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Research Article

# Cardioprotective Effects of *Malus domestica* (apple) May be Mediated Through Antiplatelet, Calcium Channel Blocking, and Antioxidant Properties

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## Abstract

Research on the cardioprotective potential of *Malus domestica* (MD or apple) has been of considerable importance, and efforts are being increasingly focused on investigating their effects on human health. However, relatively little work has been done to investigate the cardioprotective effects of crude extract of MD. In the present study, the antioxidant potential of crude methanolic extract of MD fruit part was investigated by measuring its effects on glutathione peroxidase (GPx), superoxide dismutase (SOD) enzymes, and total antioxidant status (TAS) in blood samples. Antiplatelet and calcium channel-blocking activities were also investigated. Our results reveal that crude methanolic extract MD showed a significant concentration-dependent increase in GPx, and SOD but not in TAS compared with saline solution (0.9% NaCl) used as the negative control and vitamin C as a positive control. Furthermore, crude extract of MD inhibited calcium channels and showed strong antiplatelet effects against arachidonic acid and platelet-activating factor-induced platelet aggregation. These results suggest that the cardioprotective effects of MD may be mediated through multiple pathways, including calcium antagonizing, antiplatelet, and antioxidant properties.

**Keywords**: *Malus domestica*, calcium channels, glutathione peroxidase, superoxide dismutase, total antioxidant status, platelet-activating factor, arachidonic acid.

## Introduction

An important member of the Rosaceae family, a plant family that consists of more than 100 genera and 3000 species and is the third most economically important family in the temperate regions, is Malus Domestica (MD)- better known as the apple (Dirlewanger et al. 2002). The flesh, dried form, processed form, and juices are some of the many ways in which Rosaceae plants are consumed. Flavonoids, cyanogenic glucosides, phenolic derivates, and phytoestrogens are some of the wide varieties of phytochemicals that are found in rosacea fruits (Mazur et al. 2000), where phenols bear the potential to provide health benefits and exhibit curative advantages (Macheix, Sapis, and Fleuriet 1991, Swanson 1998, Selmar 1999). Antioxidants and/or antineoplastic compounds that have been identified in these fruits. These compounds include but are not limited to L-Ascorbic acid, quercetin, kaempferol, myricetin, p-coumaric acid, gallic acid, and ellagic acid.

