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ORIGINAL RESEARCH

Protective effect of *Chuquiraga spinosa* Lessing associated with simvastatin on N-Nitroso-N-methylurea (NMU)-induced prostate cancer in rats

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Background and objective: *Chuquiraga spinosa* Lessing (ChS) has shown protective effect on N-Nitroso-N-methylurea (NMU)-induced prostate cancer in rats. Currently, statins are being studied for their pro-apoptotic and antimetastatic effects. The main objective of this research was to determine the protective effect associated with the oral administration of simvastatin and ethanolic extract of the aerial parts of ChS in the prevention of prostate cancer.

Methods: Fifty-six albino male rats were randomized into seven groups: I) negative control: physiological serum: 2 mL/kg; II) TCN: testosterone 100 mg/kg + cyproterone 50 mg/kg + NMU 50 mg/kg; III) TCN + S40 (simvastatin 40 mg/kg); IV) TCN + ChS250 (ChS 250 mg/kg); V) TCN + ChS50 (ChS 50 mg/kg) + S40; VI) TCN + ChS250 (ChS 250 mg/kg) + S40; and VII) TCN + ChS500 (ChS 500 mg/kg) + S40. The antioxidant activity was tested by using (2,2-diphenyl-1-picrylhydrazyl) (DPPH) assay. Hematology, toxicological biochemical parameters, prostate-specific antigen (PSA), histology and prostate size were evaluated as main indicators of protective effect.

Results: Triglyceride values were decreased in the groups receiving ChS, being significant (P=0.02) in IV and VII group compared to cancer-inducing group (TCN). In groups that received ChS, PSA levels (P=0.71) were significant compared with TCN group. The VII group had the lowest prostate volume by sonography. The TCN group showed multiple foci of high-grade prostatic intraepithelial neoplasia (HG-PIN) with the presence of cells in mitosis; whilst, groups V and VI had few areas of HG-PIN.

Conclusion: In experimental conditions, the ethanolic extract of *C. spinosa* in association with simvastatin showed a protective effect on prostate cancer through hypolipidemic and antioxidant activity.

Keywords: prostate cancer, anti-tumor, *Chuquiraga spinosa*, simvastatin

Introduction

Prostate cancer is the most frequently diagnosed malignancy and is the fifth leading cause of death in men worldwide and one of the leading causes of cancer death in the United States;[1,2] also considering prostate cancer presents slow growth, several strategies include monitoring initially and reducing complications associated with surgery and radiation therapy can generate eventually resistance to androgen deprivation;[3,4] In addition, these treatments can cause the risk of creating side effects.

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Table 1 Effects of *Chuquiraga spinosa* Lessing (ChS) extract associated with simvastatin on hematological parameters after 20 weeks of treatment

Experimental groups		Hematological parameters					
	Hb (g/dL)	Ht (%)	WBC ($\times 10^3\ \mu L^{-1}$)	Neutrophils (%)	Eosinophils (%)	Platelets ($\times 10^3\ \mu L^{-1}$)	RBC ($\times 10^3\ \mu L^{-1}$)
Negative control	14.9±0.7	46.6±2.3	4333±986	26.6±3.2	5.0±1.0	393.666±3.132	7.1±0.21
TCN	15.5±0.5	48.3±1.5	8966±1342	26.3±4.5	4.6±0.6	416.000±4.680	6.80±0.11
TCN + ChS250	15.4±0.3	48.0±1.0	4266±289	26.0±3.0	5.0±1.0	495.666±2.829	7.22±0.11
TCN + S40	16.2±0.6	50.3±2.3	5600±900	30.0±4.3	5.0±1.0	401.666±1.650	6.81±0.5
TCN + ChS50+ S40	15.9±0.8	49.6±2.5	4933±832	28.6±3.0	4.6±0.6	410.000±35.085	6.70±0.18
TCN + ChS250+ S40	14.9±1.2	46.3±3.7	4000±529	27.6±4.1	4.6±1.0	477.333±6.658	7.93±0.08
TCN + ChS500+ S40	15.4±1.2	48.0±3.0	5666±144	26.3±2.5	5.3±1.1	451.000±1.000	6.10±0.5
Coefficient F/*p**	1.06/0.43	1.01/0.45	2.56/0.06	0.50/0.79	0.21/0.96	2.13/0.11	6.40±0.5

