel was markedly ineffective in inhibiting the growth of Hep G2 cells. Moreover, it was observed that the peel on its own demonstrated stronger antiproliferative activity than that of the apple fruit(Eberhardt, Lee, and Liu 2000).

Methanolic and aqueous fractions of MD exhibited antibacterial activity against gram-positive and gram-negative strains such as *S. aureus, E. coli, B. subtilis,* and *P. aeruginosa* (Sun et al. 2002). Decreased oxidation of diphenylhexatrienelabeledphosphatidylcholine

which is incorporated in low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL), is seen upon the administration of apple phenolics to humans. Binding to serum albumin of the compound diphenylhexatrienelabeled propionic acid is a good indicator of oxidation in the aqueous phase of the human serum. Consumption of the apple fruit reduced the oxidation of albumin diphenylhexatrienelabeled propionic acid, reaching peak activity at 3 hours (Malaviya and Mishra 2011).

The concentration of triglycerides, high-density lipoproteins (HDL), LDL, and total cholesterol (TC) was reduced upon MD. This is mainly due to the chemicals found in the fruit that block lipid peroxidation and decrease LDL, TG, and cholesterol synthesis (Mayer et al. 2001). The polyphenol extracts of apples bear anti-inflammatory potential and confer protection in rat gastric mucosa cells *in vivo* and human gastric cells *in vitro*. By acting as singlet oxygen quenchers, hydrogen-donating compounds, metal ion chelators, and reactive oxygen species (ROS) scavengers, phenolic compounds have been determined to induce antioxidant effects (Khayat and Kargari 2011).

The presence of flavonoids, phenolic acid, and vitamins in large amounts both in pear and apples have been known to arrest inflammation in the airways and are associated with mitigating bronchial hypersensitivity and decreasing the risk of asthma (Graziani et al. 2005).

In diabetic patients, apple has been used to induce glycemic control. It was found in a clinical investigation that the consumption of apples leads to lower blood glucose levels as compared to oat cookie consumption (Woods et al. 2003). Almost total inhibition of cholera toxin catalyzed by ADP-ribosylation was observed with the use of MD extracts. This may be due to the presence of highly polymerized catechins in the fractions. The fraction with monomeric, dimeric, and trimeric catechins caused 39% inhibition, whereas the fraction with non-catechin phenols induced a mere 3.5% inhibition (*De* Oliveira, Sichieri, and Moura 2003). However, very few scientific investigations have been carried out to determine if the cardioprotective effects of apples are due to their potential impact on platelet aggregation, calcium channels, and antioxidant enzyme present in the blood. Here in this study, we decided to explore these questions. We investigated the effects of crude methanolic extract of the fruit part on arachidonic acid (AA), collagen, adenosine diphosphate (ADP), and platelet-activating factor (PAF)-induced human platelet aggregations on calcium channels, and antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and total antioxidant status (TAS).

2. Materials & Methods

2.1. Plant Material

A taxonomist in our university obtained 5 kg of MD fruit from the market after correct identification. For future reference, a sample was kept in our laboratory. After mashing, fruit pulp was put in a tightly closed container and kept at room temperature for future use.

2.2. Preparation of Crude Plant Extract

The fruit pulp was soaked in 5 liters of 70% methanol for 72 hours with occasional shaking. Two filtration cycles were carried out, first through a muslin cloth and then through a Grade 4 (20 – 25 μm) Whatman filter paper. After three repeats, the filtrate was subjected to vaporization in a rotary evaporator to produce a thick, dark-brown, semisolid mass under reduced pressure. The semisolid mass so obtained was the crude extract. The yield of the methanolic extract of MD (MD-Cr) was 16%.

2.3. Fractionation of crude extract

Distilled water was added to the crude extract of MD, followed by various non-polar solvents such as n-hexane, petroleum ether, and acetone to remove highly non-polar constituents of the MD-Cr. After repeatedly removing the non-polar fractions along with their dissolved phytocompounds, we were left with an aqueous fraction of the MD (MD-Aq).

2.4. Preparation of Platelets

Human samples were collected after obtaining ethical approval. Blood was withdrawn from normal volunteers who were medication free for a minimum of 7 days. Sodium citrate solution (9:1) 3.8 % (w/v) was added to the blood samples, after which centrifugation was performed for 15min at 20°C at 260g to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by centrifuging the rest of the blood sample at 1200g for 10 min. Phase contrast microscopy was used to determine the thrombocyte number. PRP having platelet counts between 2.5 and 3.0 x 108 mL^{-1} of plasma was used for all aggregatory experiments at a temperature of 37°C (Ahmed, Gul, Gul, et al. 2014).