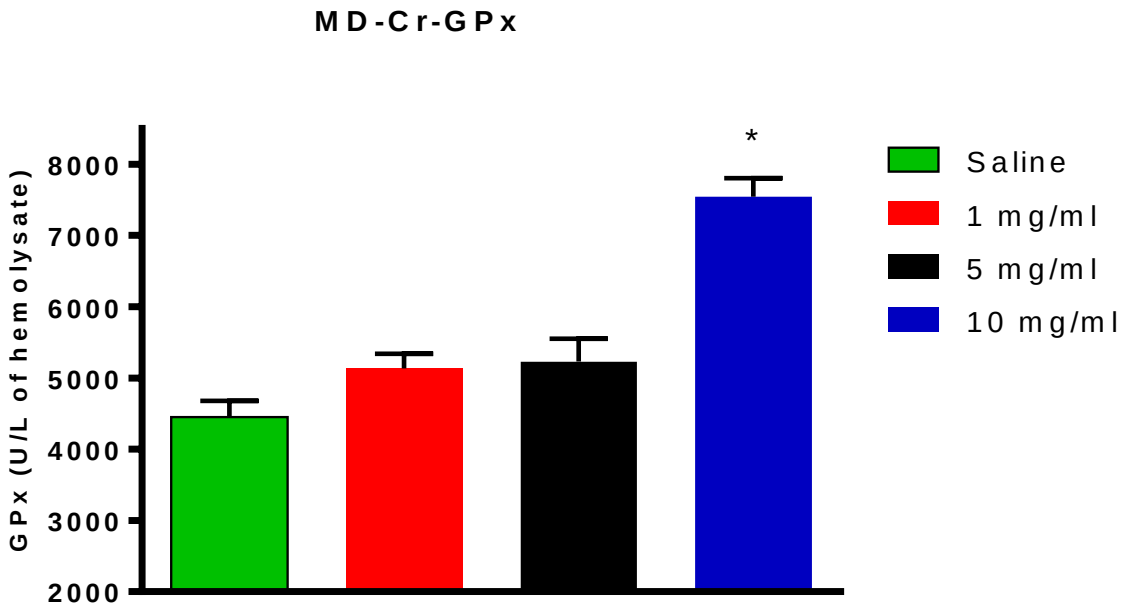


Figure 1: Graphical representation of the effect of MD-Cr on GPx levels in human blood. * represents P<0.05

The phytocompounds extracted from apples bear great medicinal value and have been used for such purposes over the course of time. The fruit and vinegar obtained from apple juice also hold curative properties (Johnston and Gaas 2006). Reduced cardiovascular disease (CVD) and reduction in coronary and total mortality have all been observed (Knekt et al. 1996). A decrease in chronic obstructive pulmonary disease symptoms (Tabak et al. 2001), and improvements in preventing the risk of thrombotic stroke (Knekt et al. 2000) have all been highly associated with apple consumption. The mechanical action of eating a fruit allows for the cleansing of both the teeth and the gums. Subsequently, apples serve as excellent dentifrices (Grieve and Leyel 1984). Increased thrombocytic activity and normalization of the nervous system are some of the many curative properties of apple cider, alongside its nutritive value and its ability to act as a source of vital energy. The smooth muscles of the heart, vascular walls, and gastrointestinal tract are all strengthened by the intake of apples. Marked improvement in gums has also been observed. The amino acids Cysteine and arginine, as well as malic acid, present in apples have the ability to eliminate, from the body, any stored toxic substances. The anti-inflammatory effects of these substances serve to counter gout, uric acid, urticarial ailments, and renal diseases (Patel et al. 2012). Khan and colleagues in 2011 reported that 3% apple juice, when formulated as Stable water in oil emulsion, exhibited anti-sebum production effects, decreased melanin level, greasiness, and erythema, causing acne when applied on hyper-pigmented human skin, subsequently enhancing the appearance of oily facial skin (Khan et al. 2011). Several of its pharmacological actions, such as antioxidant, antiarthritic, anti-cancer, cholesterol-lowering, and antidiabetic, have been reported previously (Patocka et al. 2020).

However, very few scientific investigations have been carried out to determine if the cardio-protective effects of apples are due to their potential impact on platelet aggregation, calcium channels, and antioxidant enzymes present in the blood. Here in this study, we decided to explore these questions. We investigated the actions of crude methanolic extract of the fruit

part on arachidonic acid (AA) and platelet-activating factor (PAF)-induced human platelet aggregations, on calcium channels, and antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and total antioxidant status (TAS).

