



Antioxidant capacity of *Chuquiraga spinosa* Less and *Senecio rhizomatus* Rusby and their effects on the progression of testosterone enanthate-induced prostatic hyperplasia in rats

[Capacidad antioxidante de *Chuquiraga spinosa* Less y *Senecio rhizomatus* Rusby y sus efectos sobre la progresión de la hiperplasia prostática inducida por enantato de testosterona en ratas]

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Abstract

Context: Benign prostatic hyperplasia affects men older than 50 years and can progress to atypical prostatic hyperplasia. Conventional pharmacotherapy causes adverse effects, affecting adherence and leading to the use of phytotherapy as a complementary treatment.

Aims: To evaluate the antioxidant capacity of *Chuquiraga spinosa* (ChS) and *Senecio rhizomatus* (SeR) and their effect on the progression of testosterone-enanthate-induced prostatic hyperplasia in rats.

Methods: The antioxidant capacity of the hydroalcoholic extracts of ChS and SeR was assessed by protecting erythrocytes subjected to oxidative stress with hydrogen peroxide. The progression of prostatic hyperplasia in the ventral zone of the Holtzman rat prostate gland was evaluated using an experimental design: Group I received a placebo, groups II to XI received 25 mg of intramuscular testosterone enanthate weekly for four weeks, groups III to X received extracts (ChS-250, ChS-500, SeR-250, SeR-500, ChS-250 + SeR-250, ChS-250 + SeR-500, ChS-500 + SeR-250 and ChS-500 + SeR-500 mg/kg orally), and group XI received 1 mg/kg finasteride orally for 35 days.

Results: ChS and SeR protected against oxidative stress and increased plasma membrane redox activity in erythrocytes. They significantly decreased ($p < 0.05$) prostatic index, serum PSA, and oxidative stress in prostatic tissue, with SeR-500, SeR-250, and ChS-500 mg/kg standing out. Histologically, both extracts preserved the cellular structures of the prostate and prevented progression from benign prostatic hyperplasia to atypical prostatic hyperplasia.

Conclusions: ChS and SeR extracts possess antioxidant capacity and prevent the progression of prostatic hyperplasia by regulating serum PSA and oxidative stress in prostatic tissue.

Keywords: *Chuquiraga spinosa* Less; oxidative stress; prostatic hyperplasia; *Senecio rhizomatus* Rusby.

Resumen

Contexto: La hiperplasia prostática benigna afecta a hombres mayores de 50 años y puede progresar a hiperplasia prostática atípica. La farmacoterapia convencional produce efectos adversos que afectan la adherencia, lo que lleva al uso de fitoterapia como tratamiento complementario.

Objetivos: Evaluar la capacidad antioxidante de *Chuquiraga spinosa* (ChS) y *Senecio rhizomatus* (SeR) y su efecto sobre la progresión de la hiperplasia prostática inducida por enantato de testosterona en ratas.

Métodos: Se evaluó la capacidad antioxidante de los extractos hidroalcohólicos de ChS y SeR protegiendo a los eritrocitos sometidos a estrés oxidativo con peróxido de hidrógeno. Se evaluó la progresión de la hiperplasia prostática en la zona ventral de la glándula prostática de la rata Holtzman mediante un diseño experimental: el grupo I recibió un placebo, los grupos II a XI recibieron 25 mg de enantato de testosterona intramuscular semanalmente durante cuatro semanas, los grupos III a X recibieron extractos (ChS-250, ChS-500, SeR-250, SeR-500, ChS-250 + SeR-250, ChS-250 + SeR-500, ChS-500 + SeR-250 y ChS-500 + SeR-500 mg/kg por vía oral), y el grupo XI recibió 1 mg/kg de finasterida por vía oral durante 35 días.

Resultados: ChS y SeR protegieron contra el estrés oxidativo y aumentaron la actividad redox de la membrana plasmática en los eritrocitos. Disminuyeron significativamente ($p < 0,05$) el índice prostático, el PSA sérico y el estrés oxidativo en el tejido prostático, destacando SeR-500, SeR-250 y ChS-500 mg/kg. Histológicamente, ambos extractos preservaron las estructuras celulares de la próstata y evitaron la progresión de hiperplasia prostática benigna a hiperplasia prostática atípica.

Conclusiones: Los extractos de ChS y SeR poseen capacidad antioxidante y previenen la progresión de la hiperplasia prostática regulando el PSA sérico y el estrés oxidativo en el tejido prostático.

Palabras Clave: *Chuquiraga spinosa* Less; estrés oxidativo; hiperplasia prostática; *Senecio rhizomatus* Rusby.

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INTRODUCTION

Benign prostatic hyperplasia (BPH) is a chronic disease, histologically defined as the hyperplasia of stromal cells in the transition zone and epithelial cells in the periurethral zone (Langan, 2019). Oxidative stress is a factor that should be considered as a premalignant condition capable of progressing to prostate cancer (Minciullo et al., 2015). BPH associated with lower urinary tract symptoms (LUTS) generally affects men over 50 years of age, with a higher prevalence in those over 81 years of age, reaching 90% (Egan, 2016). Conventional treatment consists of surgery or the use of α 1-adrenergic blockers or 5- α -reductase inhibitors, but in some patients, they can cause adverse reactions such as hypotension or dizziness (Kim et al., 2019), and those with cardiovascular or metabolic comorbidities have a higher risk of experiencing adverse effects (Huri et al., 2014), leading to low adherence to pharmacological treatment.

In patients with LUTS who receive primary care, phytotherapy ranks second as a complementary treatment at 23.5% (Fourcade et al., 2008). In the US, 40% of patients who do not undergo surgery use herbs alone or in combination with medications as supplements (Keehn and Lowe, 2015). Polyphenols in preclinical and clinical studies have demonstrated antioxidant and anti-inflammatory activity in BPH (Cicero et al., 2019). Polyphenols in the diet may not replace drugs in treatment, but are promising for preventing prostatic inflammatory processes, so further research is needed (Eleazu et al., 2017).

Chuquiraga spinosa Less and *Senecio rhizomatus* Rusby (family *Asteraceae*) are herbs that grow above 3300 meters above sea level, whose aerial parts are traditionally used by the population for urinary tract conditions (Tello-Ceron et al., 2019) and as anti-inflammatory agents (Beltrán and Gamarra, 2015) respectively. In *C. spinosa*, glucosylated polyphenols of quercetin, kaempferol, and isorhamnetin have been identified (Landa et al., 2009), which inhibit the enzymatic expression of COX-2 and iNOS. Therefore, they can inhibit pro-inflammatory mediators (El-Saber Batiha et al., 2020; Kang et al., 2018). In *S. rhizomatus*, polyphenols, flavonoids, and alkaloids have been identified (Justil-Guerrero et al., 2021a). The hydroalcoholic extract of *C. spinosa* has shown anti-inflammatory effects in mice and chemopreventive effects in rats with induced prostatic dysplasia (Arroyo et al., 2017; Casado et al., 2011). The antioxidant activity of both species is directly proportional to the polyphenol content; in addition, *C. spinosa* decreased induced chronic inflammation in mice (Justil-Guerrero et al., 2021b).