2.5. Measurement of Platelet Aggregation

A dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) was used to gauge the magnitude of aggregation (Aslam et al. 2008). 0.45 mL aliquots of PRP were used, and the volume was made up to 0.5 mL by adding standard drugs prepared in normal saline. Platelet agonists, AA, PAF, collagen, and ADP, were used to stimulate the aggregation process. PRP was incubated with MD-Aq for 60 seconds prior to the addition of the pro-aggregatory agents to determine the anti-aggregatory activity of the MD-Aq. Changes in light transmission vs. time data were used to quantify the extent of aggregation for 5 minutes after inducing aggregation. Once the antiplatelet activity against the agonists was established, dose-response curves were drawn, and IC_{50} values were calculated.

2.6. Measurement of Total Antioxidant Status

Kits from RANDOX, UK, were used to perform this assay on a spectrophotometer DU 800 (Beckmann, USA). Human plasma was used as a sample. The assay was done as explained by Ahmed and associates (Ahmed, Gul, Idris, et al. 2014). All the kit chemicals, MD-Aq, and finally, the substrate were added sequentially to create the reaction mixture. The absorbance of the sample at wavelength 600nm was measured using UV-spectrophotometer. Inhibition of the oxidation by metmyoglobin of 2,2' azino-bis-[3-ethylbenz-thiazoline-6-sulfonic acid] (ABTS) to ABTS+ formed the principle of this experiment. The absorbance at 600nm determines the amount of ABTS+ produced. In these conditions, the plasma antioxidants can block the absorbance at 600nm in a manner that is proportional to their concentration.

2.7. Measurement of Glutathione Peroxidase Activity

Similar to the previous experiment, a spectrophotometer DU 800 (Beckmann, USA) and kits from RANDOX, UK, were used to assay the activity of GPx. First, the chemicals, the aqueous extract, and lastly, the substrate were added to prepare the reaction mixture. The absorbance wavelength is set at 340nm. Combining the peroxidase reaction with the glutathione reductase and nicotinamide dinucleotide phosphate (NADPH) mediated reduction of oxidized glutathione is the principle of this assay (Ahmed, Gul, Idris, et al. 2014). t-Butyl-hydroperoxide (hydrogen peroxide or tert-butyl hydroperoxide) reduction was followed by the decrease in absorbance of NADPH at 340 nm.

2.8. Measurement of Superoxide Dismutase Activity

SOD activity was evaluated using RANDOX, UK kits available commercially, and the experiments were conducted on a spectrophotometer DU 800 (Beckmann, USA). Oyanagui and his colleagues outline the assay to gauge SOD activity (Ōyanagui 1984). The reaction mixture was formulated by adding all the chemicals first, then the aqueous extract, and then the substrate. The absorbance was measured at 505nm wavelength. Superoxide radicals generated from the xanthine and xanthine oxidase form a formazan dye. The extent of reaction suppression serves as the parameter to gauge SOD activity.

2.9. Calcium Channel Blocking Activity

Calcium channel-blocking experiments were performed as stated previously(Kulkarni 2004). Open access to water was provided to the guinea pigs with a 24-hour fasting period before the

