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Research article

Antioxidant Capacity and Protective Effect of Aqueous and Hydroalcoholic Extracts of *Senecio rhizomatus* Rusby "Llancahuasi" on Erythrocytes Subjected to Oxidative Stress

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ABSTRACT

Objective. To evaluate the antioxidant capacity and protective effect of aqueous and hydroalcoholic extracts of *Senecio rhizomatus* Rusby in rat erythrocytes subjected to oxidative stress with hydrogen peroxide (H_2O_2). **Methodology.** This study used an experimental design. The extracts were obtained through maceration with 96° ethanol (SeR96), 70° ethanol (SeR70), 50° ethanol (SeR50) and through infused water (SeRAc). Secondary metabolites were identified through colorimetric reactions and precipitation. In each extract, we could determine the capacity to eliminate 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), the reduction of ferric ion and the total polyphenol content. In addition, the activity on the plasma membrane redox system (PMRS) was evaluated in each extract. The protection against oxidative stress in erythrocytes was evaluated by determining the content of reduced glutathione (GSH) and malondialdehyde (MDA). **Results.** Alkaloids, flavonoids, phenolic compounds, sesquiterpene lactones and sugars were identified in all the extracts. The total polyphenols content showed a correlation with the reduction of ferric ion (r=0.885) and with DPPH radicals elimination (r = -0.899), where the one with the highest antioxidant capacity was SeR50. Thus, the SeR50 (all concentrations) and SeR70 (100 µg/mL concentration) significantly increased the PMRS activity compared to the control group. After inducing oxidative stress in erythrocytes, all the extracts maintained the GSH level and inhibited MDA formation significantly compared to the H_2O_2 group. **Conclusion.** The antioxidant capacity of hydroalcoholic extracts (96°, 70°, 50°) and aqueous infusion of *Senecio rhizomatus* Rusby is related to the content of polyphenols. They increase the plasma membrane redox system activity in rat erythrocytes and protect them from oxidative stress induced with H_2O_2, showing an increase in the concentration of reduced glutathione and a decrease in malondialdehyde.

Key words: Antioxidant, Reactive oxygen species, Plasma membrane redox system, Reduced glutathione, Lipoperoxidation.

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INTRODUCTION

Oxidative stress is the imbalance between oxidants and antioxidants, which cause cell damage.[1] It is considered the main cause of noncommunicable diseases such as diabetes which, through hyperglycemia, generates complications such as hyperlipidemia and hypertension, damaging multiple organs.[2,3] In addition, it causes endothelial dysfunction, which generates cardiovascular problems such as atherosclerosis,[4] neurodegeneration, immunosuppression and aging.[5,6] The potential role of free radicals, reactive oxygen species (ROS) and antioxidants in the etiology of chronic diseases has stimulated an increase in research over the last few years.[7] These diseases can be prevented by healthy diets and the consumption of antioxidants.[8] Thus, vegetable species, which are traditionally used by the population in diverse forms of administration, are a potential source of prevention of noncommunicable diseases.

In mammalian systems, erythrocytes have enzymatic and non-enzymatic antioxidant defenses, such as glutathione (GSH) vitamin C and vitamin E, to recognize ROS levels and limit the damage they produce.[9] In addition, these cells have a system to transfer electrons in the membranes known as the plasma membrane redox system (PMRS), which is an electron transport chain in the plasma membrane by which the cells oxidize the electron donors, mainly NADH or NADPH, and transfer the resulting electrons to the extracellular acceptors to protect them from free radicals. These defenses decrease with age, as well as with chronic diseases where PRMS is altered in erythrocytes.[10] During these conditions, some reports show that the decrease in antioxidant defenses may increase with antioxidant supplementation.[11,12] The PMRS system contains proteins that are electron donors, electron transporters, extracellular electron acceptors and oxidoreductase enzymes.[13] Lately, antioxidants have been studied in numerous epidemiological research that had related their consumption to the reduction

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of the appearance of diseases related to oxidative damage, hence, the interest has been focused mainly on the use of natural antioxidants for the improvement of human health.[14]

Polyphenols are effective against free radicals, since they strengthen the endogenous antioxidant system, reducing lipid oxidation, protein oxidation and mRNA damage, preventing inflammatory processes that are aggravating factors in noncommunicable diseases such as cardiovascular, degenerative diseases of the central nervous system, as well as respiratory diseases, cancer and diabetes.[15] *Senecio rhizomatus* Ruby belongs to the herbaceous stratum and grows in the Andes of Bolivia and Peru, specifically in La Libertad, Ancash, Junín, Huancavelica, Lima, Apurimac, Arequipa, Cuzco and Puno between 3600 and 5000 meters above sea level between the rock crevices and snow covered soils. In addition, this species is used as a medicine with the name "llancahuasha", "ticllahuasha", "llancahuasi".[16,17] One traditional form to use it is as a type of plaster for skin application when having external wounds; another form is in maceration and infusions administrated orally when having respiratory tract conditions.[18] In the ethanolic and aqueous extracts of this species, active metabolites such as tannins, flavonoids, saponins, steroids, triterpenoids and alkaloids have been identified.[18,19] However, the antioxidant capacity is still unknown, as well as the in vitro evaluation of its action on cell membrane redox systems and the protection against oxidative stress.

The redox system in mammalian erythrocytes stands out for being part of the most abundant cells in blood exposed to constant oxidation caused by free radicals due to the presence of hemoglobin, which is the main source of maintenance of the redox system since they do not have mitochondria.[20]

Therefore, the objective of this study was to evaluate the antioxidant capacity of the aqueous (SeRAc), 50° ethanolic (SeR50) and 96° ethanolic (SeR96) extracts of *Senecio rhizomatus* Rusbi as well as the protective effect on the oxidative stress induced in rat erythrocytes with hydrogen peroxide (H_2O_2), evaluating indicators of the activity of the plasma membrane redox system (PMRS), reduced glutathione content (GSH) and malondialdehyde (MDA).

