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Research Article

INHIBITION OF PROTEIN CARBONYLATION IN HUMAN PLASMA BY ACETONE EXTRACT FROM HAWTHORN (*Crataegus mexicana*)

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A B S T R A C T

In recent years, various diseases have been linked to protein oxidation, changing their biological function due to the formation of carbonyl groups. The objective of this research is to evaluate the inhibition of carbonylation by the acetone extract of hawthorn (*Crataegus Mexicana*) in human plasma proteins in vitro inducing oxidative stress by iron sulfate. The quantification of carbonyl groups was developed by spectrophotometric technique using 2,4-dinitrophenylhydrazine (DNPH).We worked with 6 clinically healthy people. Three concentrations of the acetone extract of hawthorn (C. mexicana) were used at 10, 100 and 1000 mg/L. The concentration of 1000 mg/L of the extract of hawthorn presented greater inhibition in the carbonylation of proteins with more than 50%.

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INTRODUCTION

Exposure to X-rays, ozone, smoking, air pollutants and industrial chemicals in addition of many essential metabolic processes in the human body (Lobo *et al*., 2010).A free radical is any chemical species that contain one or more unpaired electrons in its outer orbital. (Maldonado *et al*., 2010).These species have characteristics colourless, tasteless and are highly reactive, are also key to form other free radicals called reactive oxygen species (ROS) (Guoying *et al*., 2017; Llancari and Matos, 2011). Oxygen is essential for aerobic sorganisms but in large quantities becomes toxic since it is the main promoter for the generation of ROS as: superoxide anion ($O_2^{\cdot-}$),hydrogen peroxide(H_2O_2),hydroxyl radical($HO^{\cdot}$) and hydroperoxyl ($HO_2^{\cdot}$) (Maldonado *et al*., 2010; Lehmann *et al*., 2015). Under normal conditions ROS production is controlled by primary-type antioxidant systems present in the body(Mateen *et al*., 2016).The imbalance between the ROS and the antioxidant systems of the organism, establish a condition called oxidative stress that will cause inflammation and various diseases such as: cancer, insulin resistance, diabetes mellitus, cardiovascular diseases, atherosclerosis, among others (Assim and Reem, 2012; Mittal *et al*., 2014; Galván *et al*., 2014), in addition to processes such as aging because they oxidize macromolecules of the organism (proteins, lipids and damage DNA)(Mittal *et al*., 2014).

For this reason in recent years, the study of protein oxidation has been increased, defined as "the covalent modification of a protein induced by direct reactions with reactive oxygen species or indirect reactions with secondary products of oxidative stress"(Zhang *et al*., 2013), the most studied is carbonylation (Moller and Rogowska, 2011).

The carbonylation caused by ROS is defined as an "irreversible posttranslational oxidative modification of the amino acid side chains"(Hlaváckováa *et al*., 2017), they alter their function and can produce cellular senescence and cell death (Castro *et al*., 2013),There is a growing research that reported the quantification of carbonyls (Graziano *et al*., 2015), as well as various techniques to detect and quantify these carbonyl groups bound to proteins which, are the direct product of the action of free radicals, (Fedorova *et al*., 2013; Gutiérrez *et al*., 2016), the most used is the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazones bound to proteins (Levine *et al*., 1990). Hydrazones can also be quantified spectrophotometrically or detected immunochemically with anti-dinitrophenyl antibodies for greater sensitivity (Luo and

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Wehr, 2009).To prevent increased protein oxidation, cells and aerobic organisms require antioxidant systems (Zamora, 2007). Therefore, the search for functional foods or nutraceuticals has intensified (Sindhi *et al*., 2013), with greater flavonoid-type antioxidant activity, due to its ability to reduce the formation of free radicals (Pier-Giorgio, 2000). Within these foods is a great variety of fruits rich in antioxidants of this type, as is the case of the hawthorn, there are around 150 specimens worldwide, 95 of them belong to the American continent and 13 of them are native to Mexico (García *et al*., 2013). This fruit is located at altitudes ranging from 400 to 3,000 meters above sea level; both wild (between 14 and 32º north latitude) and cultivated (between 19 and 20º north latitude) (Pérez, 2014). There are several varieties of this genus within which is *Crataegus mexicana*, this variety of fruit, contains high nutritional value and antioxidant power (Méndez *et al*., 2013) in addition to demonstrate that they have the ability to protect erythrocytes against oxidative damage (Banderas *et al*., 2015).