The model of testosterone-induced hyperplasia in rats is the most used since it affects the prostatic ventral zone of rats, which is sensitive to hormonal changes and lesions that are similar to the central zone of the human prostate, known to be where morphological and immune alterations begin (Scolnik et al., 1994). Considering that inhabitants use both plant species for lower urinary tract inflammations, exhibit *in vitro* antioxidant activity, and their efficacy in the progression of prostatic hyperplasia is unknown, this research aimed to evaluate the antioxidant capacity of *C. spinosa* and *S. rhizomatus* and their effect on the progression of testosterone enanthate-induced prostatic hyperplasia in rats.

MATERIAL AND METHODS

Reagents and equipment

Hydrogen peroxide (H_2O_2); aluminum trichloride, Folin-Ciocalteu reagent, thiobarbituric acid, potassium ferricyanide; 1,10-Phenanthroline-4,7-diol; trichloroacetic acid (TCA); 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) and tetrazolium nitroblue were purchased from Sigma-Aldrich, USA. Testosterone enanthate from Bayer Pharma AG, Germany. UV-VIS spectrophotometer GENESYS® 10S, USA; analytical balance 0.0001 OHAUS Corporation, USA; LEICA DM 750 microscope with Leica ICC50 W camera, Switzerland.

Research design

The experimental intervention was considered the independent variable, while the dependent variable was measurable. The groups were randomly formed and the results of the treated groups were compared with those of the control group.

Study population

For antioxidant capacity, erythrocytes were used from male Holtzman rats at 4% in PBS. To evaluate the progression of prostatic hyperplasia, 66 male Holtzman strain rats (weighing 225 ± 25 g) from the National Institute of Health Biotechnology were used. Eleven groups of 6 rats were randomly formed.

Handling of experimental animals

The experiment was carried out following the guidelines of the "Guide for the Care and Use of Laboratory Animals" published by the National Research Council of the United States (National Research Council, 2011). The housing environment allowed the fulfillment of the five freedoms. The ambient temperature was maintained at $22 \pm 2^\circ\text{C}$, humidity

ty at $55 \pm 10\%$, noise level <20 kHz, the animals had access to 12 hour light-dark cycles and received a balanced rodent diet of the Universidad Agraria la Molina and purified water *ad libitum*.

The research protocol was reviewed and approved by the Faculty of Pharmacy and Biochemistry Ethics Committee of the Universidad Nacional Mayor de San Marcos on 21 May 2020 (Certificate number 0038/FFB-UDI-2020).

Plant species

Chuquiraga spinosa and *Senecio rhizomatus* species were collected in the province of Tayacaja in the Huancavelica department at 4000 meters above sea level. A sample of each species was sent to the Herbarium of the Museum of Natural History of the National University of San Marcos for taxonomic identification (certificates 242-USM-2019 and 243-USM-2019).

Extraction of secondary metabolites

The extraction was carried out according to (Zucolotto et al., 2012). The plant species were washed with potable water and dried in the shade for 1 day, then dried in an oven at $40 \pm 2^\circ\text{C}$ for 3 days. After grinding them with an electric mill, 5 mm particles were obtained. Maceration was performed with 70% and 50% ethanol, respectively, in a ratio of 1:10 for 7 days. Subsequently, the solvent was recovered using a rotary evaporator, resulting in hydroalcoholic extracts of *Chuquiraga spinosa* (ChS) and *Senecio rhizomatus* (SeR).

Secondary metabolite determinations

Identification of secondary metabolites was performed by phytochemical screening according to Lock (1994). After the extract was prepared with an appropriate solvent, it was divided into aliquots and subjected to specific tests for different compounds. These tests included ferric trichloride reaction for phenolic compounds, Shinoda and AlCl_3 test for flavonoids, gelatin and NaCl test for tannins, Dragendorff and Mayer tests for alkaloids, Borntrager test for quinones, Benedict and Feling A & B tests for reducing sugars, sulfuric vanillin test for terpenes, Liebermann-Burchard test for terpenoids and steroids, and Baljet A & B tests for sesquiterpene lactones. The results were observed through color changes or precipitate formation, providing a preliminary identification of the bioactive compounds present in the sample.

Total phenol content

Phenols were quantified by reducing the Folin-Ciocalteu reagent (Singleton et al., 1999). Twenty-five milligrams of extract were weighed and dissolved in

methanol at a concentration of $100 \mu\text{g/mL}$. From this solution $500 \mu\text{L}$ were taken and $250 \mu\text{L}$ of Folin-Ciocalteu reagent was added. After 5 minutes, $1250 \mu\text{L}$ of a 20% sodium carbonate solution was added to alkalize the sample. After a 2-hour incubation, protected from light, the absorbance was measured on a UV-VIS spectrophotometer at 760 nm. Three samples were analyzed for each extract. To calculate the phenol concentration in the samples, a calibration curve prepared with standard gallic acid solutions was used. Results were expressed in milligrams of equivalent gallic acid per gram of dry extract weight (mg GAE/g).

Total flavonoid content

Total flavonoids were determined by reducing AlCl_3 (Dewanto et al., 2002). Twenty-five milligrams of extract were diluted with methanol at a concentration of $100 \mu\text{g/mL}$. To 0.5 mL of the sample, 2.25 mL of H_2O and 0.15 mL of 5% NaNO_2 were added. After 6 minutes, 10% $\text{AlCl}_3 \cdot \text{H}_2\text{O}$ was added, the mixture was incubated for 5 minutes at room temperature, and then 1 mL of 1 M NaOH was added. The mixture was vortexed and the absorbance was measured using a UV-VIS spectrophotometer at 510 nm. Three samples were analyzed for each extract. To calculate the total flavonoid content in the samples, we used a calibration curve prepared with standard quercetin solutions. Results were expressed in milligrams of quercetin equivalent per gram of dry extract weight (mg QEE/g).