MATERIALS AND METHODS

The design of the present study was experimental and was developed following the "ARRIVE" guidelines for experimental research with animals. It was also approved by the Institutional Research Ethics Committee of the Norbert Wiener Private University with file No. 005-2019.

Senecio rhizomatus Rusby and the packed red blood cells (PRBC) obtained through cardiac puncture in rats were considered the population. The units of analysis were the types of extracts and erythrocytes respectively. The sample and replicates are described below:

Collection of the plant species, its taxonomic classification and how the extracts of *Senecio rhizomatus* Rusby were obtained

The plant species was collected in Parco Villanueva, in the district of Acostambo, province of Tayacaja, department of Huancavelica, between the months of May and June 2019. The taxonomic classification was done at the Natural History Museum of the National University of San Marcos (Universidad Nacional Mayor de San Marcos) according to the Cronquist System of Classification (1988). In order to obtain the dry extract, the fresh species was dehydrated in an oven at a controlled temperature (40°C ± 2°C), then the plant was pulverized and the hydro-alcoholic and aqueous extracts were obtained, as described by Casado *et al*.[21] The hydroalcoholic extracts (96°, 70°, 50°) were macerated for seven days and the aqueous ones were put in infusion for 30 minutes in a 1:10 ratio (extract: solvent). Next, with the help of a rotary evaporator, the extract was concentrated and dried in an oven at 40 ± 2°C to obtain the dry extracts.

Qualitative phytochemical profile analysis

For the identification of metabolites, the method proposed by Lock[22] was used. It allowed the identification of different compounds through different tests of color change and precipitation. In addition, fractionation methods were designed to guide the phytochemical studies for a better result.

Determining the total polyphenol content

It was carried out according to the Quispe-Mendoza *et al*. method[23] with some modifications. Gallic acid (GA) at concentrations of 1, 2, 3, 4 and 5 µg/mL was used as reference standard to make a calibration curve. For the analysis, 25 mg of the sample were weighed; then, it was dissolved in 2.5 mL of methanol. In addition, we diluted it with distilled H_2O to obtain concentrations of 100 µg/mL. From the new dilution, 500 µL were taken and 250 µL of Folin Cicalteu reagent were added (1, 2 N); then, it was shaken for 5 minutes and 1250 µL of calcium carbonate was added. After 60 minutes, the absorbance was measured with a UV-VIS spectrophotometer at 760 nm. The results were expressed as mg of gallic acid equivalents in 1 g of a dry sample (mg GA/g).

Evaluation of the capacity to reduce 2,2-diphenyl-1-picrylhydrazyl radical (DPPH)

The antioxidant capacity of the extract was based on the reduction of the DPPH radical, showing a decrease in the violet coloration according to the Umamaheswari *et al*.[24] method, where a 0.4 mM solution of DPPH in pure methanol was prepared. Then, from this solution, 100 µL was added to the different types of extracts diluted to 200 µL/mL at a concentration. After slightly shaking, the solution was left to stand at room temperature protected from light for 30 minutes. Finally, the absorbance was measured at 517 nm using a microplate reader as a way of comparison to the antioxidant capacity of ascorbic acid.

Determining the capacity to reduce ferric ion

It was done according to the Benzi *et al*.[25] method with some modifications. The method is based on the ferric iron reduction (Fe^{+3}) present in the FRAP reagent as well as in the ferrous form (Fe^{+2}) in the presence of antioxidants. The FRAP reagent was prepared on the same day of the assay by mixing acetic acid-sodium acetate buffer (pH 3.6), TPTZ (2,4,6-tri(2-pyridyl)-striazine) and $FeCl_3$. The reductive capacity of the sample forms a blue coloration of intense proportionality due to the generation of ferrous-TPTZ complex. Twenty five mg of ascorbic acid was weighed, diluted in 2.5 mL of methanol and diluted with H_2O until obtaining a solution of 100 µg/mL. From this solution, 500 µL were taken and 1.5 mL of FRAP solution was added. After leaving it to stand in the dark for 60 minutes, the absorbance was determined in a UV-VIS spectrophotometer at 593 nm. The capacity to reduce ferric ion was expressed as mg equivalent of ascorbic acid (AA) in 1 g of dry sample (mg AA/g).

Experimental animals and obtaining packed red blood cells (PRBC)

Twelve 8-week old male Hotzman rats between 200 and 220 g. of body weight acquired from the National Institute of Health (NIH) were used. For the animals' maintenance and care during the study, the guidelines of the "Guide for the care and use of laboratory animals"[26] were followed. The acclimatization was carried out one week before the beginning of the trial, 4 rats per 1200 cm^2 cage were kept with water and food at will, the cleaning and disinfection of the cage was carried out twice a week, room temperature was kept at 22°C ± 2°C, 50% relative humidity and 12 hours of light and 12 hours of darkness.

Blood was obtained in the morning on an empty stomach by means of cardiac puncture. Two mL of blood were extracted in heparinized tubes, it was immediately centrifuged at 1800 × g during 10 minutes, the plasma was eliminated leaving the PRBC which was washed 3 times with a phosphate buffered saline solution (PBS) pH 7,4. Then it was diluted at 20% with PBS. From the PRBC, three groups were formed for the PMRS, MDA and GSH assays. Each group was subdivided into three subgroups for each extract of SeR96, SeR50 and SeRAc and 1 mL of PRBC sample for each type of extract were considered for the assay with the dilutions of 100, 50, 25 and 12.5 µg/mL. Likewise, samples of 1 mL were considered for the control and H_2O_2 groups.

In Vitro Incubation of Erythrocytes with the extracts

From each extract (SeR96, SeR70, SeR50 and SeRAc), dilutions of 100; 50; 25; and 12.5 µg/mL were obtained using PBS with 0.01 % (w/v) ascorbic acid in the final solution. For the control and H_2O_2 groups, PBS with 0.01 % (w/v) ascorbic acid was used for incubation. All dilutions were obtained in triplicate. To each dilution, 1 mL of PRBC was added and it was incubated for 30 minutes at 37°C with constant shaking; at the end of incubation, erythrocytes were washed three times. Finally, the new PRBC was used for PMRS, MDA and GSH activity assays.

