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Phytochemical screening and investigation of hepatoprotective activity and plasma antioxidant capacity of *Citrus reticulata* fruit against CCl_4-induced liver injury in rat

Saliha Djidel, Amel Bouaziz*, Nihed Barghout, Assia Bentahar, Seddik Khennouf and Saliha Dahamna

Laboratory of Phytotherapy Applied to Chronic Disease, of Biology and Animal Physiology, University Ferhat Abbas Setif 1, 19000 Setif,Algeria.

Corresponding author: **Amel Bouaziz** Email : a.bouaziz@univ-setif.dz , Bouaziz_amel2011@yahoo.fr

Tel: 213557762353

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Abstract

Mandarin (*Citrus reticulata*) is a fruit consumed largely in Algeria, belonging to the Rutaceae family. The aim of this study was to screening secondary metabolites and investigate the effect of ethanolic extract prepared from the fruit on the plasma antioxidant capacity and oxidative stress markers in CCl_4 induced-damage in liver of mice. Oral administration of mandarin extract at doses of 200 and 600 mg/kg for 7 days enhanced protein levels with values of 10.26±0.181 and 12.36±0.407 mg/ml, respectively when compared to CCl_4 group (10.10±0.071 mg/ml). Mandarin ethanolic extract showed a significant increase in catalase enzyme activity at a dose of 600 mg/kg (147.9±22.69 U/g tissue compared to CCl_4 group (49.33±13.93 U/g tissue). GSH level was increased in dose dependent in the liver with 8.17±0.32, 10.35±0.27 nmol/g tissue, respectively compared to CCl_4 group (6.663±1.25nmol/g tissue). The results showed that mandarin extract at doses of 200 and 600 mg/kg was more effective in enhancing antioxidant status in liver by reducing MDA level with 38.47±6.709 and 15.42±1.495 nmol/g tissue) when compared with CCl_4 group (120.2±4.85 nmol/g tissue). Also, *C. reticulata* extract showed good plasma antioxidant activity using DPPH and reducing power assays. These results indicate that mandarin consumption can be used as hepatoprotective effect.

Keywords : *Citrus reticulata*, Protein level, Catalase, Glutathione, Malondialdehyde, DPPH, Reducing power.

Introduction

The liver is particularly exposed to oxidative stress because of the direct release of CCl_4 metabolites and cytokines, which promote the inflammatory response. CCl_4 is a xenobiotic that can cause acute and chronic tissue injuries. It activates the phase I of cytochrome P450 system, producing reactive metabolic trichloromethyl radicals (CCl°_3) and peroxy trichloromethyl radicals (Khan et al., 2012).

Free radical generation during the hepatic metabolism of CCl_4 causes lipid peroxidation, radical interaction with structural proteins, nuclear and mitochondrial DNA, and DNA mistakes. Also, CCl_4 activates the liver's resident macrophages, which produce and secrete chemoattractants and neutrophil activators, resulting in neutrophil recruitment to that site and production of reactive oxygen species, which cause inflammatory changes in hepatocytes, leading to hepatotoxicity (Johra et al., 2023).

Antioxidant defense system consists of exogenous antioxidants like vitamin C, vitamin E, carotenoids, and polyphenols, with the diet serving as the main source, and endogenous (enzymatic and non-enzymatic) antioxidants like glutathione peroxidase (GPx), glutathione dismutase (SOD), catalase (CAT), and glutathione (GSH) (Bouayed and Bohn, 2010).

Polyphenols are the most common type of antioxidants in human diets and flavonoids, which include thousands of compounds, are the largest and most thoroughly researched class of polyphenols (Lima et al., 2014).

Mandarin *(Citrus reticulata)* belonging to Rutaceae family is one of the most consumed fruits in the world because of its appealing color, pleasant flavors, and phytochemicals content, such as phenolic acids, flavonoids, carotenoids, and vitamin C (Lee and Kim, 2022).

The antioxidant properties of *C. reticulata* extracts have been studied in various *in vitro* models, which revealed that extract have free radical scavenging, chelating, reducing, and anti-lipid peroxidant activities according to various in *vitro* antioxidant assays (Bentahar et al., 2020; Chaallal et al., 2020) . However, no scientific report of this fruit *in vivo* has ever been published in the literature demonstrating antioxidant efficacy in liver tissue. The current study aimed to assess the effect of ethanolic extract from mandarin fruit on plasma antioxidant capacity and antioxidative status in CCl_4 induced damage in liver of mice by evaluation some various biomarkers.