Antioxidant capacity in erythrocytes

The activity of the plasma membrane redox system (PMRS), reduced glutathione (GSH), and malondialdehyde (MDA) was evaluated. Blood was extracted by cardiac puncture in rats, plasma was removed to obtain erythrocyte packs, which were diluted to 4% in phosphate buffer (PBS) pH 7.4. After each extract (12.5 to $100 \mu\text{g/mL}$) or ascorbic acid (20 mM) was diluted with PBS. For the PMRS assay, PBS with 0.01% ascorbic acid was used. The erythrocytes were treated by incubating with each dilution in a 1:1 ratio for 30 minutes at 37°C , then centrifuged and the supernatant was removed. PMRS activity was evaluated by the reduction of ferricyanide to ferrocyanide (Avron and Shavit, 1963); 0.2 mL of treated erythrocytes was added to 1.8 mL of 1 mM potassium ferri-cyanide, incubated at 37°C for 30 minutes, centrifuged, and 1.5 mL of supernatant was added to 0.15 mL of 1,10-phenanthroline-4,7-diol. The absorbance was determined at 350 nm ($\epsilon = 20,500 \text{ M}^{-1} \text{ cm}^{-1}$), and the activity of PMRS was expressed in terms of the ferrocyanide concentration in $\mu\text{mol/mL}/30 \text{ min}$. The content of MDA and GSH was determined in H_2O_2 -

oxidized erythrocytes (Stocks and Dormandy, 1971). To 0.2 mL of treated erythrocytes, 1.8 mL of 200 mM H_2O_2 was added. MDA was determined according to Esterbauer and Cheeseman (1990); 1.5 mL of oxidized erythrocytes was added to 1.5 mL of 20% TCA for deproteinization, then after agitation and centrifugation, 1 mL of supernatant was added to 1.5 mL of 0.67% thiobarbituric acid, incubated at 100°C for 60 minutes, and absorbance was determined at 532 nm ($\epsilon = 156,000 \text{ M}^{-1} \text{ cm}^{-1}$), and concentration was expressed in nmol/mL. GSH was determined according to (Sedlak and Lindsay, 1968); 1 mL of deproteinized sample was added to 2 mL of 0.4 M TRIS solution pH 8.9, incubated at 80°C for 20 minutes and 50 μL of 0.6 mM DTNB was added, the absorbance was determined at 412 nm ($\epsilon = 13,100 \text{ M}^{-1} \text{ cm}^{-1}$), and the concentration was expressed in nmol/mL.

Effect on the progression of prostatic hyperplasia

Group formation, induction of prostate hyperplasia, and treatment

Eleven groups of $n=6$ were formed as follows: Group I: Normal (placebo 2 mL/kg); Group II: TE + placebo 2 mL/kg; Group III: TE + finasteride 1 mg/kg; Group IV: TE + ChS 250 mg/kg; Group V: TE + ChS 500 mg/kg; Group VI: TE + SeR 250 mg/kg; Group VII: TE + SeR 500 mg/kg; Group VIII: TE + ChS 250 mg/kg + SeR 250 mg/kg; Group IX: TE + ChS 250 mg/kg + SeR 500 mg/kg; Group X: TE + ChS 500 mg/kg + SeR 250 mg/kg; and Group XI: TE + ChS 500 mg/kg + SeR 500 mg/kg. Group formation was blinded to the caregiver responsible for treatment administration. Prostatic hyperplasia was induced with 25 mg of testosterone enanthate (TE) intramuscularly (Palomino-De-La-Gala et al., 2019) in groups II to XI on days 7, 14, 21, and 28. From day 1 to day 35, groups I and II received oral placebo (1% polysorbate 80) at a dose of 2 mL/kg, while groups III to XI received oral treatment (finasteride or extract).

Obtaining serum, prostatic index, and tissue homogenate

On the last day, the rats were weighed and then anesthetized with intraperitoneal pentobarbital at 50 mg/kg. Blood samples were obtained by cardiac puncture, and exsanguination was performed (Tobar Leito et al., 2021). The serum was separated by centrifugation at 2000 rpm. Dissection was performed from the ventral part to the genitourinary area, and the ventral lobes of the prostate were obtained, washed with PBS, and weighed on an analytical balance. The prostatic index (PI) was determined by dividing the weight of the prostate by the body weight (Buncharoen et al., 2016). Sections of both lobes were homogenized with PBS to obtain a concentration of 0.1 g/mL. After centrifugation, the supernatant was

stored at -20°C , and the rest of the lobe was fixed in 10% buffered formalin and embedded in paraffin for morphological evaluation.

Prostate-specific antigen (PSA) and oxidative stress

Rat PSA was determined in serum using an enzyme-linked immunosorbent assay (ELISA) kit code ELK9631 (ELK Biotechnology, USA) according to the supplier's instructions.

Nitric oxide

Nitric oxide (NO) was evaluated in the prostatic tissue supernatant. It was determined by reducing nitrates using the Griess reagent (Palmer et al., 1987). 800 μL of sample, 700 μL of H_2O , and 100 μL of 1 M NaOH were added. After 5 minutes, 100 μL of 30% ZnSO_4 was added. The mixture was stirred until a milky suspension was obtained and centrifuged at 3000 rpm for 15 minutes. To 920 μL of the obtained supernatant, a zinc powder aliquot was added, stirred, and left to stand for 45 minutes. Then it was centrifuged again at 3000 rpm for 15 minutes, and 800 μL of supernatant and 800 μL of a mixture of 2% sulfanilamide with 0.1% N-1-naphthyl ethylenediamine was added. The absorbance was determined with a UV-VIS spectrophotometer at 540 nm. Six samples were analyzed. To calculate the NO content in the samples, a calibration curve prepared with sodium nitrite standard was used. The results were expressed in $\mu\text{mol/g}$.

Malondialdehyde

Malondialdehyde (MDA) was determined in the prostatic tissue supernatant. It was determined by reaction with thiobarbituric acid (Esterbauer and Cheeseman, 1990). A sample of 0.5 mL was mixed with 1 mL of 20% trichloroacetic acid, and then, after stirring, it was centrifuged for 10 minutes at 3000 rpm. To 1 mL of the supernatant, 1.5 mL of 0.67% thiobarbituric acid in 0.25 N HCl was added, stirred, and placed in a boiling water bath for 30 minutes. Then, it was cooled with ice water. The absorbance was determined with a UV-VIS spectrophotometer at 535 nm. Six samples were analyzed. The concentration of MDA in the samples was calculated using an extinction coefficient of $156,000 \text{ M}^{-1} \text{ cm}^{-1}$. The result was expressed as nanomoles per gram (nmol/g).

Glutathione

Glutathione (GSH) was determined in the prostatic tissue supernatant. It was determined by reaction with DTNB (Sedlak and Lindsay, 1968). To 0.5 mL of the sample, 1.5 mL of 0.02 M TRIS buffer pH 8.2 and 0.1 mL of 5.5 1-dithiobis (2-nitrobenzoic acid) 0.01 M were added, then the volume was increased to 10 mL

with absolute methanol. A blank without sample and a blank without DTNB were prepared, then the tubes were capped with a rubber stopper and allowed to stand for 15 minutes, after which they were centrifuged at 3000 rpm for 15 minutes. The absorbance was determined at 412 nm on a UV-VIS spectrophotometer. Six samples were analyzed. The GSH content was determined using the molar extinction coefficient of $13,100 \text{ M}^{-1} \text{ cm}^{-1}$. The results were expressed as nanomoles per gram (nmol/g).