Determining the Plasma Membrane Redox System (PMRS) Activity

The activity of the plasma membrane redox system was determined by quantifying the reduction of ferrocyanidine according to the Avron and Shavit method.[27] First, 0.2 mL of PRBC was taken from the dilutions of the extracts and the control group which were suspended in PBS containing 5 mM glucose and 1 mM of freshly prepared potassium ferricyanidin until obtaining a final volume of 2 mL. The suspension was incubated for 30 minutes at 37°C and then centrifuged at 1800 RPM. The supernatant was collected to determine the ferrocyanidine content using the 4,7-diphenyl-1,10-phena-nthroline disulfonic acid disodium salt and measured at an absorbance of 535 nm (e = 20,500 $M^{-1}.cm^{-1}$). The results were expressed in µmol of ferrocyanidine / mL of PRBC / 30 min.

Inducing oxidative stress

The oxidative stress was induced to the groups with extracts and to the H_2O_2 group, according to the method described by Stocks and Dormandy[28], incubating 0.5 mL of PRBC with 0.5 mL of H_2O_2, 200 mmol during 30 minutes with constant shaking. At the end of the incubation, it was shaken before using it for the determination of MDA and GSH.

Determining the malondialdehyde (MDA) content

For determining the MDA content in erythrocytes, the Esterbauer and Cheeseman[29] method was used: 0.2 mL of packed red blood cells from the previous process were suspended in 3 mL of PBS containing 0.5 mM glucose. From the new suspension, 0.2 mL was taken and 1 mL of 20% trichloroacetic acid (TCA) was added, shaken and centrifuged at 2000 RPM. Then, we added 2 mL of 0.67% thiobarthybituric acid. After that, we boiled it for 20 minutes at above 90°C temperature, and then, it was cooled with ice. Finally, the absorbance was measured at 532 nm. The MDA concentration was calculated using an extension coefficient (e = 156 000 $M^{-1}.cm^{-1}$). The MDA concentration is expressed in nmol. mL^{-1} of the packed red blood cells.

Determining the reduced glutathione level (GSH)

The GSH content was determined following the Beutler and Sedlak *et al.*[30,31] method. It is based on the reduction of 5.5 $^{1-}$ Dithiobis (2-nitrobenzoic acid) (DTNB) due to the sulfhydryl group (SH) to the yellow nitro mercaptobenzoic acid anion. We used 0.1 ml of the sample and diluted it with 0.9 ml of PBS. One ml of 20% trichloroacetic acid (TCA) was added and left to stand for 20 minutes before centrifugation at 3000 RPM for 10 minutes. Then, 0.25 ml of the supernatant was taken and added to 0.75 ml PBS; subsequently, 2 ml of 0.0006 M of DTNB was added and incubated for 10 minutes. The absorbance was read at 412 nm. The concentration was calculated using the extension coefficient (e = 13 100 $M^{-1}.cm^{-1}$). The GSH concentration is expressed in mg/mL of packed red blood cells.

Data collection techniques, instruments, and procedures

The techniques to determine the biochemical parameters (DPPH, MDA, GHS, PRMS) are based on chemical reactions with typical coloring where the absorbances were measured with UV-VIS spectrophotometer equipment, at different wavelengths according to each analytical technique. The obtained results were recorded in a virtual data collection sheet and then processed in their respective spreadsheets.

Data processing and analysis

The mean and standard deviation of each group was determined taking into account that the variables are quantitative. In the first stage, the homogeneity of variance was determined using the Shapiro Wilk's normality test. In the second stage, we used the ANOVA test to verify the variance. In the third stage, the Tukey test was applied to evaluate the difference between the groups. The correlation between the antioxidant capacity variables and the phenolic compounds was evaluated with the Pearson's test. All the analyses were evaluated with a significance level of $p<0.05$ or $p<0.01$. The data were processed through Excel software and SPSS Version 24.

RESULTS

Taxonomic classification of the species and the obtaining of the hydroalcoholic extracts

The plant species was identified as ***Senecio rhizomatus* Rusby**, according to certificate No. 243-USM-2019 issued by the Natural History Museum of the National University of San Marcos. The obtained extracts were SeR 96, SeR70, SeR50 and SeRAc as shown in Figure 1, the performance was 23%, 37%, 48% and 46% respectively.

Qualitative analysis of the phytochemical profile

Alkaloids, flavonoids, phenolic compounds, sesquiterpene lactones and sugars were identified in all extracts. On the other hand, quinones, tannins, amino groups and reducing sugars were only in the SeR70, SeR50 and SeRAc extracts. Also, triterpenes and steroids were identified only in the SeR96, SeR70 and SeR50 extracts as summarized in Table 1.

Antioxidant capacity of the extracts

The hydroalcoholic (96°, 70°, 50°) and aqueous extracts which was proportional to the concentration in each extract (Figure 2). In addition, it was evidenced that the elimination expressed in IC50 was inversely proportional to the reduction of ferric ion, with a correlation of -0.903 with a $p < 0.01$ significance level. The extract with the best DPPH radical elimination and the highest ferric ion reduction was SeR50 (Table 2 and Figure 3).

Polyphenol content

The total polyphenols expressed in mg GA/g of dry extract was proportional to the elimination of ferric ion expressed in mg AA/g of dry extract and inversely proportional to the elimination of DPPH radical expressed in IC50, which had a correlation of 0.885 and -0.899 respectively with a significance level of $p < 0.01$.