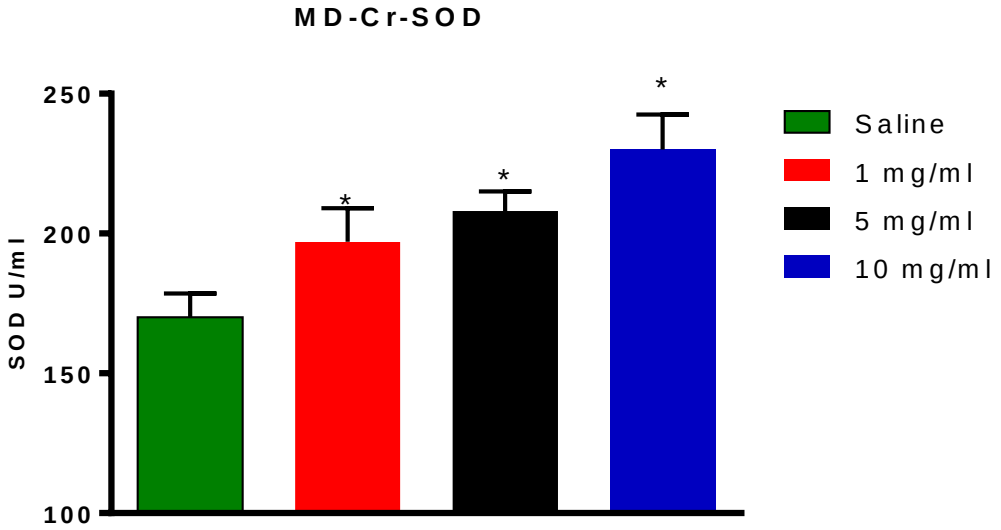


Figure 2: Graphical representation of the effect of MD-Cr on SOD levels in human blood.
*represents P<0.05

## Material & Methods

### Fruit Material

5kg of MD fruit was obtained from the market. A taxonomist from the Department of Botany, Faculty of Sciences, University of Karachi, Pakistan, was responsible for identifying the desired variety. For future reference, a specimen has been kept in the laboratory. Mashed fruit pulp, at room temperature, was stored in a tight container for future use.

### Crude Extract Preparation

The mashed fruit was soaked in 5 Liters of 70% methanol for a duration of 3 days with periodic shaking. Sequential filtration was performed using a muslin cloth and a qualitative (20-25µm) Grade 4 Whatman filter paper. After three repeats, using a rotary evaporator, the combined filtrate was evaporated to a dark brown, dense semi-solid mass under reduced pressure (to prevent thermal decomposition of the phytochemicals). This is referred to as the crude extract (MD-Cr). The yield was 16%.

All reagents were of the highest quality and purity. Distilled water and normal saline were used to prepare the stock solutions and dilutions, respectively. A maximum of 20µL of the stock solution was used to induce aggregation. A 2mg/vial, which is 3.83mM, was diluted to 38.3µM in saline (1:100) to prepare the PAF solution. To induce aggregation 5-10 µL of the dilution was added to Platelet Rich Plasma (PRP).

### Animals

500-750 grams of both male and female local guinea pigs were used for the experiments in accordance with ethical guidelines for animals of the University of Karachi. The specimens were housed and bred in controlled conditions (23–25 °C) temperature, regular exposure to light and dark cycles, tap water ad libitum, and a

standard diet) in the University of Karachi animal house.

**Preparation of Platelets**

Blood samples were obtained via venipuncture from the healthy volunteers who were medication free for seven days. A sodium citrate solution 3.8% W/V was added to the blood samples, and the resultant solution was subjected to centrifugation to obtain PRP for 15 minutes at 20 degrees Celsius with a rotation of 260g. PRP having platelet levels between 2.5 and 3.0 x $10^8$ $mL^{-1}$ of plasma were determined using Phase Contrast Microscopy, and at 37 degrees Celsius, all aggregation studies were performed (Ahmed et al. 2014).