Superoxide dismutase

The activity of superoxide dismutase (SOD) was determined in the prostatic tissue supernatant. It was determined by reducing nitroblue tetrazolium (NBT) to formazan blue (Beauchamp and Fridovich, 1971). Solutions of 50 mM potassium phosphate pH 7.8, 0.3 mM NBT, 0.04 mM riboflavin, 0.1 mM EDTA, and 13 mM methionine were prepared. In a test tube, 2.6 mL of buffer, 0.3 mL of each solution, and 0.1 mL of the sample were mixed. The tubes were incubated at 25°C for 15 minutes, covered with aluminum foil, and then exposed to fluorescent light for 10 minutes. The absorbance was measured at 560 nm with a UV-VIS spectrophotometer. The SOD activity was calculated by dividing the difference in absorbance of the samples with and without SOD by the control absorbance, then multiplying by the dilution factor. Six samples were analyzed. Results were expressed in units per milligram (U/mg) of protein.

Catalase

Catalase (CAT) activity was determined in the prostatic tissue supernatant. It was determined by the reduction of dichromate/acetic acid to chromic acetate (Sinha, 1972). To 100 μL of supernatant, 2.9 mL of 0.05% H_2O_2 was added. The absorbance was immediately measured with a UV-VIS spectrophotometer at 240 nm every 15 seconds for 2 minutes. The reaction was stopped by adding 2 mL of dichromate-acetic acid reagent. Six samples were analyzed. CAT activity was determined by dividing the absorbance difference per minute by the milligrams of protein per milliliter and then multiplying by 7500. Results were expressed in units per milligram (U/mg) of protein.

Evaluation of prostate histology

The prostate sections, with a thickness of 5 μm , were deparaffinized, rehydrated with a graded series of alcohol, and stained with hematoxylin-eosin (Zhou et al., 2009). To achieve simultaneous visualization of the right and left lobes of the gland within the same section, a frontal cutting plane of the ventral prostate was selected. Morphological evaluation was conducted using an optical microscope (Scolnik et al., 1994).

Each section was assigned a score based on acinar morphology, cellular crowding, intraluminal papillary projections, loss of cellular polarity, and hyperplastic nodules (Table S1 in supplementary data). This scoring system and histopathological characteristics allowed the evaluation of prostatic hyperplasia progression into three categories: normal, benign prostatic hyperplasia, and atypical prostatic hyperplasia (Table S2 in supplementary data).

Data processing and analysis

For antioxidant capacity, prostatic index, PSA and oxidative stress, mean, standard error of the mean (SEM), and normality were calculated using the Shapiro-Wilk test. Analysis of variance and differences between groups was determined using the Tukey test. In the categories of progression of hyperplasia, the variance between groups was determined using the Kruskal-Wallis test, and comparisons with the control group were made using the Dunn test. For all tests, a significance level of 0.05 was assumed. The analyses were processed using SPSS version 24 software.

RESULTS

Phytochemical analysis

The yield obtained from ChS and SeR was 17% and 48%, respectively. The identified secondary metabolites and the content of polyphenols with total flavonoids are shown in Table 1.

Antioxidant capacity in erythrocytes

The PMRS activity in erythrocytes and the content of GSH and MDA in the erythrocytes subjected to oxidative stress are shown in Fig. 1. When evaluating the ChS-treated groups in Fig. 1A, all treatments are observed to increase the level of PMRS compared to the normal group. The highest increase was observed in the 100 $\mu\text{g}/\text{mL}$ group (1.71 times the normal level). Furthermore, treatments at 25 to 100 $\mu\text{g}/\text{mL}$ showed significant differences ($p < 0.05$) compared to the normal group. Regarding the level of GSH, Fig. 1C shows that all treatments significantly increased ($p < 0.05$) the level of GSH compared to the control group. The highest increase was observed in the 100 $\mu\text{g}/\text{mL}$ group (1.97 times the control level). Regarding the level of MDA, Fig. 1E shows that all treatments significantly reduced ($p < 0.05$) the level of MDA compared to the control. The greatest reduction was observed in the 100 $\mu\text{g}/\text{mL}$ group (0.29 times the control level). In general, when evaluating ChS treatment, the 100 $\mu\text{g}/\text{mL}$ group showed the most significant changes in reducing MDA and increasing PMRS and GSH.

Table 1. Identification and content of the phytochemical components of ChS and SeR.

Identification				Content		
Phytochemical components	Reagents	ChS	SeR	Phytochemical components	ChS	SeR
Phenolic compounds	Ferric trichloride	Presence	Presence	Total phenols ⁺	130.13 ± 1.02	103,48 ± 1.44
Flavonoids	Shinoda/AlCl ₃	Presence	Presence	Total flavonoids ⁺⁺	93.37 ± 1.72	82.71 ± 1.44
Tannins	Gelatin/NaCl	Absence	Presence			
Quinones	Borntranger	Absence	Presence			
Reducing sugars	Benedict/Feling A & B	Absence	Presence			
Terpenes	Sulfuric vanillin	Presence	Presence			
Alkaloids	Mayer/Dragendorff	Presence	Presence			
Triterpenes and steroids	Lieberman–Burchard	Presence	Presence			
Sesquiterpene lactones	Baljet A & B	Presence	Presence			

⁺Phenols content expressed in milligrams of gallic acid (GA) per gram of dry extract. ⁺⁺Flavonoid content expressed in milligrams of quercetin (QE) per gram of dry extract. ChS: *Chuquiraga spinosa*; SeR: *Senecio rhizomatus*.