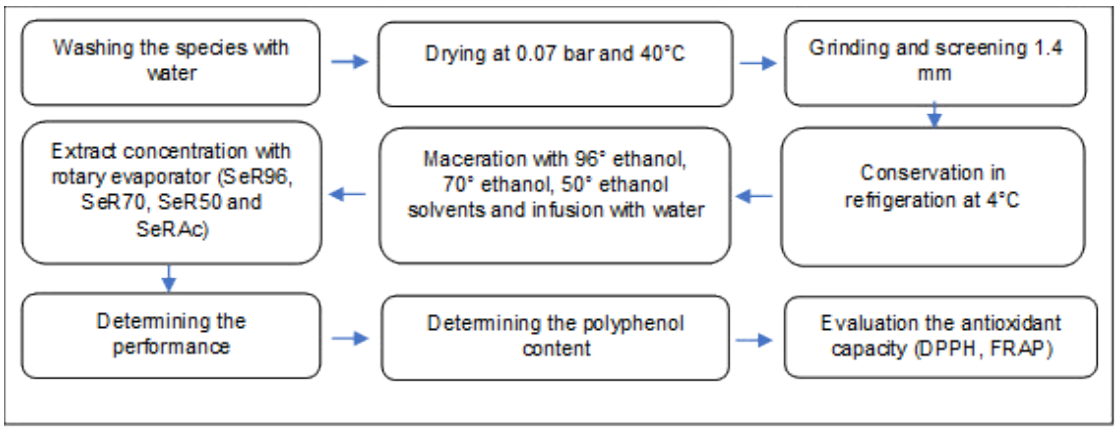

Figure 1: Flowchart of how the extracts of Senecio rhisomatus Rusby were obtained, SeR96: 96° Ethanolic extract; SeR70: 70° Ethanolic extract; SeR50: 50° Ethanolic extract and SeRAc: Aqueous extract.

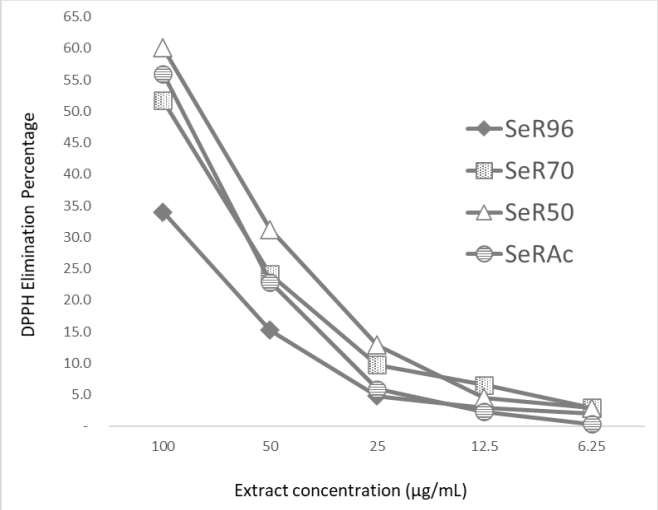

Figure 2: Percentage of the elimination of DPPH radical in SeR96, SeR70, SeR50, SeRAc extracts at concentrations of 100, 50, 25, 12.5 and 6.25 µg/mL; DPPH: 2,2-diphenyl-1-picrylhydrazyl.

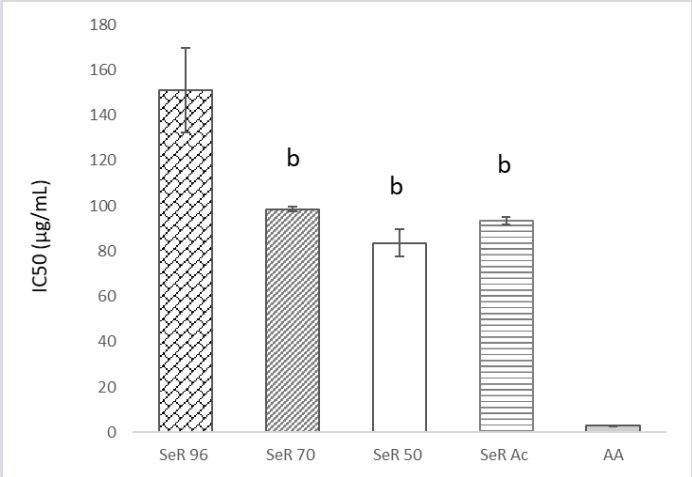

Figure 3: Elimination of DPPH radical expressed in inhibitory concentration 50 (IC50) of the SeR96, SeR70, SeR50, SeRAc extracts and AA, where AA: ascorbic acid; b: $p < 0.01$ compared to the SeR96 group.

Table 1: Identification of phytochemical constituents of *Senecio rhizomatus* Rusby extracts.

Active Metabolites	Reagents	SeR 96	SeR 70	SeR 50	SeR Ac
Alkaloids	Mayer	+	+	+	+
	Dragendorff	+	+	+	+
	Wagner	+	+	+	+
Flavonoids	Shinoda	+	+	+	+
	Aluminum trichloride 1%	+	+	+	+
Quinone	Borntrager	-	+	+	+
Phenolic compounds	Ferric trichloride 1%	+	+	+	+
Saponins	Sample + water dest 1%	-	-	-	-
Tannins	Gelatin	-	+	+	+
Triterpenes and steroids	Liebermann - Burchard	+	+	+	-
Sesquiterpene lactones	Baljet A and B	+	+	+	+
Free amino group	Ninhidrina	-	+	+	+
Sugars	Molish	+	+	+	+
Reducing sugars	Benedict	-	+	+	+
	Feling A and B	-	+	+	+

Table 2: Elimination of DPPH radical, ferric ion reduction and total polyphenols of Senecio rhizomatus Rusby extracts.