Notes: Values expressed as mean ± SD (n=6). *ANOVA Test. Tukey test. In Table 1, no statistical significance was observed between normal control and TCN control group in all measured parameters except for a significant ($p<0.001$) increase in white blood count (WBC) in the TCN + ChS250 compared to TCN (Table 1). TCN + ChS250+ S40-treated animals showed a significant ($p<0.001$) decrease in WBC, platelets, and increase in red blood count (RBC) ($p<0.001$) as compared to TCN and normal groups. Concerning creatinine content in blood, no significant changes were observed between animals treated with TCN and extract associated with simvastatin at all tested doses.
Abbreviations: TCN (Inductor cancer), Testosterone 100 mg/kg + cyproterone 50 mg/kg + NMU 50 mg/kg; ChS50, ChS 50 mg/kg; ChS250, ChS 250 mg/kg; ChS500, ChS 500 mg/kg; S40, Simvastatin 40 mg/kg; Hb, hemoglobin; Ht, hematocrit; WBC, white blood count.

Cholesterol is the main sterols and structural component of cell membranes; its biosynthetic pathway is indirectly related to cell-growth processes.[5] Statins, which are a class of lipid-lowering drug reduce not only serum cholesterol but also mevalonate synthesis by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA). Mevalonate is a precursor of several major products regulating the cell cycle, including dolichol, geranyl pyrophosphate (GPP), and farnesyl-pyrophosphate (FPP).[6] Dolichol, GPP, and FPP have a stimulatory effect on DNA synthesis and is linked to several tumor cell proteins, for instance, isoprenylating the intra-cellular G-proteins Ras and Rho, which are involved in cell proliferation, differentiation, and apoptosis.[7]

Syväläa et al, studied the effectiveness of the combination of simvastatin and enzalutamide in prostate cancer cells, observing a reduction on growth and signaling, with large induction of autophagy.[8] Pennanen et al, evaluated the association of simvastatin and metformin, demonstrating a synergism by reducing the cytosolic ATP, induction of necrosis and autophagy on tumor cell lines of prostate cancer.[9] Murtola et al, determined that a lipophilic statin (Simvastatin) exhibits a greater inhibition of cell growth compared to one hydrophilic (Rosuvastatin).[10] Miyasawa et al, determined the expression of annexin A10, which is associated to inhibit the proliferation, migration, and invasion into cells of human prostate cancer.[11]

The World Health Organization (WHO) states that about 65% of the population worldwide prefer to use medicinal plants, approximately 60% of the agents used against cancer derived from medicinal plants and other natural resources.[12] The National Cancer Institute of the United States (NCI) identified 3000 plants with anticancer properties, the majority of tropical origin (70%). Traditionally *Chuquiraga spinosa* is used as a treatment of urinary diseases in northern Peru.[13]

On the other hand, *C. spinosa* (Family: Asteraceae) known as "huamanpinta" in Peru has shown protective effect on N-methyl-N-nitrosourea (NMU)-induced prostate and gastric cancer in rats as well as cytotoxicity on prostatic carcinoma (DU-145).[14]

Cave et al, indicate that the presence of polyphenols can generate beneficial effects in reducing the risk of cancer due to its anti-inflammatory and antioxidant capacity.[15] Prevention of cancer diseases can be favorable from the economic approach because it allows spending less on specialized human resources, centers and expensive treatments that are usually prolonged, so more investment is required.

The main objective of this research was to determine the protective effect in the prevention of prostate cancer associated with the oral administration of simvastatin and the ethanolic extract of the aerial parts of *C. spinosa* Lessing (ChS).

Materials and methods

Chemicals

NMU; Ferric chloride ($FeCl_3$); Folin-Ciocalteu; 2,2′-diphenyl-1-picrylhydrazyl (DPPH); thiobarbituric acid; and trichloroacetic acid were purchased from Sigma Co. (St. Louis, MO, USA). Sulfuric acid; sodium carbonate (Na_2CO_3); sodium hydroxide (NaOH); were obtained from

Table 2 Effects of *Chuquiraga spinosa* Lessing (ChS) extract associated with simvastatin on some toxicological biochemical parameters after 20 weeks of treatment