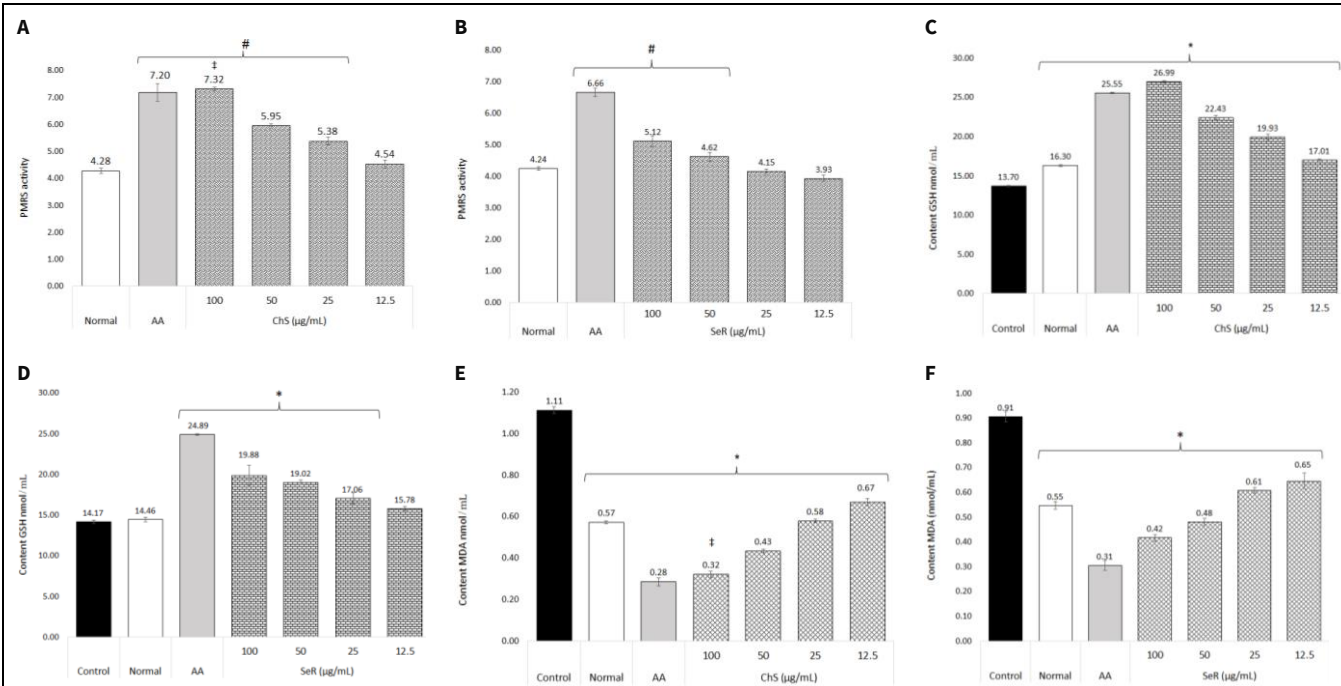


Figure 1. Antioxidant capacity of *Chuquiraga spinosa* (ChS) and *Senecio rhizomatus* (SeR) against erythrocytes subjected to oxidative stress (A) and (B): PMRS activity of erythrocytes; (C) and (D): GSH content in erythrocytes induced to stress with H₂O₂; (E) and (F): MDA content in stress-induced erythrocytes with H₂O₂. Data represent mean ± SEM (n = 3). *Comparison with the control group (p<0.05). #Comparison with the normal group (p<0.05). †Comparison with the AA group (p>0.05). AA: Ascorbic acid; MDA: malondialdehyde; GSH: reduced glutathione; PMRS: plasma membrane redox system.

Additionally, compared to AA, better activity was observed in PMRS and GSH, but less effect was observed on the reduction of MDA.

When evaluating the SeR-treated groups, Fig. 1B shows a significant increase (p<0.05) in PMRS activity in the 50 and 100 μg/mL groups compared to the normal group. The highest increase is observed in the 100 μg/mL group (1.20 times the normal level). For

GSH in Fig. 1D, the groups treated with concentrations of 25 to 100 μg/mL show a significant increase (p<0.05), with the 100 μg/mL group showing the most notable effect (1.40 times the control level). Regarding MDA in Fig. 1F, all treated groups show a significant reduction (p<0.05), with the 100 μg/mL group showing the greatest reduction (0.46 times the control level). In general, when evaluating the SeR-treated groups, the 100 μg/mL group exhibited the

most significant changes, indicating a notable effect on reducing MDA and increasing PMRS and GSH; however, it was observed that it was not more effective than the AA-treated group.

Evaluation of the effect on prostatic index, PSA, and oxidative stress

Table 2 shows the evolution of body weight, prostatic index (PI), and PSA level in serum in serum in different experimental groups. The normal group presents the lowest PI values (1.24 ± 0.16) and PSA values (0.49 ± 0.02 pg/mL). The control group (TE) has the highest PI values (2.64 ± 0.22) and PSA values (1.04 ± 0.04 pg/mL). Treatment with finasteride significantly reduces these values (PI: 1.59 ± 0.15 ; PSA: 0.54 ± 0.01 pg/mL), with inhibitions of 75.31% and 48.28%, respectively. Treatments with ChS 250 and ChS 500 also show reductions in PI and PSA, with greater inhibitions in ChS 500 (70.78% in PI and 41.99% in PSA). Similarly, treatments with SeR 250 and SeR 500 show high inhibitions, which are more pronounced in SeR 500 (80.29% in PI and 61.90% in PSA). The ChS and SeR combinations, although less effective than individual treatments of finasteride and SeR, still show significant reductions compared to the control. In summary, finasteride and SeR 500 are the most effective treatments to reduce PI and PSA, with inhibitions reaching up to 80.29% and 61.90%, respectively.

Table 3 presents the evaluation of oxidative stress performed on prostatic tissue. The effects of various

treatments on oxidative stress biomarkers and antioxidants were observed in different experimental groups. Compared to the control group treated with testosterone enanthate (TE), the groups treated with finasteride, ChS, and SeR (both individually and in combination) showed a significant reduction in malondialdehyde (MDA) and nitric oxide (NO), as well as an increase in levels of reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD). Treatment with SeR-500 treatment demonstrated the greatest reduction in MDA (57.91%) and a significant increase in GSH (114.07%), CAT (120.98%), and SOD (104.13%). The ChS and SeR combinations also improved these biomarkers, although the magnitude of the effects varied according to the specific dose and combination, with the ChS-500 + SeR-500 combination standing out for its notable increases in antioxidants. In general, these treatments showed positive effects in mitigating TE-induced oxidative stress, with ChS-500, SeR-250 and SeR 500 mg/mL being particularly noteworthy.

Evaluation of the effect on prostatic histopathological alteration in rats

Fig. 2 shows the description and histopathological score of the ventral prostate zone of rats. Extracts of ChS-500, SeR-250, and SeR-500 mg/kg presented mild architectural atypia, and those treated with the combination of ChS and SeR presented moderate architectural atypia.

Table 2. Body weight, prostatic index, and prostate-specific antigen (PSA).

Group (n)	Initial weight	Final weight	Prostatic weight	Prostatic index (PI)		PSA	
	(grams)	(grams)	(grams)	PI	Inh. (%)	pg/mL	Inh. (%)
Normal	215.67 ± 13.02	263.83 ± 13.09	0.33± 0.03* [#]	1.24 ± 0.16* [#]	-	0.49 ± 0.02*	-
Control (TE)	211.67 ± 12.46	247.33 ± 17.23	0.65 ± 0.03 [#]	2.64 ± 0.22 [#]	-	1.04 ± 0.04 [#]	-
Finasteride	211.00 ± 18.72	277.67 ± 14.17	0.44 ± 0.06*	1.59 ± 0.15*	75.31	0.54 ± 0.01*	48.28
ChS 250	211.17 ± 18.94	275.83 ± 32.59	0.54 ± 0.04* [#]	1.96 ± 0.13* [#]	49.11	0.76 ± 0.01* [#]	27.63
ChS 500	216.83 ± 14.06	280.33 ± 27.70	0.46* ± 0.06	1.65 ± 0.16*	70.78	0.61 ± 0.00*	41.99
SeR 250	217.83 ± 16.79	297.50 ± 53.14* [#]	0.49 ± 0.12* [#]	1.66 ± 0.52*	69.92	0.52 ± 0.01*	49.78
SeR 500	213.50 ± 13.30	286.50 ± 26.05* [#]	0.43 ± 0.04*	1.52 ± 0.19*	80.29	0.40 ± 0.01* [#]	61.90
ChS 250 + SeR 250	214.17 ± 17.14	257.33 ± 37.94	0.49 ± 0.04*	1.90 ± 0.38* [#]	53.36	0.67 ± 0.03* [#]	35.99
ChS 250 + SeR 500	215.83 ± 9.60	269.33 ± 31.81	0.53 ± 0.05* [#]	1.98 ± 0.35* [#]	47.42	0.85 ± 0.04* [#]	18.89
ChS 500 + SeR 250	217.67 ± 9.38	267.83 ± 34.62	0.53 ± 0.06* [#]	1.98 ± 0.22* [#]	47.20	0.93 ± 0.02* [#]	11.30
ChS 500 + SeR 500	217.67 ± 13.39	272.00 ± 25.60	0.54 ± 0.06* [#]	1.99 ± 0.15* [#]	46.97	0.66 ± 0.03* [#]	37.03