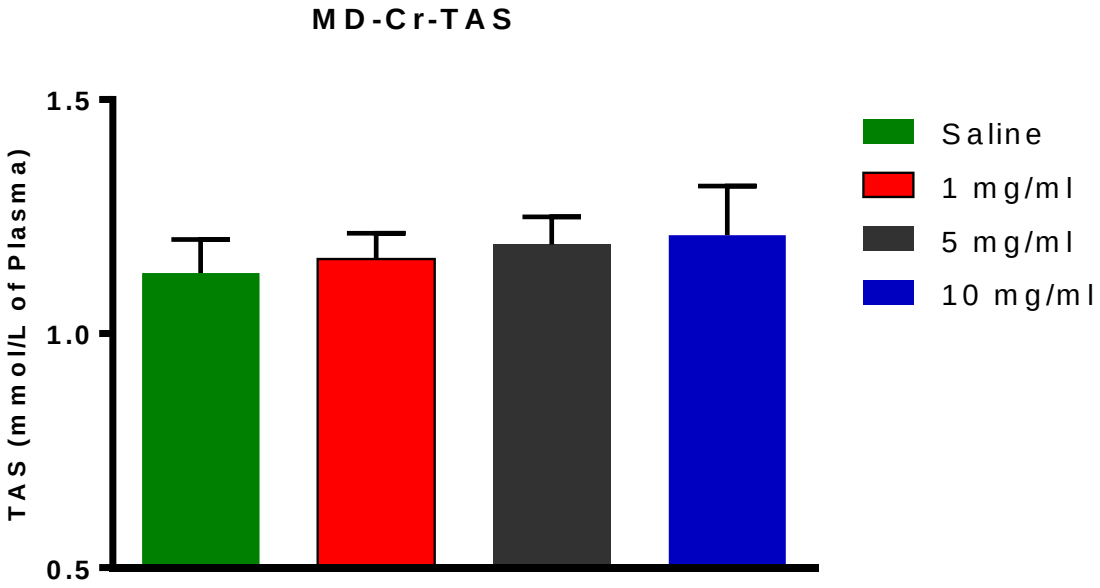


Figure 3: Graphical representation of the effect of MD-Cr on TAS levels in human blood.

**Measurement of Platelet Aggregation**

Using 0.45mL aliquots of PRP, a Dual-channel Lumi- aggregometer (Model 400 Chronolog Corporation, Chicago, USA) apparatus was used to measure aggregation (Aslam et al. 2008). 0.5mL was the final volume makeup using the standard drugs dissolved in normal saline and/or appropriate non-aggregating medium or crude extract. AA/PAF were employed to carry out platelet aggregation. The effect of the crude extract to prevent aggregation was quantified at multiple doses through incubation with PRP for 1 minute, after which aggregating agents were added. Light Transmission versus Time data was used to determine the extent of aggregation 5 minutes after adding all the reagents and/or standard drug/crude extract. Dose-response curves with associated $IC_{50}$ values were generated once antiplatelet responses were established.

**Measurement of Total Antioxidant Status**

Spectrophotometer DU 800 (Beckmann, USA) for conducting assays and commercially available kits from RANDOX, UK, were used to measure TAS levels. The assay protocol was adopted from Mitrevky and associates (Ahmed et al. 2014). Chemicals were added, then the crude extract, and then the substrate. The core principle of the technique is the ability of plasma antioxidants to prevent 2,2' azino-bis-[3-ethylbenz-thiazoline-6-sulfonic acid] (ABTS)

from undergoing oxidation into ABTS+ by metmyoglobin. The amount of ABTS+ produced is monitored by reading the absorbance at 600nm. Under these reaction conditions, the antioxidants in the plasma cause suppression of the absorbance at 600nm to the degree that is proportional to their concentration. A concentration vs absorbance data was generated

**Measurement of Glutathione Peroxidase Activity**

Kits from RANDOX, UK, and spectrophotometer DU 800 (Beckmann, USA) were used to obtain GPx levels. First, all the chemicals, then the extract, and finally, the substrate were added to the reaction mixture. For GPx measurement maximum wavelength was set at 340nm. In this assay, GPx is measured by coupling the peroxidase reaction with the reduction of oxidized glutathione by glutathione reductase and NADPH (Ahmed et al. 2014). t-Butyl-hydroperoxide (hydrogen peroxide or tert-butyl hydroperoxide) reduction was followed by a decrease in absorbance of NADPH at 340nm. The activity was evaluated using GSH as the co-substrate (Ahmed et al. 2014).