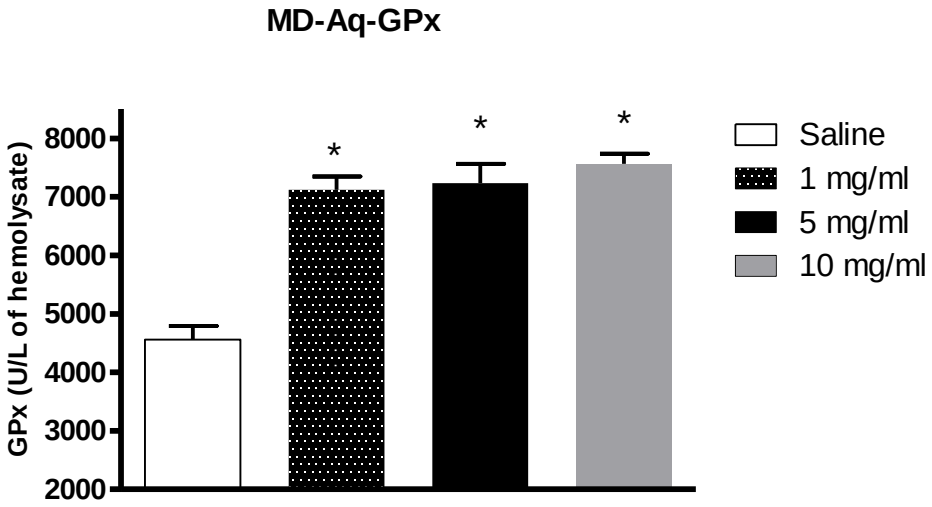

Figure 1: Graphical representation of the effect of MD-Aq on GPx levels in human blood. * represents P<0.05

experiment. The abdomen was cut, and the ileum was isolated after the animals were sacrificed through cervical dislocation. The ileum preparations were mounted in Tyrode's solution in 10 mL tissue baths, maintained at 37°C, and aerated with a mixture of 5% carbon dioxide in oxygen (carbogen). The tissues were incubated for 0.5 hours and subjected to a 1 g preload. A sub-maximal dose of 0.3 μm of acetylcholine (Ach) was administered to obtain a control response. The tissue was considered stable when two Ach doses, given consecutively, produced the same response. Potassium chloride (KCl) was used as an agonist (80 mM final bath concentration.

2.10. Statistical Analysis

The mean ± standard deviation (SD) of the mean was used to express all data. $p < 0.05$ was deemed as statistically significant.

3. Results

MD-Aq showed dose-dependent improvement in the GPX activity (See figure 1). There was a big elevation in GPx levels to 7121 (U/L of hemolysate) with the smallest dose of 1 mg/ml. However, with higher doses of 5 and 10 mg/ml, only slightly more elevation was observed. GPx levels at all three doses were not markedly different from each other but were significantly higher than the effect of saline. The standard used (vitamin C at 100 μg/ml) produced GPx levels of 8769 (U/L of hemolysate).

MD-Aq has no significant effect on SOD activity. SOD activity increased at all three doses but did not reach statistical significance (see Figure 2). The highest SOD levels were observed with 10 mg/ml and were 185 U/L compared to 174 U/L for saline. The standard used (vitamin C at 100 μg/ml) produced SOD levels of 223 U/ml.

MD-Aq improved TAS levels in a dose-dependent fashion. However, at the first two doses of 1 and 5 mg/ml, elevation in TAS levels was not marked and did not reach statistical significance. TAS levels were highest at 10 mg/ml of MD-Aq, reaching 1.75 mmol/L of plasma (See figure 3). Thus only at the highest dose of MD-Aq, TAS levels were significantly high than the effect of saline. The standard used (vitamin C at 100 μg/ml) produced TAS levels of 1.80 mmol/L of plasma.

Application of MD-Aq against KCl-induced contraction in the guinea pig ileum also showed a dose-dependent response (see Figure 4). Only about 10% inhibition was observed at the first dose, but at the next two doses, the inhibitory response reached 50%. At 10 mg/ml, close to 90%

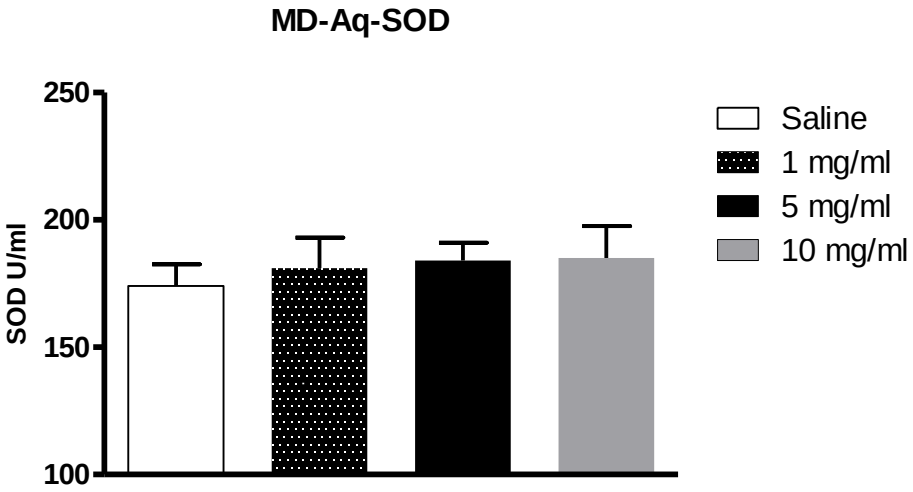

Figure 2: Graphical representation of the effect of MD-Aq on SOD levels in human blood. * represents P<0.05