Each column represents the mean ± SEM (n = 6). *Comparison with the TE Control group (p<0.05). [#] Comparison with the TE + Finasteride group (p<0.05). PSA: prostate-specific antigen; Inh.: Inhibition; TE: testosterone enanthate; ChS: *Chuquiraga spinosa*; SeR: *Senecio rhizomatus*.

Table 3. Markers of oxidative stress in prostatic tissue.

Group (n)	Malondialdehyde		Nitric oxide		Reduced glutathione		Catalase		Superoxide dismutase	
	(nmol/g)	Inh. (%)	(μmol/g)	Inh. (%)	(nmol/g)	Inc. (%)	(U/mg protein)	Inc. (%)	(U/mg protein)	Inc. (%)
Normal	14.30 ± 0.28* [#]	-	0.11 ± 0.00*	-	13.52 ± 0.15*	-	38.43 ± 0.36*	-	32.31 ± 0.53* [#]	-
Control (TE)	38.96 ± 0.33 [#]	-	0.28 ± 0.01 [#]	-	6.13 ± 0.08 [#]	-	19.08 ± 0.29 [#]	-	14.84 ± 0.27 [#]	-
Finasteride	22.62 ± 0.42*	41.95	0.13 ± 0.00*	51.97	12.60 ± 0.11*	105.75	38.52 ± 0.52* [#]	101.91	25.10 ± 0.32*	69.19
ChS250	27.85 ± 0.25* [#]	28.53	0.16 ± 0.00* [#]	41.93	9.35 ± 0.11* [#]	52.72	35.42 ± 0.24* [#]	85.64	23.84 ± 0.26*	60.65
ChS500	20.55 ± 0.35*	47.27	0.15 ± 0.00*	46.09	11.97 ± 0.23*	95.33	39.16 ± 0.20*	105.28	29.12 ± 0.37* [#]	96.26
SeR250	18.28 ± 0.28* [#]	53.09	0.19 ± 0.01* [#]	32.11	11.98 ± 0.29*	95.57	41.98 ± 0.87* [#]	120.06	23.36 ± 0.35* [#]	57.47
SeR500	16.40 ± 0.26* [#]	57.91	0.12 ± 0.00*	54.84	13.11 ± 0.21*	114.07	42.16 ± 0.19* [#]	120.98	30.29 ± 0.36* [#]	104.13
ChS250+ SeR250	25.06 ± 0.87*	35.69	0.16 ± 0.01*	43.38	8.47 ± 0.17* [#]	38.26	34.32 ± 0.2* [#] 0	79.92	23.25 ± 0.67* [#]	56.69
ChS250+ SeR500	30.10 ± 2.03* [#]	22.75	0.18 ± 0.00* [#]	36.44	6.98 ± 0.27 [#]	13.97	27.66 ± 0.25* [#]	45.00	16.95 ± 0.32* [#]	14.26
ChS500+ SeR250	31.55 ± 1.26* [#]	19.03	0.23 ± 0.00* [#]	18.41	6.29 ± 0.10 [#]	2.63	24.41 ± 0.19* [#]	27.95	15.44 ± 0.18 [#]	4.06
ChS500+ SeR500	26.27 ± 0.55*	32.59	0.14 ± 0.00*	50.09	8.15 ± 0.47* [#]	33.03	31.88 ± 0.48* [#]	67.08	16.02 ± 0.27 [#]	7.95

Each column represents the mean ± SEM (n = 6). *Comparison with the TE control group (p<0.05). [#]Comparison with the TE + finasteride group (p<0.05). TE: Testosterone enanthate; Inh.: Inhibition; Inc.: Increase; ChS: *Chuquiraga spinosa*; SeR: *Senecio rhizomatus*.

Table 4. Evaluation of the progression of hyperplasia in the ventral zone of prostatic tissue.

Treatment	Normal	BPH	APC	P-value*	Comparison**
	(n)	(n)	(n)		
Normal	6	0	0	-	-
Control TE	0	4	2	-	-
TE + Finasteride	2	4	0	-	0.013
TE + ChS 250	0	6	0	-	0.237
TE + ChS 500	0	6	0	-	0.237
TE + SeR 250	0	6	0	0.046	0.237
TE + SeR 500	2	4	0		0.013
TE + ChS 250 + SeR 250	0	6	0		0.237
TE + ChS 250 + SeR 500	0	5	1	-	0.554
TE + ChS 500 + SeR 250	0	6	0	-	0.237
TE + ChS 500 + SeR 500	0	5	1	-	0.554

n: number of rats; BPH: Benign prostatic hyperplasia; APH: Atypical prostatic hyperplasia. *Kruskal-Wallis test. **Comparison with TE Control group (Dunn's test). ChS: *Chuquiraga spinosa*; SeR: *Senecio rhizomatus*. TE: Testosterone.

Table 4 represents the evaluation of the progression of prostatic hyperplasia, which was determined by histopathological scoring and histological description. Progression of prostatic hyperplasia was categorized into three groups: normal, BPH, and APH. Significant differences (p = 0.013) were observed in the groups treated with finasteride and SeR 500 mg/kg compared to the TE Control group.

DISCUSSION

In Table 1, it is observed that the phytochemical components of ChS and SeR are similar. Additionally, tannins, quinones, and reducing sugars were identi-

fied in SeR. These findings coincide with research on *Senecio rhizomatus* (Puelles et al., 2010) and *Chuquiraga spinosa*, where flavanols derived from kaempferol, quercetin, and isorhamnetin were also identified (Landa et al., 2009). Species of the *Senecio* genus have been found to contain hepatotoxic pyrrolizidine alkaloids (Brugnerotto et al., 2021), which can regulate inflammatory signaling pathways (Lin et al., 2022).