Experimental group	Toxicological biochemical parameters								
	AST (UI/dL)	ALT (UI/dL)	AP (UI/dL)	TB (mg/dL)	IB (mg/dL)	Total proteins (g/dL)	Albumin (g/dL)	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)
Negative control	213.9±8.6	86.3±3.4	330.3±33.5	0.9±0.2	0.5±0.1	6.8±0.1	3.9±0.1	75.3±7.3	104.0±12.4	31.0±1.7
TCN	248.3±8.9	106.6±12.6	314.6±99.0	0.7±0.1	0.4±0.1	7.0±0.3	4.0±0.1	106.3±43.0	177.3±57.5	29.0±2.0
TCN + ChS250	248.2±22.7	108.3±5.8	314.0±52.0	0.8±0.1	0.5±0.1	7.1±0.5	3.9±0.1	75.3±7.3	87.0±6.5 *	31.0±1.7
TCN + S40	217.5±45.0	108.3±16.4	394.0±71.3	0.8±0.1	0.5±0.0	6.7±0.5	3.9±0.1	80.0±10.1	100.0±28.8	32.3±4.1
TCN + ChS50+ S40	258.3±33.5	114.3±18.0	423.3±134.3	0.7±0.1	0.5±0.1	7.3±0.2	4.0±0.0	92.0±9.6	100.6±26.3	29.3±1.5
TCN + ChS250+ S40	246.6±26.2	110.6±9.2	336.3±39.2	0.7±0.0	0.5±0.1	7.0±0.3	4.0±0.1	86.6±8.0	98.3±12.6	33.0±1.0
TCN + ChS500+ S40	255.5±26.7	94.3±10.0	453.3±147.0	0.8±0.1	0.5±0.1	7.0±0.1	4.0±0.1	77.0±11.5	81.6±22.4*	30.9±1.8
Coefficient F/p*	1.29/0.32	2.09/0.12	1.12/0.39	1.17/0.37	1.27/0.33	0.82/0.57	0.53/0.77	1.56/0.23	3.78/0.02	1.99/0.13

Notes: Values expressed as mean ± SD (n=6). *ANOVA Test. Tukey test. Table 2 depicts the effects of ChS extract associated with simvastatin on some toxicological biochemical parameters. Rats that received only TCN presented a significant ($p<0.001$) increase in the transaminases (ALT and AST) values as compared to normal rats. TCN + ChS500 + S40 showed a significant increase in both enzymes activities, ALT ($p<0.001$) and AST ($p<0.001$). TCN + S40 group showed a significant decrease in AST activity ($p<0.001$) as compared to TCN group. On fasting lipid levels, a statistically significant ($p<0.001$) reduction in the TG level was found with TCN + S40 and TCN + ChS500+ S40 group as compared to TCN group. It was also observed with TCN + ChS500+ S40 a significant decrease in the HDL cholesterol ($p<0.05$) levels as compared to TCN group.

Abbreviations: TCN (Inductor cancer), Testosterone 100 mg/kg + cyproterone 50 mg/kg + NMU 50 mg/kg; ChS50, ChS 50 mg/kg; ChS250, ChS 250 mg/kg; ChS500, ChS 500 mg/kg; S40, Simvastatin 40 mg/kg; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein; ALT, alanine transaminase; AST, activity, aspartate transaminase; AP, alkaline phosphatase; IB, indirect bilirrubin; TB, total bilirrubin.

Merck Co. (Darmstadt, Germany). Other solvents used were of analytical grade and purchased from Merck Co. (Darmstadt, Germany). Simvastatin was purchased from Medifarma Co (Lima-Peru).

Plant collection

The plant was collected in April 2018 from Huanta, Ayacucho, Peru. The plant material was botanically identified by Dr. Mario Benavente, from the Universidad Nacional Mayor de San Marcos (Lima-Peru). A voucher specimen (25-USM-2018) was deposited at the National Herbarium of Natural History of the UNMSM.

Extract preparation

The fresh, whole plant (2 kg) was collected and shade dried to obtain 500 g dry sample which was later powdered in a blade mill and used for solvent extraction. For sample preparation, 500 g of dried sample was extracted once (3000 mL for each) with 96% ethanol at 22°C for 72 hrs and concentrated using a rotary evaporator under reduced pressure at 40°C to yield the ChS extract (19.5%). The extract was stored in amber vial until further use.

Qualitative phytochemical screening of the ethanolic extract

Qualitative phytochemical screening was done using standard procedures described by Evans[15] to determine the phytochemicals present. The extract (5 mg) was dissolved in 10 mL of the respective solvents used for their extraction. The solution was made ready for qualitative phytochemical analysis for carbohydrates, phenols, anthracene derivatives, saponins, tannins, flavonoids, alkaloids, and terpenes.

Evaluation of antioxidant activity

The antioxidant activity of the ethanolic extract was evaluated by the method proposed by Herrera et al.[16]

1,1-diphenyl-2-picrylhydrazyl (DPPH) was used at a concentration of 20 µg/mL in methanol and the extract at concentrations of 1.0, 10.0, and 50.0 µg/mL in methanol was used in the reactions.