Sample	Elimination of DPPH radical[#]	Reduction of ferric ion[+]	Total Polyphenols [++]
SeR96	$150.97^{c} \pm 10.74$	$3.07^{d} \pm 0,25$	$43.38^{c} \pm 0.60$
SeR70	$98.39^{b} \pm 0.59$	$81.31^{c} \pm 0,69$	$59.92^{a} \pm 0.40$
SeR50	$83.55^{b} \pm 3.51$	$89.31^{b} \pm 0,29$	$76.69^{b} \pm 0.56$
SeRAc	$93.45^{b} \pm 1.02$	$55.77^{a} \pm 0,88$	$63.05^{a} \pm 1.94$
AA	$2.91^{a} \pm 0.01$	-	-

Table 3. Dose response of Senecio rhizomatus Rusby extracts on the activity of the plasma membrane redox system, malondialdehyde content and glutathione content in erythrocytes subjected to oxidative stress with H_2O_2

Sample	Concentration (µg/mL)	PMRS[#]	GSH (µg/mL)	MDA (nmol/mL)
H_2O_2	-	-	21.40 ± 1.10	0.95 ± 0.03
Control	-	3.11 ± 0.04	$34.63^{a} \pm 0.45$	0.11 ± 0.02
SeR96	100	3.56 ± 0.14	$37.77^{b} \pm 2.70$	$0.54^{b} \pm 0.00$
	50	3.40 ± 0.09	$34.70^{a} \pm 1.75$	$0.58^{b} \pm 0.00$
	25	3.25 ± 0.09	$33.03^{a} \pm 1.01$	$0.57^{b} \pm 0.01$
	12.5	3.25 ± 0.11	$33.36^{a} \pm 1.63$	$0.80^{b} \pm 0.01$
SeR70	100	$3.90^{b} \pm 0.03$	$38.05^{b} \pm 2.36$	$0.52^{b} \pm 0.01$
	50	3.35 ± 0.04	$36.88^{b} \pm 2.34$	$0.52^{b} \pm 0.00$
	25	3.28 ± 0.14	$35.94^{b} \pm 2.99$	$0.56^{b} \pm 0.01$
	12.5	3.28 ± 0.10	$35.85^{b} \pm 2.54$	$0.66^{b} \pm 0.03$
SeR50	100	$4.59^{b} \pm 0.07$	$40.87^{b} \pm 2.36$	$0.39^{b} \pm 0.01$
	50	$4.02^{b} \pm 0.05$	$39.69^{b} \pm 2.34$	$0.42^{b} \pm 0.01$
	25	$3.94^{b} \pm 0.17$	$38.76^{b} \pm 2.99$	$0.45^{b} \pm 0.03$
	12.5	$3.69^{a} \pm 0.15$	$37.72^{b} \pm 2.07$	$0.53^{b} \pm 0.05$
SeRAc	100	3.80 ± 0.14	$34.49^{a} \pm 2.93$	$0.55^{b} \pm 0.01$
	50	3.40 ± 0.11	$34.02^{a} \pm 1.43$	$0.56^{b} \pm 0.01$
	25	3.30 ± 0.09	$33.41^{a} \pm 2.14$	$0.61^{b} \pm 0.03$
	12.5	3.24 ± 0.07	$33.12^{a} \pm 2.03$	$0.76^{b} \pm 0.03$

#DPPH radical elimination capacity expressed in IC50 (µg/mL); +Ferric ion reduction expressed in milligrams of ascorbic acid (AA) per each gram of dry extract; ++Polyphenol content expressed in milligrams of gallic acid (GA) per each gram of dry extract. In each column, the average values (n=3, + EEM) with different letters indicate significant difference ($p < 0.01$) based on Tukey's multiple comparison test.

PMRS activity in erythrocyte membranes, MDA and GSH content.

The SeR70, SeR50 and SeRAc extracts showed an increase in PMRS activity in erythrocyte membranes, which depended on the concentration of the extracts, being the SeR50 extract the one with the highest activity as shown in Figure 4.

Regarding MDA content, all the extracts and concentrations significantly decreased the MDA release ($P < 0.001$) due to the erythrocytes subjected to oxidative stress compared to the H_2O_2 group (Figure 5). Moreover, they maintained high levels of GSH compared to the H_2O_2 group as shown in Figure 6.

#Redox activity of the *Senecio rhizomatus* Rusby extracts in the erythrocyte plasma membrane determined in nmol of ferrocyanidin / mL of PRBC / 30 min. In each column (average n=3, ± EEM) letter "a" ($p < 0.05$) and letter "b" ($p < 0.01$) when compared to the control group for PMRS assay and with the H_2O_2 group for MDA and GSH assays.

DISCUSSION

In the present research, the antioxidant capacity of the SeR96, SeR70, SeR50 and SeRAc extracts of *Senecio rhizomatus* Rusby was evaluated, as well as their relationship with the polyphenol content, the increase of the activity of the erythrocyte plasma membrane redox system, and the preservation of its integrity in the oxidative stress induced with H_2O_2.

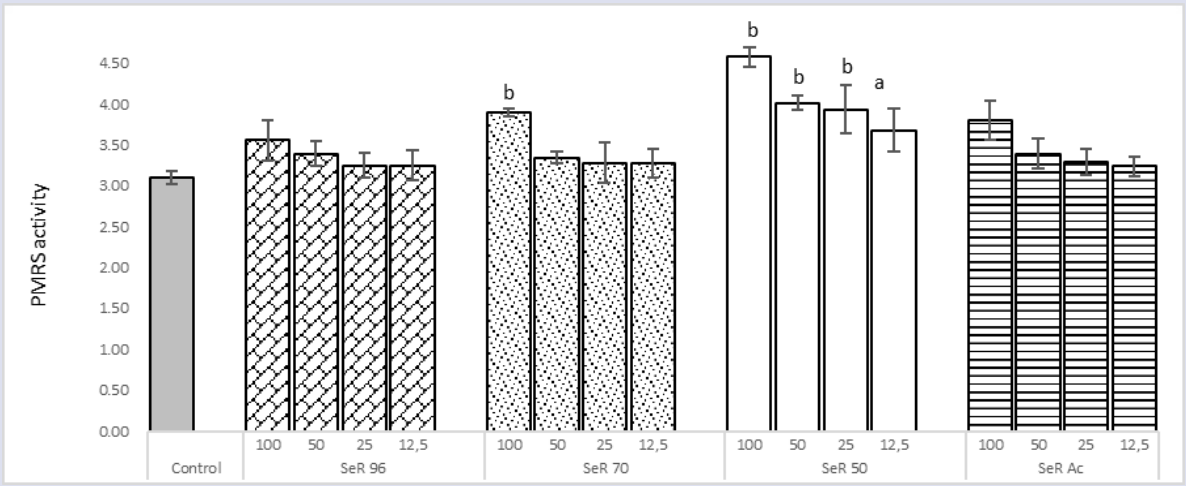

Figure 4: Dose response of SeR96, SeR70, SeR50 and SeRAc extracts compared to erythrocytes PMRS activity, determined in nmol of ferrocyanidin / mL of PRBC / 30 min., where PMRS: Plasma membrane redox system; PRBC: packed red blood cells; a: $p < 0.05$ and b: $p < 0.01$ compared to the control group.