The objective of this study is to investigate the inhibition of carbonylation by the acetone extract of the hawthorn fruit *(Crataegus mexicana)* of the principal plasma proteins *in vitro* induced with a pro-oxidant agent ($FeSO_4$).

MATERIAL AND METHODS

Chemical Reagents

Water nanopure, *n*-hexane, dichloromethane, ethyl acetate, acetone, ethanol, methanol, distilled water, hydrochloric acid (HCl), 2,4-dinitrophenylhydrazine (DNPH), mixture of Lowry, guanidine hydrochloride (HCl-guanidine), trichloroacetic acid (TCA), ferrous sulfate ($FeSO_4$), Folin-Ciocalteu reagent, phosphate buffered saline (PBS).

Obtaining Epidermis

10 kg samples of hawthorn fruit (*Crataegus mexicana*) were collected in San Pablo del Monte, Tlax., Mexico, latitude: 19.126491; length: -98.176197; altitude: 2,287 meters, the samples were washed by hand only with water, then the epidermis was obtained by dehydrating under normal conditions in the shade.

Obtaining the Extract

The dehydrated epidermis 30 g was macerated in 300 mL of each solvent according to its polaritystarting with *n*-hexane, dichloromethane, ethyl acetate, acetone, ethanol and methanol using the same biological sample, in amber bottle in the dark for 72 hours. Finally the solvent is evaporated with the help of a rotary evaporator. Of the 6 extracts we worked with the acetone extract since in previous studies the highest antioxidant activity of the aforementioned extract was reported (Méndez *et al*., 2013).

Subjects Selection and Clinical Examination

In this study 6 healthy men were selected. Their average age was 22 years old. The exclusion criterial were that no one of the participants had a chronic disease (diabetes mellitus, cardiovascular disease, anemia). Another exclusion criteria was that they weren´t under drug treatment, having history of alcoholism and/or smoking, and neither excessive exercise which could produce oxidative stress. All the participants were explained what the study consisted of and later voluntarily signed their consent. The study was approved by the Institutional Ethical Committee for Human Research, Faculty of Health Sciences and Nutrition in the State University at Tlaxcala.

Blood Sampling

This experimental study was carried out with three repetitions on three different days. We extracted 5 mL of blood samples from 6 male patients of 22 years of age, clinically healthy with fasting of 10-12 h, in non-heparinized sterilized tubes and immediately placed on ice. Subsequently, the plasma was separated from the red cells by centrifugation at 3000 rpm for 10 minutes obtaining an approximate of 2 mL of plasma with which we worked.

Quantification of Protein

The protein count was measured using Lowry's method (Lowry *et al*., 1951). 5 µL of supernatant of the blood sample was placed in an ELISA plate in triplicate and 20 µL of distilled water, 150 µL of the Lowry mixture was added, incubating at 25 ° C during 10 minutes with orbital agitation. Afterward, 25 µL of Folin reagent (Folin and Ciocalteu, 1927) dissolved in water (1:1) was added and incubating 30 minutes at 25 °C. For the blank, 200 µL of PBS was used and the absorbance at 540 nm was measured. Finally, the values were substituted in the following formula to obtain the protein content in the plasma.