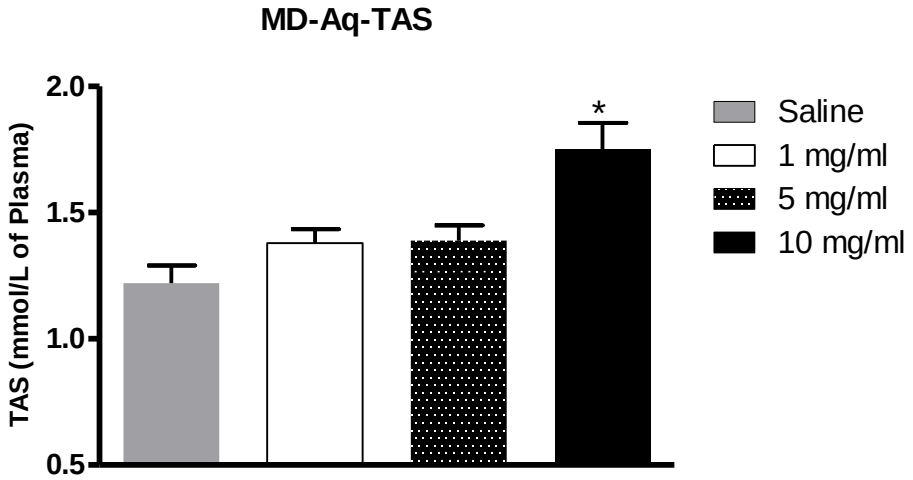

Figure 3: Graphical representation of the effect of MD-Aq on TAS levels in human blood. * represents P<0.05

inhibition was observed, compared with the effect of 1mM Verapamil (about 93%).

MD-Aq inhibited AA-induced human platelet aggregation in a dose-dependent manner. At each dose of 1, 5, and 10 mg/ml, a significantly higher inhibition was observed as compared to the saline effect. However, the maximum effect obtained at the highest dose was around 73% (Figure 5,6). On the other hand, Aspirin, a standard drug, showed almost complete inhibition of AA-induced platelet aggregation.

MD-Aq also inhibited PAF-induced human platelet aggregation but much less potently than AA-induced aggregation. Unlike against AA-induced aggregation, where about 73% inhibition was observed at 10 mg/ml, the maximum inhibition observed by MD-Aq was 17%. None of the doses of MD-Aq produced inhibition against PAF-induced aggregation that was significantly higher than that produced by saline treatment (See figure 7). The MD-Aq was not effective against collagen and ADP-induced platelet aggregation.

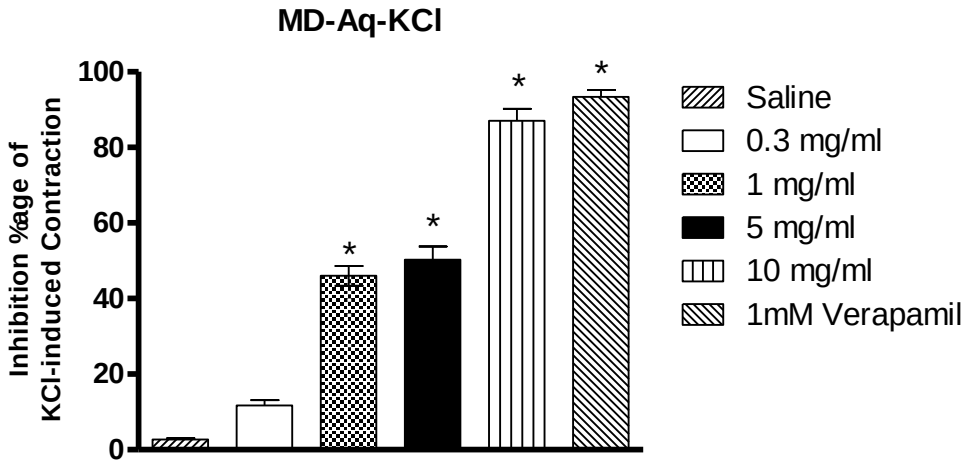

Figure 4: Graphical representation of the effect of MD-Aq on KCl-induced contraction in guinea pig ileum. * represents P<0.05