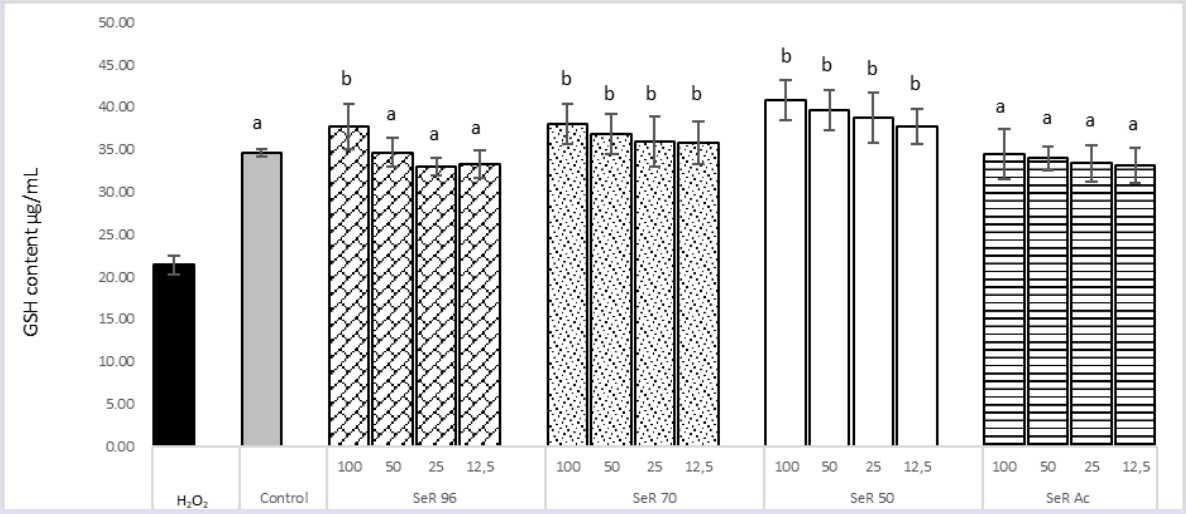

Figure 5: Dose response of the SeR96, SeR70, SeR50 and SeRAc extracts compared to GSH content in inducedoxidative stress with H2O2 in red blood cells. GSH concentration is expressed in mg/mL of PRBC. Where GSH: Glutathione, H2O2: hydrogen peroxide 200 mmol; a: $p < 0.05$ and b: $p < 0.01$ compared to the H2O2 group.

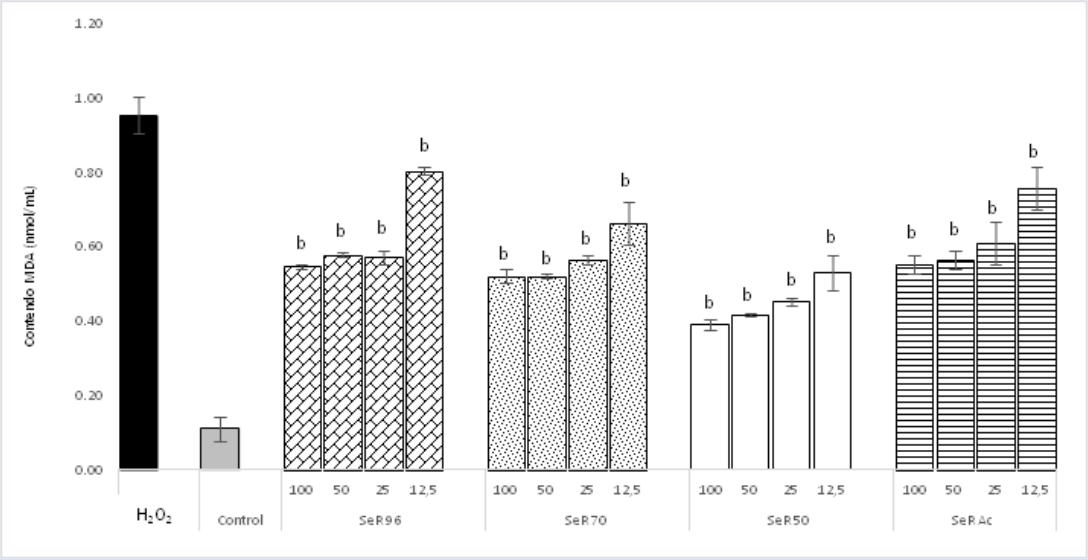

Figure 6: Dose response of the SeR96, SeR70, SeR50 and SeRAc extracts compared to MDA released due to H_2O_2-induced stress in red blood cells. MDA concentration is expressed in nmol/mL of PRBC. MDA: Malondialdehyde, H_2O_2: hydrogen peroxide 200 mmol; b: $P < 0.01$ compared to the H_2O_2 group.