$$\text{Protein } (\mu g/mL) = \frac{\text{A540nm} - 0.05012}{0.0028} * 40$$

Determination of Carbonyls Groups

The determination of carbonyl groups was obtained by Levine's method (Levine *et al*., 1990). Five eppendorf tubes with a capacity of 1.5 mL were used for each individual. Sample one was the control, which only contained plasma and 2,4-DNPH. Sample two; plasma plus pro-oxidant, ferrous sulfate ($FeSO_4$) 20 µM. Sample three; plasma plus acetone extract at the final concentration of 10 mg/L and 20 µM $FeSO_4$. Sample Four; plasma plus acetone extract at a final concentration of 100 mg/L and 20 µM $FeSO_4$.Sample five; plasma, acetone extract at a final concentration of 1000 mg/L and ($FeSO_4$) 20 µM. All samples were allowed to incubate at 25 ^oC for 1 hour. Afterward, 100 µL of each sample was taken and separately placed in 5 eppendorf tubes, 400 µL of 10 mM 2,4-dinitrophenylhydrazine (2,4-DNPH), in 2N HCl, was added, it was incubated for 1 hour, in darkness and with agitation. Then the protein was precipitated with 500 µL of 20% TCA (Trichloroacetic Acid) and separated by centrifugation for 10 minutes at 4 °C and 7,500 rpm. The supernatant was discarded and the pellet was resuspended in 500 µL of 10% TCA, allowed to stand for 10 minutes and centrifuged for 10 minutes at 4 °C and 7,500 rpm. The precipitate was washed three times with ethanol-ethyl acetate solution 1:1 (v/v) at 4 °C, for 15 minutes and centrifuged at 10,500 rpm. Finally, the precipitated protein was suspended in 250 µL of 6 M guanidine hydrochloride and pH 2.3. The washing process of the protein precipitate leads to loss of protein, so that the final protein concentration was determined with a 1:10 dilution, using the ultraviolet method, against a serum target treated with 2N HCl (without 2,4-DNPH).The molar extinction coefficient for 2,4-DNPH at 360 nm is 0.022 $\mu M^{-1}cm^{-1}$ (Candia *et al*., 2011) which was used for the determination of the carbonyl concentration in nmol mL^{-1}, according to the following equation:

$$\frac{\text{nmol}}{\text{mL}} = \frac{\text{Absorbance at 360 nm}}{(0{,}022\ \mu\text{M}^{-1}\,\text{cm}^{-1})\ (250\ \mu\text{L}/100\ \mu\text{L})}$$

The absorbance obtained was corrected with a standard curve of bovine serum albumin (0.10 mg mL^{-1} - 1.0 mg mL^{-1}) dissolved in 6 M guanidine hydrochloride at pH 2.3, with the following equation:

$$\text{Protein concetration } \left(\frac{\text{mg}}{\text{mL}}\right) = \frac{[\text{Absorbance 280 nm} - \text{Intercept Y}]}{\text{Slope}} * 2.5 * 10$$

Finally, to determine the carbonyl concentration in nmol mg^{-1} the following equation was used:

$$\text{Carbonyls } (nmol\ mg^{-1}) = \frac{\text{Carbonyls nmol mL}^{-1}}{Concentration\ of\ protein\ in\ the\ precipitate\ \text{mg mL}^{-1}}$$

Statistic Analysis

The data were expressed as means ± standard error of the mean (SEM).A one-factor analysis of variance (ANOVA) was used to compare the antioxidant capacity of hawthorn epidermis extracts. The Tukey test was also used to compare the mean differences of the treatments with respect to the control. In all cases, a level of significance of $p < 0.05$ was used. Each experiment was performed in triplicate. The Info Stat-Statistical software was used for the analyzes.

RESULTS AND DISCUSSION

Of the 10 kg of hawthorn, 115 g of dehydrated epidermis were obtained.

Extracts

From the 30 g of epidermis with 300 mL of each solvent the following post-evaporation weights were obtained and are shown in table 1.

Table 1 Weight obtained by extract.

Solvent	Evaporation temperature	Weight
n- Hexane	40 °C	686.5 mg
Dichloromethane	40 °C	1.91 g
Ethylacetate	40 °C	1.23 g
Acetone	40 °C	729 mg
Ethanol	40 °C	1.36 g
Methanol	40 °C	4.77 g

The effects of $FeSO_4$ and acetone extract at different concentrations (10, 100, 1000 mg/L.)are shown in table 2.

this type of oxidation. (Nyström, 2005; Fedorova *et al*., 2013). Glutamic semialdehyde, is a product of the oxidation of arginine and proline, while the aminoadipicsemialdehyde, the oxidation of lysine.