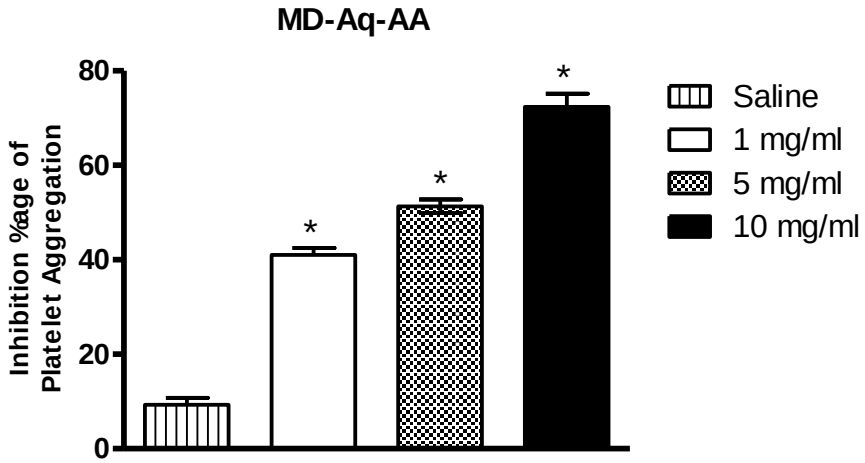

Figure 5: Graphical representation of the effect of MD-Aq on AA-induced human platelet aggregation. * represents P<0.05

4. Discussion

Medicinal plants, fruits, and vegetables have repeatedly shown strong pharmacological activities that are useful in many diseases (Afzal et al. 2022, Saghir et al. 2022, Naseer et al. 2022). One of the world's most widely grown fruit trees is the apple. Despite extensive research into the chemical constituents of apples (Wu et al. 2007, Ceymann et al. 2012), data regarding the cardioprotective effects of apples are scarce. Detailed scientific research information not only on the pharmacological activities of apples but on the disparity in the content and composition of phenolic compounds would serve as a vital stepping stone in the use of apples as a viable source of phenolic compounds in medical practice. Cosmetics and dietary supplements rich in phenolic compounds are also isolated from apples. Phloretin glycosides, catechins, phenolic acids, and some quercetin glycosides were identified as the main phenolic compounds in a few investigations (Picinelli, Dapena, and Mangas 1995, Bonarska-Kujawa et al. 2011, Mayer et al. 2001). Not only are these compounds useful in

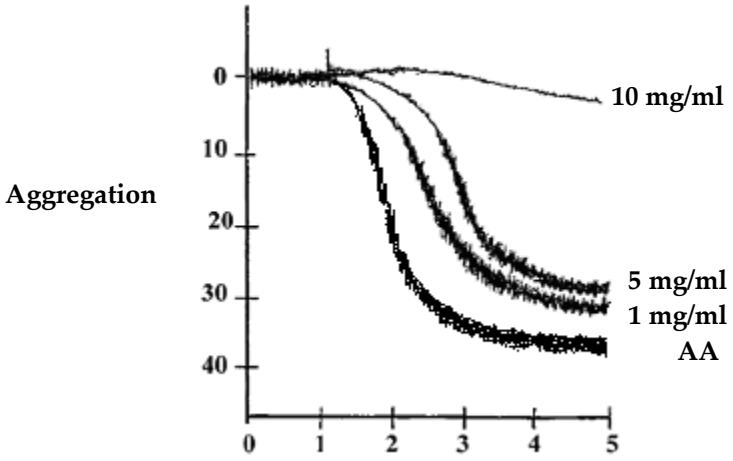

Figure 6: A scan of the effect of MD-Aq on AA-induced platelet aggregation in human blood.