Alkaloids, flavonoids, phenolic compounds, sesquiterpene lactones and sugars were identified in all types of extracts. In SeR70, SeR50 and SeRAc extracts, quinones, tannins, amino groups and reducing sugars were identified. In addition, in SeR96, SeR70 and SeRAc extracts, triterpenes and steroids were also identified (Table 1). The type, performance and quality of secondary metabolites with pharmacological activity are related to factors such as the habitat of the plant species or the polarity of the solvents used in the extraction.[21,32] The results of this study showed that the increase of the polarity of the solvents increases the variety of the metabolites and the performance of the extracts. The studies on the identification and quantification of secondary metabolites of this species are scarce and this could be due to the complicated access to its habitat since the geographical distribution is between 3500 and 5000 meters above sea level in the Andes of Ecuador, Peru and Bolivia.[17,33] In addition, the ethnobotanical evidence shows that the population uses it in infusion or as a poultice for treatment of ovarian inflammation, menstrual cramps, respiratory tract infections and external wounds.[34] In the studies of Puelles *et al.*[18] and Soto[19], flavonoids, saponins, steroids and alkaloids were also identified in the 96° ethanolic extract of *Senecio rhizomatus*. Moreover, polyphenols, flavonoids, tannins, terpenes and pyrrolizidinic alkaloids were identified in the 85° methanolic fraction in the *Senecio Vulgaris* species that has the same habitat; these metabolites confer antioxidant, antimicrobial and antifungal activity.[35] Pyrrolizidine alkaloids have also been identified in *Senecio jacobaea* and *Senecio Brasiliensis* species, where their anti-inflammatory, immunomodulatory and cytotoxic effect have been demonstrated.[36,37] In the case of *Senecio nulans, Senecio chiquianensis, Senecio hyoseridifolius, Senecio tphrosiodies*, secondary metabolites have been identified in ethanolic extracts and essential oils (terpenes, phenolic compounds, flavonoids, pyrrolizidinic alkaloids, lactones), showing good antibacterial activity against staphylococcus aureus bacteria.[38-41] It is necessary to explore the characterization of secondary metabolites of *Senecio rhizomatus* in order to guide *in vitro* and *in vivo* pharmacological research that would favor the preservation and rational use of the species.

All the extracts eliminated the DPPH radical dependently on their concentration (Figure 2) and the SeR50, SeR70 and SeRAc extracts had the best IC50 (Table 2). In addition, they reduced the ferric ion, being the SeR50 the one that showed more potency. When evaluating the relationship between the power to eliminate the DPPH radical expressed in IC50 and the power of ferric ion reduction in the extracts, a correlation of (r = -0.903) was evidenced. Regarding the content of polyphenols, SeR50 presented a higher quantity and when evaluating the relationship with the elimination of DPPH and reduction of ferric ion, correlations of r= -0.899 and r = 0.885 were shown respectively. The elimination of the DPPH radical showed the capacity to transfer electrons or donate hydrogens and the ferric ion reduction test showed the redox potential, which allows the stabilization of free radicals in hydrophilic fractions[42,43], which would avoid the formation of reactive oxygen species (O_2-, H_2O_2, •OH), nitrogen (NO) and lipoperoxides that damage diverse cellular structures.[6] The correlation between DPPH and FRAP assays with the content of polyphenols shows that the antioxidant power is mainly due to the presence of compounds of hydrophilic nature such as polyphenols, phenolic acids, flavonoids, tannins, alkaloids. The antioxidant potential of several medicinal plants has been attributed to the oxidizing and reducing power of phenolic compounds acting as oxygen sensors, electron donors and as reducers.[44,45]

The *Senecio* species are cosmopolitan; in Peru there are about 97, some of them already evaluated, that have shown antibacterial effects.[46] The 96° ethanolic extract of *Senecio rhrizomatus* is effective against the growth of *Staphylococcus aureus.*[19] However, the antioxidant capacity and polyphenol content has not yet been determined. In other endemic species of Europe, such as *Senecio racemosus* and *Senecio nemorensis,* total polyphenols (139.43 and 136.05 mg GAE/g) have been determined and the elimination of DPPH radical expressed in IC50 (24.40 and 18.81 µg/mL) presented correlation of -0.987,[47] demonstrating that the antioxidant potential is due to the presence of phenolic compounds. Also, 5 mg GAE/g of polyphenols as well as the antioxidant capacity expressed in IC50 of DPPH (12.1 µg/mL) were determined in the 50 methanolic extract of *Senecio longiflorus*, demonstrating correlation between polyphenols and antioxidant capacity and the antimicrobial activity with a lower cytotoxic activity in mammalian cells.[45] In the present study, it was demonstrated that the antioxidant capacity of the hydrophilic fraction is related to the content of polyphenols, however, it is also necessary to evaluate the relationship with the content of alkaloids as well as to evaluate the antioxidant power in lipophilic fractions.

The maintenance of PMRS activity in eukaryotic cells allows protection against external free radicals by transferring electrons from the intracellular space to the extracellular space in a system involving antioxidant enzymes, reduced substrates and low molecular weight substances.[10,48] In the present investigation, all the types of extracts increased PMRS activity dependently on the concentration; however, only the SeR50 (all concentrations) and SeR70 (100 µg/mL concentration) were observed to be significantly different from the control group (Figure 4). These results are consistent with the increase in GSH content where all extract concentrations are significantly different from the H_2O_2 group (Figure 5). Finally, the MDA content released in the erythrocyte membrane as a result of the lipoperoxidation of the membrane phospholipids decreased significantly with the treatment of all the extracts compared to the H_2O_2 group (Figure 6). Flavonoids, phenolic acids and vitamins from plant species improve the reduction capacity of the erythrocyte membrane against the aggression of free radicals, restoring fluidity and stability,[49-51] Polyphenols form an inactive redox complex with iron, protecting the erythrocyte membrane from lipid peroxidation,[52] and in silico studies it has been demonstrated that they act as cofactors of the cytochrome b5 reductase enzyme, favoring the stability of PMRS.[13,20] This explains that the capacity to reduce ferric ion of the polyphenols of the *Senecio rhizomatus* demonstrated in this study is reflected in the affinity on the iron of hemoglobin. Thus, it is able to form reduced stable compounds, increasing the activity of the PMRS of erythrocytes; however, it is still necessary to identify the type and structure of the main polyphenols of this species to know the mechanisms by which it increases the activity of the PMRS.