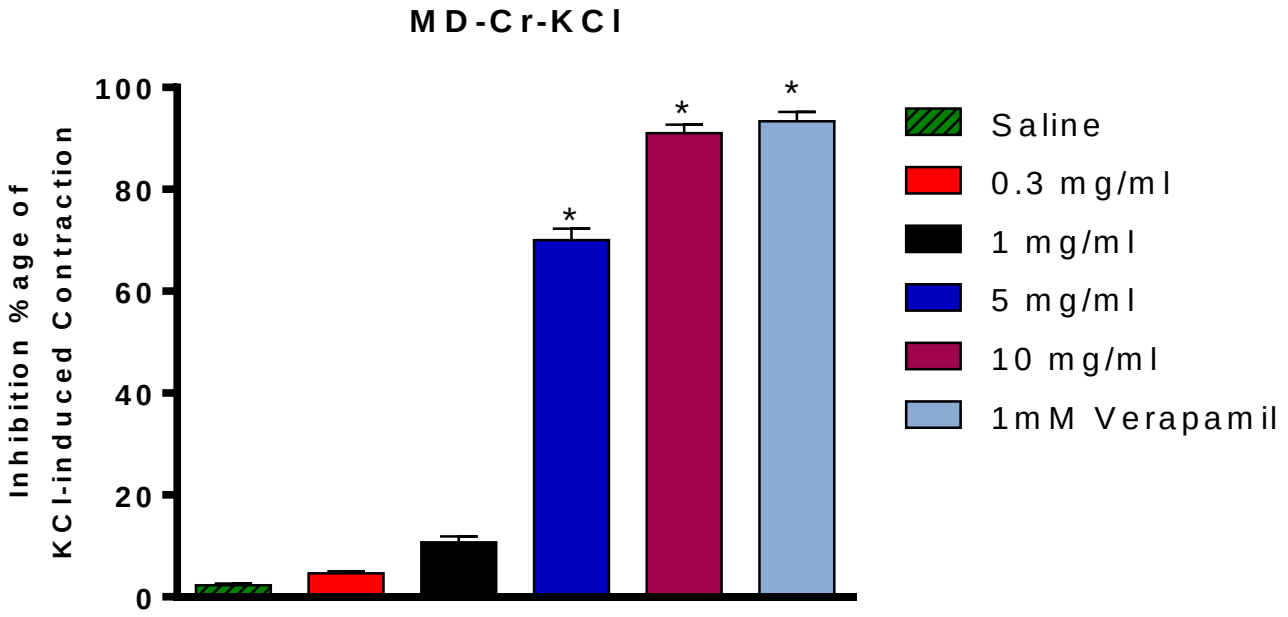


Figure 4: Graphical representation of the effect of MD-Cr on KCl-induced contraction in guinea pig ileum. * represents P<0.05

**Measurement of Superoxide Dismutase Activity**

The assay for measuring SOD was conducted as per the protocol delineated by Oyanagui and his colleagues (Oyanagui 1984). The sequence for preparing the reaction mixture is as follows 1) Adding all the reagents, 2) adding the crude extract, 3) adding the substrate. Kits from RANDOX, UK, and spectrophotometer DU 800 (Beckmann, USA) were used to measure the activity of SOD. The absorbance wavelength was set at 505nm. Superoxide radicals are generated from Xanthine and Xanthine Oxidase to form formazan dye which is essentially 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride. The extent of the inhibition of this reaction determines the extent of SOD activity. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of the INT under the condition of the assay.

**Isolated Tissue Experiments for Calcium Channel Blocking Activity**

The animals were deprived of any food 24 hours prior to the experiment but were given free access to water. The abdomen was cut, and the ileum was extracted after the animals were terminated via cervical dislocation. Preparations were mounted in 10mL tissue baths aerated with carbogen, acclimatized to 37 degrees Celsius containing Tyrode's solution. Subjected to incubation for 30 minutes with a 1-gram preload, control and sub-maximal responses (with acetylcholine 300mM) were recorded. When two consecutive doses yielded equal responses, the tissues were deemed stable. Once stabilization was achieved against 80 mM final bath concentration of KCl, which was used as the agonist, the ability of MD-Cr to block Calcium channels was investigated.

**Statistical Analysis**

All values are expressed as Mean ± Standard Deviation (SD), where a p-value of <0.05 was termed statistically significant.