2. Materials and Methods

2.1. Plant material.

Mandarin fruits were obtained from a market in Setif region, located in Northeastern Algeria. The fruits purchased were of good quality, without damage. The fruits were washed with distilled water, peeled, and the seeds removed.

2.2. Animals

Abino rats weighing 180-200 g were obtained from the Pasteur Institute in Algiers, Algeria, and acclimatized for one week before the experiments. The animals were housed in an air-conditioned animal room with unlimited access to water and food. The experimental protocol in rats was carried out following revision and permission by the Animal Ethics Committee of the Institute of Nature and Life Sciences, University Ferhat Abbas, Setif 1, Algeria.

2.3. Preparation of fruit Extract

The extraction method was conducted by Markham (1982). The consumed part of the fruits (1kg) was carefully prepared by cutting them into small pieces. Subsequently, the pieces were ground in a mixer and homogenized before being mixed with five liters of ethanol (80:20, v/v). The mixture was kept at room temperature to maximize the extraction of bioactive molecules. The resultant solution was then filtered in order to extract the supernatant. A crude ethanol extract was obtained by subjecting the supernatant to evaporation in a vacuum rotary evaporator at a regulated temperature of 40°C. After that, this crude extract was dried and kept in a safe place at 4°C until it was needed.

2.4. Phytochemical screening

Using Haddouchi et al. (2016) method, the presence or absence of some compounds from the chemical families of secondary metabolites was determined. Phytochemical tests based on color, turbidity, or precipitation responses reactions.

2.5. *In vivo* antioxidant activity of extract

2.5.1. Treatment of animals

For a period of 7 days, the treatment was administrated orally via an orogastric tube to four groups of six each (Bouaiz et al., 2018) with minor modification. Group 1 was given normal saline 0.9 % and served as CCl_4 treated group . group 2 treated with vitamin C (100 mg/kg) and used as standard. The extract treated groups (3 and 4) received the fruit extract at doses of 200 and 600 mg/kg, respectively. On the seven day, all animals were given CCl_4 (2 ml/kg, IP as 50:50 solution in olive oil), which induced a damage in liver. Animals were anesthetized after the delivery of carbon tetrachloride. Blood was collected from the vena cava and the

serum was separated for determination of plasma antioxidant activity. Livers immediately removed and cleared with ice-cold saline and kept at -20°C.

2.5.2. Preparation of homogenate

After being cut into small pieces, the liver tissue was submerged in a 1.15 M KCL buffer. Fragments were homogenized to obtain 10% of homogenate and the resultant mixture was centrifuged for 10 min at 4°c and 4000 rpm. The supernatant was used for the determination of MDA as a marker of lipid peroxidation, total protein content, reduced glutathione (GSH) levels and catalase activity.

2.5.3. Determination of total protein level

Tissue protein content was measured by the method of Gornall et al., (1949), employing the Biuret reagent. Bovine serum albumin was used as a reference standard. 1 ml of biuret reagent was mixed with 25 µl of sample solution. After 10 min of incubation at 37°C, the absorbance at 540 nm was measured. Total protein level was calculated through the formula:

Total protein = (Abs of sample/Abs of standard)×

Where n is the standard concentration.

2.5.4. Catalase activity assay

Clairborne (1985) method was utilized to estimate the activity of catalase. The procedure recorded the reduction in ultraviolet (UV) absobance of the peroxide solution as a result of catalase-induced enzymatic hydrolysis of H_2O_2. 50µl of tissue supernant was added to a cuvette containing 2950 µl of H_2O_2 solution (19mM). the absorbance was measured at 240nm. Results were expressed as the quantity of enzyme that decompose 1 µM of H_2O_2 per minute at 25 °C. Consequently, the specific activity was expressed in terms of units per gram of tissue, in accordance with the provided formula below:

U/g tissue = (2.3033/ T) × (log A1/A2) /g tissue

A1: Absorbance at t_0.

A2: Absorbance at t_1.