Animals

Fifty-six male Holtzman rats with an average weight of 200 g ±20 g, from the National Institute of Health, acclimated for 5 days in large-ventilated cages and food ad libitum were used. The study was conducted in the Bioterio of the Faculty of Medicine, Universidad

Nacional Mayor de San Marcos (Lima-Peru). Rats were maintained in periods of 12 hrs' light/dark cycle and a temperature of 21±2°C.

Induction of prostate cancer in rats

Tumor induction was carried out following the method of Bosland and Prinsen[17] with slight modifications. Rats received cyproterone acetate daily (50 mg/kg body weight in sesame oil) by intraperitoneal injection for 18 consecutive days, 1 day after the final dose of cyproterone acetate, rats received daily subcutaneous injections of testosterone propionate (100 mg/kg in sesame oil) for 3 days; next day, each rat received a single intraperitoneal injection of NMU (50 mg/kg body weight in sterile saline, pH 5.0). The groups were named according to the treatment and dosed in mg/kg.

Experimental groups

Rats were randomized into seven groups with eight rats per group; according to the following experimental design: 1) Negative control: PS 2 mL/kg; 2) TCN: Testosterone 100 mg/kg + cyproterone 50 mg/kg + NMU 50 mg/kg; 3) TCN + ChS250 (ChS 250 mg/kg); 4) TCN + S40 (Simvastatin 40 mg/kg); 5) TCN+ ChS50 (ChS 50 mg/kg) + S40; 6) TCN + ChS250 (ChS 250 mg/kg) + S40; 7) TCN+ ChS500 (ChS 500 mg/kg) + S40. The treatment was received by 20 weeks. At the end of the experimental period, the rats were weighed. Blood samples were obtained to assess biochemical and hematological indicators. The animals were sacrificed by pentobarbital anesthetic (100 mg/kg).

Hematological and biochemical

Drawing blood in rats was by intracardiac puncture, the animals were previously subjected to a state of anesthesia using pentobarbital 30 mg/kg.

Hematological parameters were determined spectrophotometric method; total leucocyte was counted in a Neubauer chamber; total cholesterol by the modified method of Roeschlau et al,[18] cholesterol high-density lipoproteins was determined based on the method of Trinder;[19] triglycerides were estimated by the enzymatic method GPO-PAP, as described by Høstmark et al.[20] Alanine aminotransferase by using the method of Mohun et al;[21] alkaline phosphatase activity was evaluated by the method of Jacoby et al;[22] the determination of urea according to the cleavage of urea with urease (Berthelot reaction) according to Fawcett and Scott.[23]

The amount of specific antigen (prostate-specific antigen, PSA) was quantified using an ELISA kit available commercially (Diagnostics Biochem, Dorchester, ON, Canada) against a standard curve (0.2–50 ng/mL PSA).

Prostate ultrasonography

After the tumor induction, a high-level system (CHISON D600 VET, JIANG SU, CHINA) was used with a linear 10 MHz transducer. Before the examinations, rats were shaved in the lower abdomen and then placed in the supine position on a heating pad. A B-mode test was performed to determine the site and size of the tumor within the prostate.

Statistical analysis

Data are presented as mean ± standard deviation (SD in triplicate from three independent in vitro experiments and for each in vivo experimental group. Statistical analysis of data with SPSS software version 21.00 were realized using the one-way analysis of variance followed by Tukey's post hoc test for multiple comparisons. Statistical significance of differences was considered at a value <0.05.

Ethical considerations

This research was approved by the Ethics Committee of the Faculty of Medicine of San Marcos, Acta 0310 (4 November 2017). During the study, the specifications proposed by the Institutional Committee for the Care and Use of Animals (CICUA, ILAR) were followed, and the current regulations of the Animal Protection Act (Law 27,265) were respected.

Results

The qualitative phytochemical analyses revealed that ChS ethanolic extract contains, tannins, alkaloids, terpenes, quinones, flavonoids, and phenols as main phytochemical components. Moreover, it can be observed that the efficacy concentrations of ChS extract which result in 50% of the scavenging (EC50) are 25.4 µg/mL (DPPH). In Figure 1, percentage uptake of DPPH was dependent doses 10.0 µg/mL (41.5%) 50.0 µg/mL (68.1%), and 100.0 µg/mL (85.0%).