The stability of PMRS in erythrocytes maintains the necessary amount of ascorbate to face reactive oxygen species (ROS).[53] For this, there is a recycling mechanism where, after being oxidized to dehydroascobate, at an intracellular level, the hydrophilic antioxidant GSH reduces it again to ascorbate. Furthermore, it protects the integrity of the intracellular alpha tocopherol, which has the function of attenuating the lipoperoxidation; in this way, the GSH mediates the redox changes regulating the physiological function of the erythrocyte.[54,55] In this research, it was observed that erythrocytes treated with the extracts maintained the content of GSH equivalent to the control group when exposed to oxidative stress and it did not decrease significantly as it happened with the H_2O_2 group (without treatment). This finding shows that polyphenols, due to their demonstrated antioxidant capacity, would maintain the amount of ascorbate and, in the same way, the system would maintain its stable recycling protecting the membrane of erythrocytes from the fenton reaction due to the H_2O_2 action on the hemoglobin's iron.

The erythrocyte membrane has a high content of saturated fats and is, hence, exposed to lipoperoxidation, which makes it even more vulnerable due to the presence of iron, which produces the hydroxyl radical (•OH). Also, this damages the cellular components,[56] reducing cell fluidity, compromising the deformation of the cell, and disturbing the oxygen supply to the tissues, producing hemolysis.[57] The increase of the GSH concentration in the erythrocyte membrane after the treatment with the extracts allows us to hypothesize that the metabolites would be strengthening the ascorbate recycling, the protection of the intracellular alpha tocopherol, preventing the reactive oxygen species from generating and avoiding lipoperoxidation. That is why a decrease of the DMA concentration is observed in the treatment groups compared to the H_2O_2 group.

CONCLUSION

The antioxidant capacity of hydroalcoholic extracts (96°, 70°, 50°) and aqueous infusion of *Senecio rhizomatus* Rusy is related to the content of polyphenols. They increase the activity of the plasma membrane redox system of rat erythrocytes and protect them from oxidative stress induced by H_2O_2, showing an increase in the concentration of reduced glutathione and a decrease in malondialdehyde.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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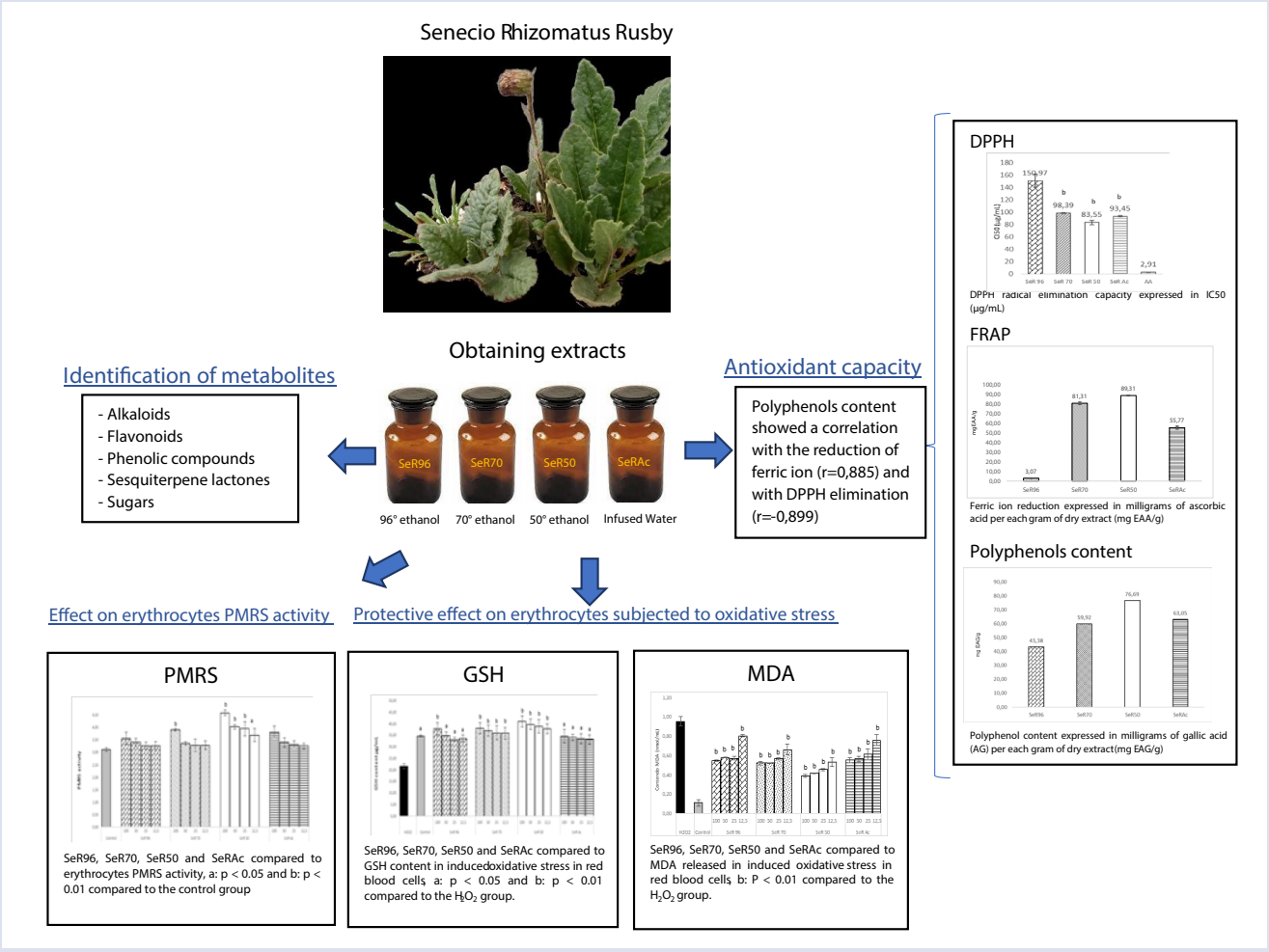
GRAPHICAL ABSTRACT



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