2.5.5. Assessment of reduced glutathione level

the total reduced glutathione (GSH) content was investigated. Ellman's reagent (5,50-dithio-bis (2-nitrobenzoic acid); DTNB) reacts with sulfhydryl groups to produce 2-nitro-5-mercaptobenzoic acid (Huo et al., 2011). In brief, 50 µl of supernatant were diluted in 10 ml phosphate buffer (0.1 M, pH 8). Then, to 3 ml of the mixture of dilution, 20µl of DTNB (0.01 M) were added. The yellow color developed was read at 412 nm after 5 min. GSH (0.4-20

µmol/ml) was used to draw the calibration curve and the results were expressed as µmol/g of tissue (Adjadj et al., 2016).

2.5.6. Assessment of Malondialdehyde (MDA) levels

MDA levels were estimated by the method of Ohkawa et al., (1979). The pink color that is produced as a result of the reaction between TBA and MAD molecules. 250 µl of 20% TCA and 500 µl of 0.67 % TBA were combined with 250 µl of homogenate. The mixture was then allowed to cool afer being heated for 15 minutes in a water bath. 4 ml of n-butanol were added to each sample and centrifuged at 3000 rpm for 15 min. the absorbance was measured at 32 nm. The MDA quantity was evaluated from a standard curve od 1.1.3.3 tetraethoxypropane and the results were expressed as nmol/g of tissue.

2.5.7. Effect of extracts on plasma antioxydant capacity using DPPH assay

The capacity of plasma to scavenge the DPPH radicals was tested using the method of Burits and Bucar (2000) with slight modification. 25 µl of plasma was mixed with 625 µl of DPPH solution (0.004%). After 30 minutes of incubation, followed by centrifugation, the absorbance at 517 nm was measured, and the plasma antioxidant ability was calculated using the equation below:

% scavenging activity = [(Abs control -Abs sample)/Abs controls] X 100

A control: is the absorbance of the blank solution

A sample: is the absorbance in the presence of extact.

2.5.7. Effect of extracts on plasma antioxydant capacity using reducing power assay

The method described by Chung et al., (2005) was used to determine the reducing power. Plasma (0.1 ml) was combined with 0.1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 0.1 ml of 1% potassium ferricyanide. The resulting mixture was then incubated at 50°C for 20 minutes. Following this, 0.250 ml of 1% trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 minutes. A 0.250 ml sample from the upper layer was mixed with 0.250 ml of distilled water and 0.5 ml of ferric chloride (0.1%). The absorbance at 700 nm was measured.

2.6. Statistical analysis

To conduct statistical tests, we used Graph Pad Prism (Version 7.00). Mean ± standard deviation of mean (SEM) was used to express results obtained *in vivo*. One-way analysis of variance (ANOVA) and the Dunnets test were employed to estimate the signifof the results at a 5 % probability level.

3.Results and discussion

3.1. Phytochemical analysis

Phytochemical screening of showed that *C. reticulata* extract fruit contains polyphenols, flavonids, tannins, terpenois, phenols and saponins (Table 1).

Table 1: Phytochemical screening of ethanolic extract prepared from *C. reticalata* fruit.

Compounds	*C. reticulata* extract
Flavonoids	+
Tanins	++
Poyphenols	+++
Quinons	+
Terpenoids	+
Intraquinons	+++
Saponins	+

3.2. Determination of antioxidant status in the liver tissue

3.2.1. Total proteins level

The pretreatment of mice with *C. reticulata* ethanolic extract increased total proteins to 10.26±0.181 and 12.36±0.407 mg/ml at the doses of 200 and 600 mg/kg, respectively when compared to CCl_4 group (10.10±0.071 mg/ml). These results remain lower than that of Vit C as standard with a value of 18.61±1.885 mg/ml (Figure 3).