The induction of oxidative stress in erythrocytes with H₂O₂ was evidenced by increasing the level of MDA and decreasing the level of GSH in the control groups (Fig. 1C-F). H₂O₂ penetrates the erythrocyte

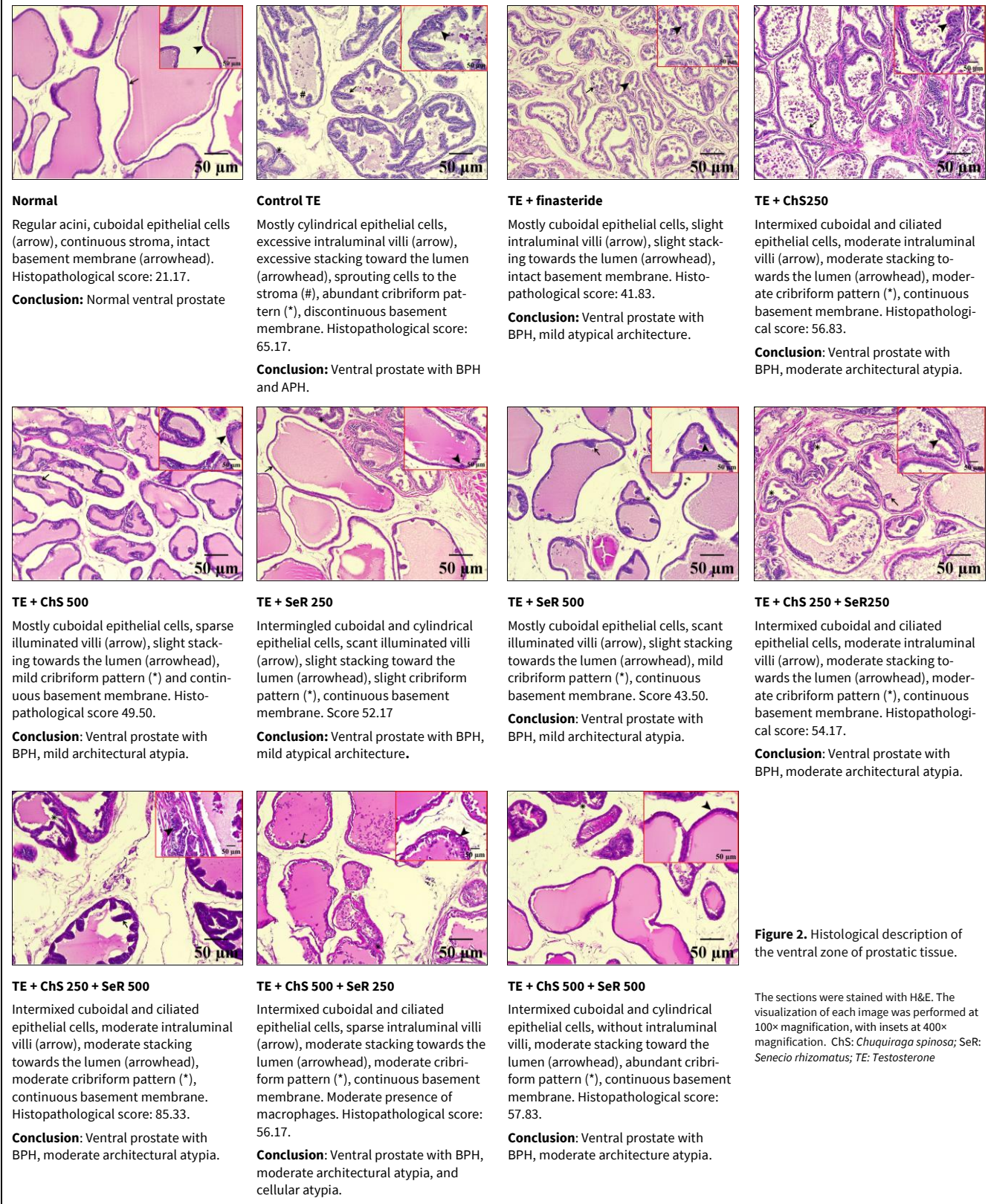


Figure 2. Histological description of the ventral zone of prostatic tissue.

The sections were stained with H&E. The visualization of each image was performed at 100× magnification, with insets at 400× magnification. ChS: *Chuquiraga spinosa*; SeR: *Senecio rhizomatus*; TE: Testosterone

membrane, disrupts internal redox balance, and promotes hemoglobin oxidation to methemoglobin, generating an oxidative cycle that leads to lipid peroxidation and GSH oxidation (Remigante et al., 2022).

Treatment of erythrocytes with ChS and SeR increased PMRS activity (Fig. 1A-B), elevated GSH, and significantly decreased MDA levels ($p<0.05$) (Fig. 1C-F). The PMRS system reduces extracellular oxidized ascorbate, mainly using intracellular ascorbate as a

substrate, while GSH replenishes intracellular ascorbate (Kennett and Kuchel, 2006). Therefore, the increase in PMRS activity and GSH levels observed in this study would prevent erythrocyte membrane lipid peroxidation, resulting in decreased MDA levels. This can be attributed to the antioxidant capacity of the polyphenols present in both extracts. As is known, quercetin is incorporated into the plasma membrane of erythrocytes and enhances the scavenging of free radicals (Han et al., 2021); furthermore, it increases GSH levels and reduces erythrocyte membrane lipid peroxidation (Remigante et al., 2022).

In the control group receiving only TE, there was an increase in IP and serum PSA levels (Table 2), while MDA levels increased, NO levels increased, and GSH, CAT, and SOD levels decreased significantly in prostatic tissue ($p < 0.05$) (Table 3). Histologically, alterations in the prostatic ventral lobes were observed, including epithelial alterations, irregularity of the acinar, and accumulation toward the stroma (Table 4). These alterations are characteristic of BPH and APA (Scolnik et al., 1994) and are due to the transformation of testosterone into dihydrotestosterone (DHT) by 5- α reductase in the cytoplasm. DHT then transcriptionally activates the androgen receptor (AR), positively regulating PSA mRNA in androgen-dependent prostatic cells (Likos et al., 2022) and increases inflammatory mediators in the prostatic epithelium and stroma (De Nunzio et al., 2016). Excess PSA alters the structure of the prostatic extracellular matrix, leading to an imbalance in paracrine signaling factors that promotes fibromuscular stromal proliferation (Fuhrman-Luck et al., 2014). Inflammatory and paracrine events mediated by AR result in increased IP due to chronic inflammation, cell hyperplasia, and reduced apoptosis (Vickman et al., 2020).

The oxidative stress and increased NO observed in the TE control group (Table 3) have also been described in both animals and humans. Furthermore, oxidative stress has been shown to activate AR, increase estrogen receptor expression, mediate the proliferative response, and damage prostatic DNA (Vital et al., 2016). Prostatic lipid peroxidation produces aldehyde compounds such as malondialdehyde (MDA) due to decreased levels of superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT) (Zabaiou et al., 2016). MDA can form adducts with functionally important proteins, such as apoptotic proteins, leading to their functional impairment (Barrera et al., 2018). In addition, excess NO can interact with radicals to produce peroxynitrite, which generates atypical protein nitrosation involved in physiological alteration. In this context, GSH can capture NO from nitrosylated proteins, preventing oxidative stress (Yoon et al., 2021).