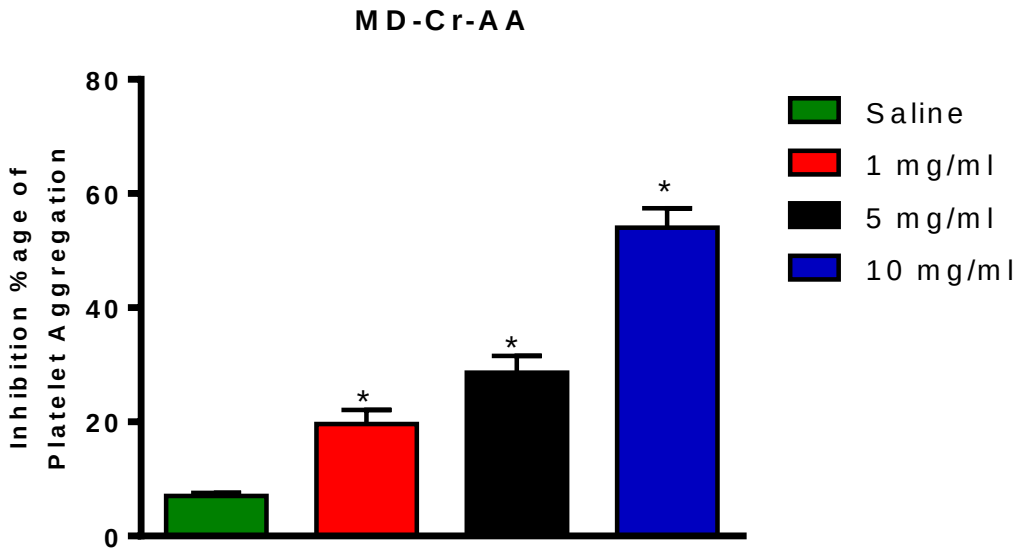


Figure 5: Graphical representation of the effect of MD-Cr on AA-induced human platelet aggregation. * represents P<0.05

**Results**

Figure 1 illustrates that a dose-dependent increase in GPx levels is observed upon exposure to MD-Cr. Doses of 1,5 and 10mg/ml induced GPx enhancement, yet a statistically significant increase was only seen in the latter dose (throughout the article, statistical significance is considered at $P < 0.05$). Vitamin C at 100µg/ml was used as the Standard, producing GPx levels of 8769 (U/L of hemolysate).

A positive trend was also seen between SOD levels and doses of MD-Cr. Contrary to the GPx results, where a statistically significant increase was seen at high doses only, the rise in SOD levels upon exposure to MD-Cr was much higher at every dose compared to treatment with normal saline (See figure 2). The same concentration of Vitamin C used to test GPx was taken as the Standard and yielded SOD levels of 223U/mL.

Statistically significant elevation of TAS was not achieved even at the maximum dose of MD-Cr (See figure 3). 1.80mmol/L of plasma TAS levels were seen in the presence of the Standard used (vitamin C at 100µg/ml).

$Ca^{2+}$ channels are responsible for carrying contraction of the guinea pig ileum, and the effect of Md-Cr on ileum contraction due to Potassium Chloride (KCl) was studied. The First two doses did not produce much effect. Interestingly, high doses of MD-Cr 5mg/mL and 10mg/mL induced statistically significant contraction comparable to $Ca^{2+}$ blockade induced by the standard 1mM Verapamil (See figure 4).

Regarding the AA-induced platelet aggregation, doses of 1mg/mL, 5mg/mL, and 10mg/mL produced significant inhibition of platelet aggregation. Figure 5 shows that the maximum inhibition of 54% was seen at the highest dose Aspirin, the Standard, carried out completed platelet inhibition.