There are two types of reaction of carbonylated proteins the primary one that is detectable by DNPH and the secondary one that is generated from lipoperoxidation,its decomposition forms several species of carbonyls (three to nine carbons in length), the most reactive and cytotoxic are; β-unsaturated aldehydes (4-hydroxy-*trans*-2-nonenal and acrolein), di-aldehydes (malondialdehyde and glyoxal) and keto-aldehydes (4-*oxo*-*trans*-2-nonenal) (Yuichiro *et al*., 2010).

It is estimated that the reaction, which is carried out in this study, is primary due to the proteins obtained in the blood plasma (albumine, globulins, fibrinogen) while the formation of carbonyls groups occurs mainly in the proline side chains , arginine and lysine with more than fifty percent due to the characteristics of its chemical structure to intervene in this process; however, more studies are required to confirm this theory and to explain why.

It was shown that ferrous sulfate is an inducer (sample two) of the formation of carbonyl groups in plasma proteins because it is a free radical generating pro-oxidant and that they were detected by the presence of 2,4-dinitrophenylhydrazine. On the other hand, the acetone extract decreased the formation of these groups and increased the protection of antioxidant activity to avoid further injuries (sample five), this could be related to the amount of flavonoid antioxidants that is present in the hawthorn, the which is mainly responsible for neutralizing free radicals and their effects, however, studies that identify which metabolites are responsible for this protection are required.

In this study, it was shown that there is a significant difference between the induction of the prooxidant versus the addition of the antioxidant (sample two/sample five) and it can be affirmed that the probability of exposure to pro-oxidant factors during life, increase the production of free radicals and as a consequence the formation of carbonyl groups,in this study it was demonstrated that ferrous sulfate carbonilized in more than 50% the plasma proteins of the participants as can be seen in table 2 (sample 2) and that by inducing hawthorn epidermis extract decreased the formation of these groups, in the

Table 2 Effects of $FeSO_4$ and acetone extract at different concentrations (10, 100, 1000 mg / L.)

Participant	Total protein, mg/mL	DNPH mg/mL	DNPH + Prooxidant	DNPH + E. Acetone 10 mg/L + Prooxidant	DNPH +E. Acetone 100 mg/L + Prooxidant	DNPH + E. Acetone 1000 mg/L +Prooxidant
1	6.32	0.0343±0.02[a]	0.0672±0.03[b]	0.0658±0.01[c]	0.0635±0.05[d]	0.0377±0.02[e]
2	8.18	0.0612±0.03[a]	0.08509±0.06[b]	0.0841±0.04[c]	0.1083±0.01[d]	0.0457±0.04[e]
3	9.57	0.0467±0.04[a]	0.0811±0.05[b]	0.0702±0.03[c]	0.0604±0.02[d]	0.0136±0.05[e]
4	7.7	0.0409±0.05[a]	0.083±0.02 [b]	0.0691±0.04[c]	0.0542±0.05[d]	0.0241±0.01[e]
5	4.74	0.0404±0.01[a]	0.0525±0.06 [b]	0.0387±0.03[c]	0.0237±0.04[d]	0.0196±0.03[e]
6	3.68	0.0362±0.05[a]	0.0592±0.06 [b]	0.057±0.02 [c]	0.054±0.03[d]	0.0352±0.02[e]

The amount of carbonyl groups in plasma proteins after different treatments. The values are expressed as nmol mg^{-1}.
Different letters represent a significant difference in * $p < 0.05$ (Tukey analysis).
E. Acetone (Extract of acetone).
Prooxidant (ferrous Sulfate (20 μM)).

as a source of carbonyl groups that occur mainly in the proline, arginine and lysine residues, since they are more susceptible to

) no
low
amount of free radical entrapment, however the concentration

of 1000 mg/L showed a good protection against these reactive species, inhibiting the formation of these carbonyl groups.

CONCLUSION

From the results obtained, we can conclude that the acetone extract from the epidermis of the hawthorn (*Crataegus mexicana*) protected the proteins in the blood plasma *in vitro*, against the effect of carbonylation, because the fruit of hawthorn contains metabolites with antioxidant activity, in addition to having many nutritional properties such as vitamins, minerals and fiber.

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