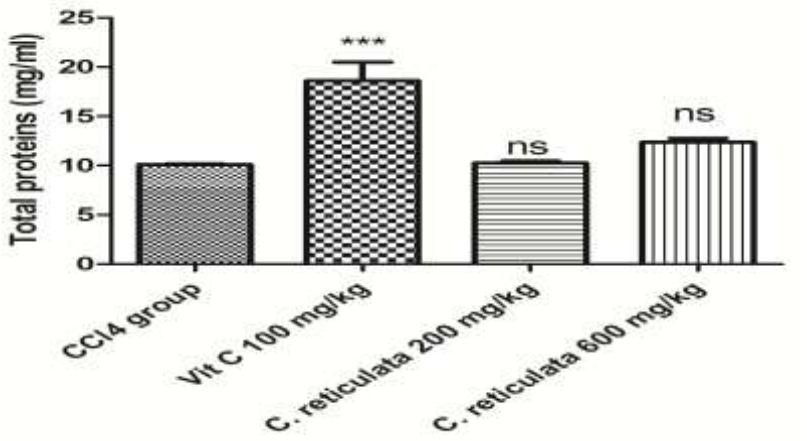

Figure 1: *Effect of C. reticulata on total proteins level in CCl_4-induced liver damage of rat. Vit C was used as standard and results were presented as mean±SEM (n=6). (^{ns} p>0.05; *** p<0.001) compared to CCl_4 treated group.*

3.2.2. Evaluation of catalase activity

As shown in Figure 2, daily oral admistration of *C. reticulata* extact at a dose of 200/kg increase no significantly the catalase activity (p≥0.05) with a value of 89.98±13.65 U/g tissue compared to CCl_4 treated group (49.33±13.93 U/g tissue). However, the treatment with the extract at the dose of 600 mg/kg significantly enhanced the catalase activity (147.9±22.69 U/g tissue).

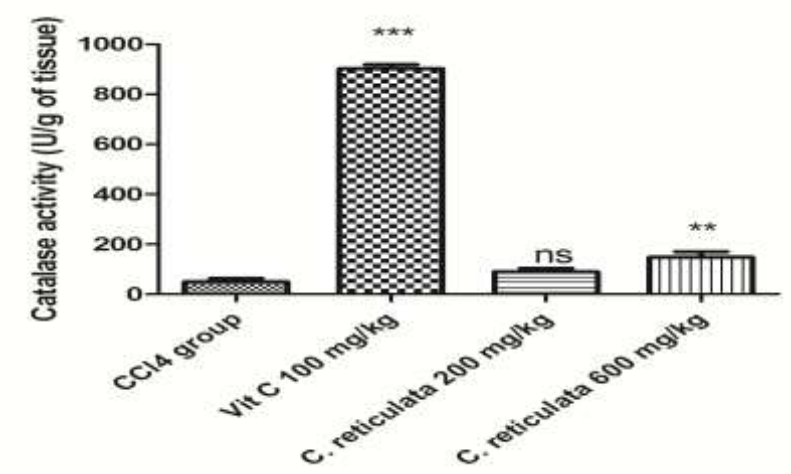

Figure 2: *Effect of C. reticulata and Vit C on catalase activity in CCl4-induced liver damage of rat. Results were expressed as mean±SEM (n=6). (^{ns} p>0.05; ** p<0.01; *** p<0.001) compared to CCl_4 treated group.*

3.2.3. GSH level estimation

In the present study, it was observed that tow doses of *C. reticulata* ethanolic extract (200 and 600 mg/kg) and Vit C increased the GSH levels with values of 8.17±0.32, 10.35±0.27 and 10.08±0.06 nmol/g tissue respectively compared to CCl4 treated group (6.66±1.25nmol/g tissue) (Figure 3).

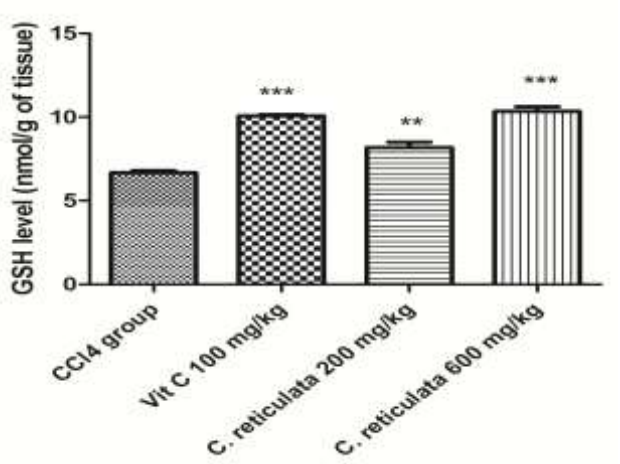

Figure 3: *Effect of C. reticulata and Vit C on GSH level in CCl4-induced liver damage of rat. values were expressed as mean±SEM (n=6). (** p<0.01; *** p<0.001) compared to CCl_4 treated group.*