Unlike AA-induced platelet inhibition, PAF-induced inhibition was much more potent. The first dose of Md-Cr was able to produce 70% inhibition at doses of 1mg/mL, which is 16% greater than the inhibition of platelet aggregation produced by AA at the highest dose of Md-Cr (See figure 6). At all three doses of 1mg/mL, 5mg/mL, and 10mg/mL, SM-induced platelet inhibition was statistically significant and comparable to Aspirin. Figure 7 summarizes all the pharmacological activities of MD-Cr investigated in this study.

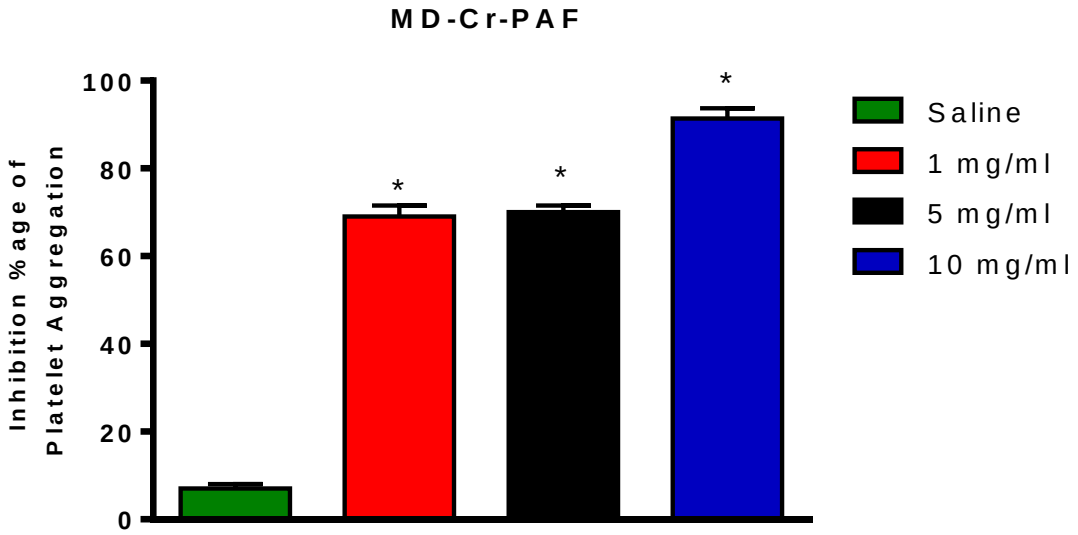


Figure 6: Graphical representation of the effect of MD-Cr on PAF-induced human platelet aggregation. * represents P<0.05

**Discussion**

Many research groups have determined that the total content of phenols and their derivates from apples is between 110 to 357mg/100g of fresh apple (Podsędek et al. 2000). This demonstrates that MD is a viable source of phenolic compounds (Eberhardt, Lee, and Liu 2000). Also, one study determined that the significant antioxidant activity of peeled and unpeeled apples exhibited antineoplastic effects and inhibited the in-vitro growth of human cancer cells (Eberhardt, Lee, and Liu 2000). Interestingly, ascorbic acid, commonly known as the water-soluble vitamin C, only contributed to less than 0.4% of the total antioxidant effect, thus suggesting that phenolics and other factors

are the main players in this regard. Un-peeled apples showed a greater extent of anti-proliferative and antioxidant activity with reference to peeled apples. Burda et al., 1990 & Escarpa, 1998 have shown that the peel of the apple, in comparison to the flesh of the fruit itself, has a higher concentration of total phenolic compound (Escarpa and González 1998, Burda et al. 1990). In light of the aforementioned facts, it is reasonable to conclude that the apple peels possess more bioactivity than the flesh.

In line with findings described by the investigations of Kim et al. (2010) alongside Bashir and Gilani (2008) conducted with various other plant extracts, our extract MD-Cr fraction also enhanced GPx levels (Kim et al. 2010). The elevation in SOD levels points to MD also consisting of several chemicals that can increase SOD content. This is consistent with the results of other studies conducted on different plants, including Lee et al. (2010) and Gupta et al. (2010), which reported similar antioxidative activities. MD-Cr, however, did not significantly affect the levels of TAS (Gupta et al. 2010, Lee et al. 2010).