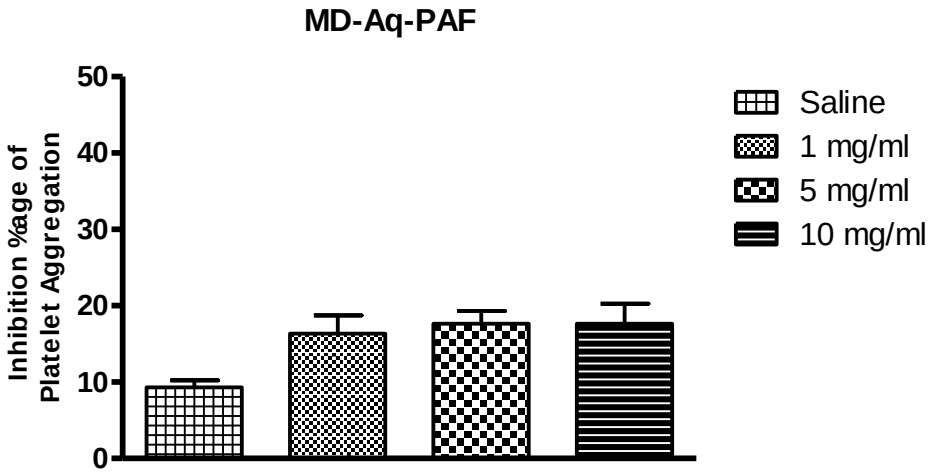

Figure 7: Graphical representation of the effect of MD-Aq on PAF-induced human platelet aggregation. * represents P<0.05

treating various ailments, but have a vital role in the plant's defense against various forms of stress and fungal infections (Hua et al. 2014, Schovánková and Opatová 2011, Veberic et al. 2005).

In our investigation, MD-Aq showed dose-dependent improvement in the GPX activity comparable to the effect of vitamin C. This GPx enhancing effects of MD are not unique. They have been reported earlier for several other medicinal plants, fruits, and vegetables and for the MD itself, albeit for a different solvent-based fraction of MD (Rukhsana Nawaz 2022). MD-Aq improved TAS levels in a dose-dependent fashion. Although the pharmacological effect was not high in the first two doses, the third dose produced a significant pharmacological effect. Similar effects have been previously reported for several other medicinal plants (Gul et al. 2013).

Calcium channel-blocking activities have been reported for several medicinal plants. In our study, the application of MD-Aq against KCl-induced contraction in the guinea pig ileum also showed a dose-dependent inhibition. A 10 mg

dose produced a response comparable to the standard calcium channel blocker-Verapamil. Many previous investigations have shown strong calcium channel-blocking effects of the crude extracts and phytocompounds isolated from medicinal plants (Gul et al. 2013).

Natural inhibition of platelet aggregation, either endogenous or exogenous, has been well documented (Gul et al. 2013). MD-Aq inhibited AA-induced human platelet aggregation in a dose-dependent manner but was much less potent against PAF-induced aggregation. However, MD-Aq was not effective against collagen and ADP-induced platelet aggregation. Similar pharmacological effects of medicinal plants have been reported previously(Ahmed, Gul, Idris, et al. 2014) (Gul et al. 2013).

It has been reported that apples and other healthy fruits and vegetables offer pharmacological benefits through various mechanisms such as scavenging free radicals, inhibiting free radical production, and enhancing the synthesis of antioxidant enzymes to protect the body from damage (Fernandez-Panchon et al. 2008, Halliwell, Rafter, and Jenner 2005, Balasundram, Sundram, and Samman 2006). Phenolic compounds also have other pharmacological activities, including antimicrobial, anti-inflammatory, antineoplastic, cardiotonic, and other activities (Duthie, Duthie, and Kyle 2000, Middleton, Kandaswami, and Theoharides 2000). Phloridzin is abundant in apples (Petkovsek et al. 2010, Gosch et al. 2009) and has antidiabetic potential (Masumoto et al. 2009). Therefore, it is likely that the pharmacological effects of MD-Aq observed in the current study may also be due to the phenolic components of MD-Aq.

5. Conclusions

Our investigation showed that MD-Aq has significant cardioprotective potential. We found that MD-Aq could elevate GPX and TAS levels, is inactive at SOD, and is excellent as a calcium channel blocker, a more potent blocker of AA-induced aggregation than PAF-induced aggregation. Our study rationalizes the traditional use of MD against several cardiovascular diseases and points to MD-Aq as a source of raw material o make drugs from to help treat various cardiovascular diseases.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

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Study Approval

The study was approved by the Department of Pharmacy, Kohat University of Science & Technology, Kohat, Pakistan.

Consent Forms

NA.

Data Availability

All the data related to this manuscript are available with the authors**.**

Author Contributions

Main idea and conceptualization, and initial draft by FA, literature collection, and review by FS & TS, graphics, language and grammar by BR, analysis and proofreading by FS and TS, review editing, ebuttals and final draft by FA.

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