3.2.4. Evaluation of lipid peroxidation

C. reticulata ethanolic extract had the ability to inhibit lipid peroxidation in liver tissue, lowering the MDA level, which is considered the final product of this process (Figure 4). Both groups treated with 200 and 600 mg/kg showed a strong ability to reduce MDA levels (38.47±6.709 and 15.42±1.495 nmol/g tissue) when compared with CCl_4 group (120.2±4.85 nmol/gtissue) (P<0.001). Vit C reduced the MDA amount to 19.73±3.177 nmol/g tissue.

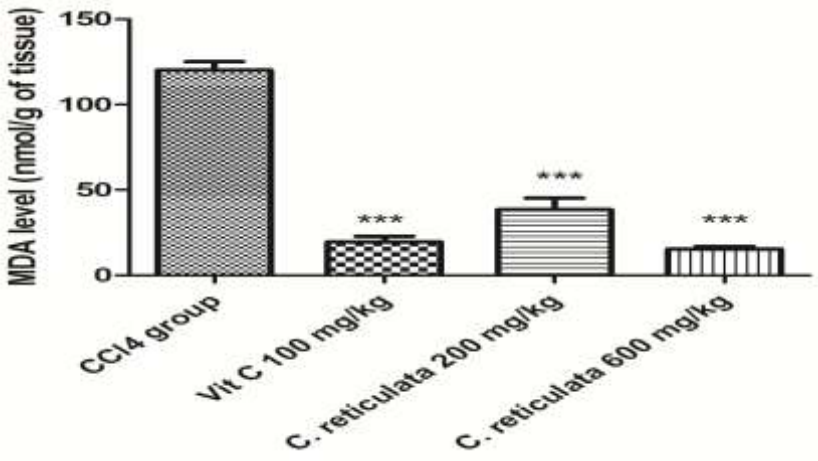

Figure 4: *Effect of C. reticulata and Vit C on MDA level in CCl_4-induced liver damage of rat. values were expressed as mean±SEM (n=6). (*** p<0.001) compared to CCl_4 treated group.*

3.3.Plasma antioxidant capacity

3.3.1. Effect of *C. reticulata* extract using DPPH radical

Daily oral administration of fruit extract prepared from *C. reticulata* (200 and 600 mg/kg/day) during 7 days augmented significantly the plasma capacity to scavenge DPPH radicals with

values of 47.26±2.94 and 64.95±2.88%, respectively compared to CCl_4 treated group (23,52±1.77).Vitamin C was used as standard that gave a value of 57.74±3.24% at dose of 100 mg/kg (Figure 5).

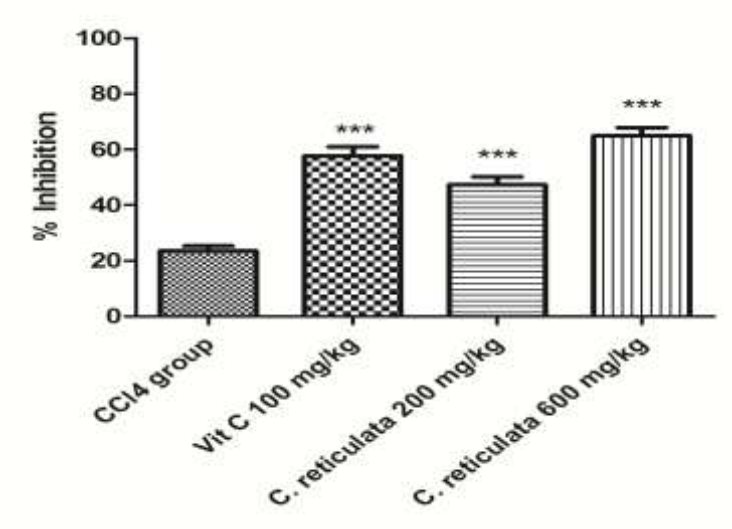

Figure 5: *Effect of C. reticulata and Vit C on plasma antioxidant capacity using DPPH radical scavenging. Data were expressed as mean±SEM (n=6). (***P<0.001) compared to CCl_4 group.*

3.1.2. Effect of extract on plasma reducing power

As seen in Figure 6, the administration of *C. reticulata* ethanolic extract revealed electron donation ability, but only the reducing power of the dose 600 mg/kg (0.32±0.02) was significantly higher than that of CCl_4 treated group (0.09±0.02). Vit C as positive standard had a high absorbance at 700 nm with a value of 0.67±0.02.