Many plants possess calcium channel-blocking effects (Brankovic et al. 2009, Shah, Bhulani, et al. 2010, Shah and Gilani 2010, Shah, Gowani, et al. 2010, Perez-Hernandez et al. 2008, Gilani et al. 2009, Gilani et al. 2007). Calcium Channels Blockers (CCBs) are commonly used in cardiovascular diseases, particularly in cases of primary hypertension. Unlike other vasodilators, they are most effective as blood pressure-lowering agents in hypertensive than in normotensive rats and humans. Buddleja crispa has also been deemed to demonstrate calcium channel-blocking effects (Gilani et al. 2009). In geriatrics, CCBs are considered the treatment of choice over Beta-Blockers. In the current investigation, MD-Cr showed significant calcium channel-blocking potential.

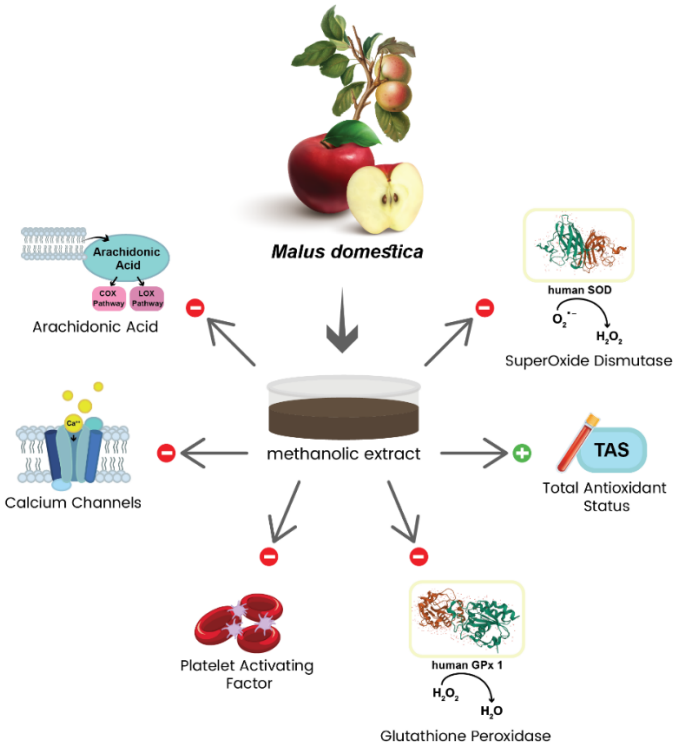


Figure 7: Summary of the pharmacological activities of MD-Cr investigated in this study.

Pro-inflammatory markers such as AA and PAF were greatly suppressed by Md-Cr.

Furthermore, partial inhibition of AA-induced aggregation was observed, which is corroborated by many research articles (Kamruzzaman et al. 2010, Bukhari et al. 2010, Shah et al. 1997, Shah et al. 1998, Shah et al. 1999). MD-Cr markedly inhibited PAF-induced human platelet aggregation. Similar activities have been reported from other plants previously (Amrani et al. 2009, Bukhari et al. 2010, Shah et al. 1997, Shah et al. 1998, Shah et al. 1999).

## Conclusions

It is observed that phytocompounds present in MD-Cr, at high doses, can elevate the levels SOD, GPx, blocks calcium channels, and potently inhibit PAF-induced aggregation but only partially block AA-induced aggregation. However, they proved to be ineffective on TAS levels. These results indicate that conventional cardioprotective actions of apples may be realized by affecting multiple pathways such as oxidant enzymes, platelets, and calcium channels.

## Conflict of Interest

The authors declare that they have no competing interests.


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## Consent Forms

Not applicable

## Authors Contribution

RN Conceptualized the study, HI and THA wrote the initial manuscript, did the experimental analysis, and RN supervised the whole project and wrote the final manuscript.


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