In the groups treated with finasteride and extracts, mean levels of IP and PSA were significantly lower ($p < 0.05$) than in the TE control group (Table 2). ChS contains quercetin, which inhibits the transcriptional activity of AR (Yuan et al., 2010), and kaempferol, which inhibits the expression of the target gene AR for PSA, suppresses cell proliferation, and reduces the prostatic index (Da et al., 2019; Wang et al., 2021). Regarding SeR, it contains flavonoids, which are capable of reducing PSA levels (Csikós et al., 2021). The combination of ChS and SeR did not enhance the effect on IP and PSA, ruling out the possibility of synergy. A relevant finding was that ChS 500, SeR 250, and SeR 500 mg/kg extracts reduced IP and PSA similarly to finasteride ($p > 0.05$), which selectively inhibits 5- α reductase II, decreasing the level of DHT that activates AR in epithelial cells (Bauman et al., 2014) and reduces PSA production. Therefore, it could be inferred that the polyphenols in both extracts interact with AR or the enzyme 5- α reductase II.

The treated groups reduced oxidative stress. SeR 500, SeR 250, and ChS 500 mg/kg stood out, with SeR 500 mg/kg showing the greatest reduction in MDA and NO by 57.91% and 54.84%, respectively, and increased GSH, CAT, and SOD by 114.07%, 120.98%, and 104.13%, respectively (Table 3). The combination of both extracts did not show synergy. The effect of ChS on oxidative stress could be attributed to the presence of quercetin-3-O-rutinoside and isorhamnetin-3-O-glucoside, which inhibit lipid peroxidation and protein degradation (Barreca et al., 2016), as well as the glucosylated flavonoids of kaempferol, quercetin, and isorhamnetin (Landa et al., 2009), which decrease iNOS expression and activation of NF- κ B in inflamed prostatic tissue (Sciarra et al., 2008). SeR's antioxidant activity on prostatic tissue could be attributed to the presence of flavonoids, which prevent oxidative and nitrosative stress (Kumar et al., 2020).

As mentioned above, testosterone can be converted to DHT or estrogens. Excess of these products abnormally activates the androgen receptor (AR) and the estrogen receptor alpha (ER α) abnormally, respectively, triggering inflammation, oxidative stress, and alteration of the structure of the ventral prostatic tissue (Atawia et al., 2013), which can progress to atypical hyperplasia (HPA) (Scolnik et al., 1994). In this research, in Fig. 2 and Table 5, it can be observed that the induced hyperplasia groups, despite receiving treatment, progressed to BPH or HPA; however, these treatments preserved the cellular structures and prevented the progression of hyperplasia to an advanced state like HPA. When comparing between groups, those who received finasteride 1 mg/kg and SeR 500 mg/kg significantly regulated prostatic gland alteration ($p = 0.013$) compared to the TE control group.

This protection could be attributed to the presence of polyphenols in extracts; several studies have demonstrated that polyphenols may be involved in the regulation of prostatic tissue alteration (Mitsunari et al., 2021).

The increase in PMRS activity in erythrocytes by extracts is an important finding; however, it is necessary to identify the structure of the plasma membrane to which the extract components adhere. On the other hand, this research demonstrated efficacy in reducing PSA; however, the limitation is that it is unknown whether this action is due to the inhibition of 5- α reductase II or the regulation of AR in prostatic cells.

CONCLUSION

Hydroalcoholic extracts of *Chuquiraga spinosa* and *Senecio rhizomatus* have antioxidant capacity by protecting the plasma membrane of erythrocytes against oxidative stress. Furthermore, the administration of both extracts alone or in combination does not protect against BPH; however, they prevent progression to BPA by regulating prostatic index, PSA, and oxidative stress, thus preserving the structure of the ventral prostatic zone in rats in a model of testosterone-enanthate-induced prostate hyperplasia.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Justil-Guerrero HJ	Arroyo-Acevedo JL	Rojas-Armas JP	Justil-Guerrero CJ	Rondón-Ramos MC	Huarcaya-Rojas JY
Concepts or ideas	x	x	x	x	x	x
Design	x	x	x			
Definition of intellectual content	x	x	x			
Literature search	x	x	x	x	x	x
Experimental studies	x	x	x	x	x	x
Data acquisition	x			x	x	x
Data analysis	x	x	x	x	x	x
Statistical analysis				x	x	
Manuscript preparation	x	x	x			
Manuscript editing	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x

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Supplementary data

Table S1. Cumulative score of histopathological findings in the ventral prostate of rats (Scolnik et al., 1994).

Magnification 10×	Score
Luminal shape: Regular (1); Villous (3); Papillary (4); Cribiform (5)	
Acinar shape: Tubular (1); Branched (3); Irregular (5)	
Interacinar space: Long or moderate (1); Back-to-back glands (5)	
Stroma: Fine (1); Abundant (3); Severe smooth muscle fibrosis/hyperplasia (5)	
Magnification 40×	
Epithelial shape: Flattened (1); Cuboidal (1); Columnar (3); Hexagonal (5)	
Number of layers: Monolayer (1); Oligo, 2-3 (3); Pluri > 5 (5)	
Incidence of number of layers: Focal (3); Diffuse (5)	
Cellular alignment: Polar (1), apolar (3)	
If there is stacking of epithelial cells towards the lumen add (3)	
If there is budding of epithelial cells towards the stroma add (5)	
If periacinar groups of epithelial cells are found, add (3)	
If isolated groups of epithelial cells are found, add (5)	
Lesion distribution:	
Unilobar: isolated (2), multiple (6)	
Bilobar: isolated (4), multiple (8)	
Nuclear shape:	
Round: regular (1), irregular (5)	
Small (2), large (2), small and large in the same acinus (5)	
Mitosis per field: absent (0); isolated 1-2 (2); abundant 3-5 (5); excessive > 6 (10)	
Basement membrane:	
Intact (1), Disrupted (5)	
Thin (1), Thick (5)	
Total score	

Table S2. Histopathological characteristics in the ventral prostate of rats related to their total score (Scolnik et al., 1994).

Criteria	Normal	BPH	APH
Acinar regularity	Present	Absent	Absent
Stroma	light	Variable	Variable
Cell shape	Cuboidal	Cuboidal/cylindrical	Cylindrical
Cell polarity	Present	Present	Absent
Nuclear form	Round	Round/ovoid	Irregular
Mitosis	Absent	Isolated	Isolated/abundant
Villi acinar	Absent	Light	Extensive
Cribriform pattern	Absent	Absent	Present
Cell stacking towards the lumen	Absent	Moderate	Excessive
Cell budding towards the stroma	Absent	Light	Excessive
Basal membrane	Intact	Focal interruption	Discontinuous
Total score	≤ 40	41 - 61	> 61
BPH: Benign prostatic hyperplasia, APH: Atypical prostatic hyperplasia.			