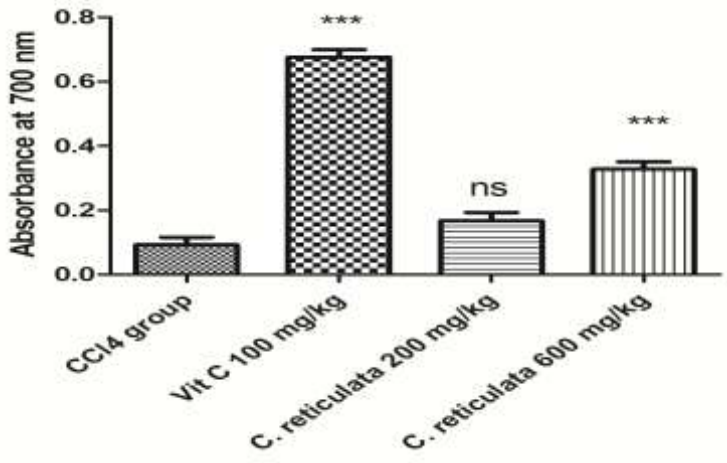

Figure 6: *Effect of C. reticulata and Vit C on plasma antioxidant capacity using reducing power. Data were expressed as mean±SEM (n=6). (^{ns} p>0.05; ***p<0.001) compared to CCl_4 group.*

4. Discussion

Fruits and vegetables contain high levels of biologically active secondary metabolites. These compounds are responsible for numerous health benefits. The main components found in the antioxidant properties of fruits and vegetables are polyphenols, flavonoids, carotenoids, Vitamin C, Vitamin E and glutathione (Rahaman et al., 2023). In our results, chemical profile in *C. reticulata* ethanolic extract showed the presence of many types of secondary metabolites such as polyphenols, terpenoides and intraquinons. Yield extraction of various plant compounds depends on the solvent polarity, which determines both the quantity and type of antioxidant molecules extracted. The time, temperature, sample ration to the solvent and chemical composition of the plant play an important roles in the extraction method (Mokrani and Madani, 2016). Bentahar et al., (2020) reported that ethanolic extract of mandarin had a total phenolic and tannins contents with values of 127.33 ± 2.32 and 47.65 ± 1.36, respectively. *Citrus* juices, in particular mandarin juice, are high in ascorbic acid which is an important antioxidant and a good indicator of mandarin juice quality (Selli et al. 2004). Mandarin essential oils, known for their fresh juic fragrance, are used in *Citrus* juice products as a natural flavoring agent. They contain volatile compounds like aldehydes, limonene, ketones, esters, alcohols, terpenes, β-myrcene, 3-carene, and α-pinene (Musara et al., 2020). Kelebec and Selli (2014) identified and quantified 11 phenolic compounds in mandarin juices, including hydroxybenzoic acids (3), hydroxycinnamic acids (5) and flavanones (3). Most fruit varieties contain significant levels of sugars that could be used in pharmaceutical and nutraceutical applications. The main dietary sugars are galctose, glucose and fructose (Hussain et al., 2023).

CCl_4 is a hepatotoxin agent that causes oxidative damage and hepatic injuries only after metabolic activation. When CCl_4 is metabolized, it produces free radicals, which can damage the hepatocyte membrane and organelles such mitochondria (Fatima et al., 2023). In the present study, we observed a significant decrease of proteins levels in CCl_4-Induced liver injury. Whereas, the pretreatment of rats with mandarin fruit extract elevated the protein content. The decrease of liver proteins in CCl_4 treated group can be explained by the fact that most proteins have groups (SH, OH) which react easily with the free radicals (Dong et al., 2016).

Catalase is a very important antioxidant enzyme. It is found in almost all aerobic organisms. Catalase converts two hydrogen peroxide molecules into a single molecule of oxygen (Nandi et al., 2019). In the current study, the increase in catalsase activity observed after

administration ethanolic extract of mandarin fruit could be attributed to the high levels of polyphenols in these extracts. Several studies have found that various polyphenols possess a positive effect on antioxidant enzyme activities (SOD, CAT, and GPx) (Kardum *et al.*, 2014; Wu *et al.*, 2015). Polyphenols can induce antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase that decompose hydroperoxides, hydrogen peroxide and superoxide anions, respectively, and inhibit the expression of enzymes such as xanthine oxidase (Tsao, 2010).

Glutathione, a tripeptide, acts as a non-enzymatic antioxidant. It is involved in the elimination of endogenous reactive oxygen species (ROS) (Lushchak, 2012). However, a decrease in its levels increases the sensitivity of cells to various damages, leading to tissue disorders. Many studies confirm that polyphenols such as heperin and rotenone works on the effective stimulation of the genes that express the enzymes GPX, CAT, and SOD, and it can also regulate the generation of GSH (Chen et al., 2012). The current study found that mandarin fruit treatment increased the level of GSH. Similarly, Jaiswa et al., (2015) reported that the lemon fruit extract enhanced the GSH level in the liver and kidney of rats after chronic carbofuran exposure. Lipid peroxidation of cell membranes is a crucial pathophysiological process in diseases and oxidative stress conditions. MDA is a major reactive aldehyde produced from the peroxidation of polyunsaturated fatty acid present in the biological membranes. It was hypothesized thatCCl_4- induced hepatotoxicity is mainly due to the lipid peroxidation of hepatocyte membranes by free radical derivatives of CCl_4 (Srilaxmi et al., 2010). The observation of elevated levels of hepatic MDA in CCl_4 treated group in the present study is consistent with this hypothesis. The treatment with mandarin peel extract relieved oxidative stress manifested by a decrease in malondialdehyde (MDA) levels with a significant elevation in antioxidant capacity (TAC), hepatorenal contents of superoxide dismutase (SOD), and reduced glutathione (GSH) (Bashandy et al., 2020).

The scavenging DPPH assay is a quick, easy and simple way to test antioxidant abilities. The DPPH radical is dark purple in solution when it is first reduced and then transformed into DPPH-H with light yellow (Baliyan et al., 2022). This method is effective to estimate oxidative stress in plasma or organs (Katalinic et al., 2005). In present study, mandarin fruit ethanolic extract increased the plasma antioxidant capacity using DPPH assay. This important effect could be attributed to the presence of phenolic compounds. Many studies have found a strong correlation between the phenolic content and antioxidant capacity of different juices (Floegel et al., 2011; Kelebek and Selli, 2014). The increase in plasma antioxidants appears to

be due to the high levels of externally derived antioxidants like ascorbic acid, phenolic compounds, carotenoids, and flavonoids (Chen et al., 2015).

The reducing capacity of a compound can be a good indicator of its potential antioxidant activity. This assay operates on the principle that substances with reduction effect react with potassium ferricyanide (Fe^{+3}) to form potassium ferrocyanide (Fe^{+2}). The complex formed reacts with ferric chloride to develop a another ferric-ferrous complex with an absorption maximum at 700nm (Bhalodia et al., 2013). De Graft-Johnson et al., (2007) investigated the correlation between the reducing power and the polyphenol structure. The same study confirmed that catechin, 3,4-dihydroxycinnamic acid, 3,4-dihydroxyhydrocinnamic acid, 3,4-dihydroxyphenylacetic acid, ferulic acid, gallic acid and quercetin distinct ability to reduce Fe 3+ ions at submicromolar concentrations that may well transpire in human plasma after ingestion of fresh fruits, vegetables, chocolate.

Conclusion

Mandarin fruit (*Citrus reticulata*) contains phytochemicals belonging to different classes such as pigments (flavonoids), phenolic compounds and micronutrients (vitamin C), which can minimize oxidative damage. The richness of biomolecules marks a primary interest in research and which arouses the interest of several researchers. Also, the results showed that treatment of rats with *C. reticulata* ethanolic extract increased catalase activity and GSH levels. It was found that mandarin extract was more effective in inhibition of lipid peroxidation that reduce MDA levels in liver tissues. On the other hand, it was showed a strong plasma antioxidant activity. Eating mandarin could be a good hepatoprotective agent.

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All coauthors are aware and approve the contents of the submitted manuscript.

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