Discussion

Many researches have demonstrated that statins to be beneficial as anticancer agents.[24] The anti-cancer effects of this kind of drugs may be due to various biological processes, such as inhibition of cell proliferation, induction of apoptosis, inhibition of angiogenesis as well as stop

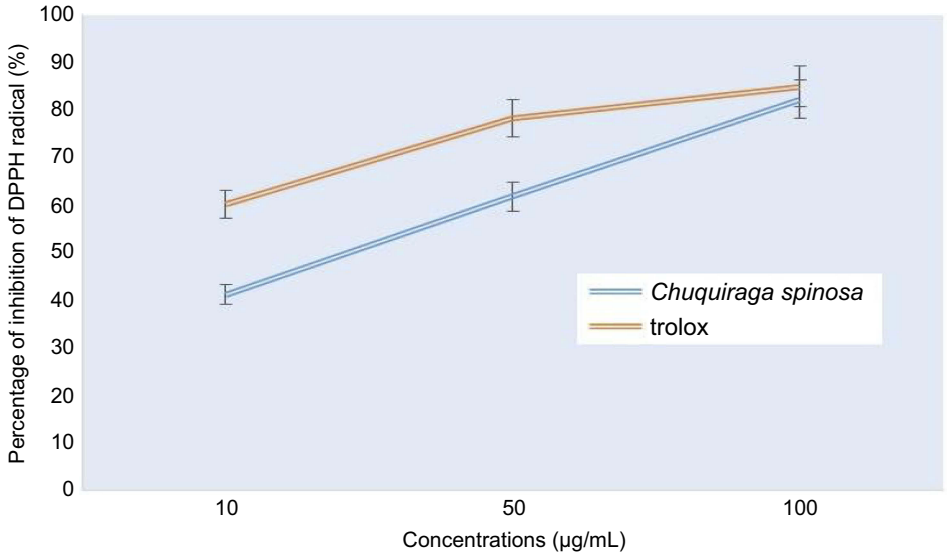


Figure 1 Antioxidant effect of *Chuquiraga spinosa* Lessing on DPPH radical.

of metastasis, improvement of the immunity system or targeting some receptor to combat malignant cells.[25]

In the development of strategies for the chemoprevention of prostate cancer, a series of animal models have been developed to evaluate such strategies, thus emerging sequential regimes as described by McCormick DL et al (1998)[26] in their research with the use of NMU induces an incidence of 3% of dorsolateral adenocarcinoma that increases to 18% in the NMU + Testosterone association. In the research of Bosland, M.C. and Prinsen, MK (1990),[27] the experimental groups that received injections of MNU, after the pre-treatment with Cyproterone and Testosterone, presented a combined incidence of adenocarcinomas and carcinomas in situ of the dorsal side of 30% with a low incidence of focal atypical hyperplasia. In our study, we found in the TCN group, an incidence of 25% of the high-grade intraepithelial neoplasia, confirming the adequate development of the animal model.

The hematological parameters are identified within the normality between the groups, there are no toxic effects with the association between ChS and Simvastatin (Table 1). Concerning the hepatic and lipid profile, a decrease in the total cholesterol and triglycerides levels is evidenced in the ChS+ simvastatin groups compared to the TCN group, being only a significant difference for triglycerides (Table 2). Statins inhibit cell cycle proliferation, induce apoptosis, inhibit angiogenesis and metastasis, delay the progression of intraductal pancreatic neoplasia in KC transgenic mouse model, avoid hepatocellular carcinogenesis, inhibit azoxymethane-induced colonic preneoplastic lesions in obese C57BL/KsJ-db/db mice.[27] In our study, the synergistic effect in the reduction of triglyceride levels when using ChS + simvastatin, which is associated with its antioxidant capacity would explain the results of the promising ones.

PSA is one of the most used biomarkers that has revolutionized the management of prostate cancer, only a destruction of the basement membrane of the epithelial cells of the prostate can cause excessive leakage of PSA into the bloodstream;[28] In our study, there were no differences between the levels of PSA, which contradicts the results of previous investigations where the PSA in the TCN group reached 1.2±0.2 ng/mL (Figure 2), so it was analyzed in a complementary way. The dimensions of the prostate, in various studies an inverse relationship between prostate volume and the incidence of prostate cancer has been demonstrated, we can see a smaller volume in the TCN + H500+ group S40 and TCN + H250+ S40; with several foci of high-grade intraepithelial neoplasia compared to the TCN + H50+ S40 group, which has a higher volume and few focal points of PIN AG (Figure 3), these findings associated with the absence of PIN in the group receiving a tamponade and few centers of BG-PIN in the groups receiving simvastatin, evidence the chemoprotective effect of *C. spinosa* at low doses (Table 3).

Previous phytochemical studies have revealed that *C. spinosa* contains nine types of flavonoids (quercetin-3-O-glucuronide, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, kaempferol-3-O-glucuronide, kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside, isorhamnetin-3-O-glucuronide, isorhamnetin-3-O-rutinoside, and isorhamnetin-3-O-glucoside) and a phenolic compound (p-hydroxyacetophenone),

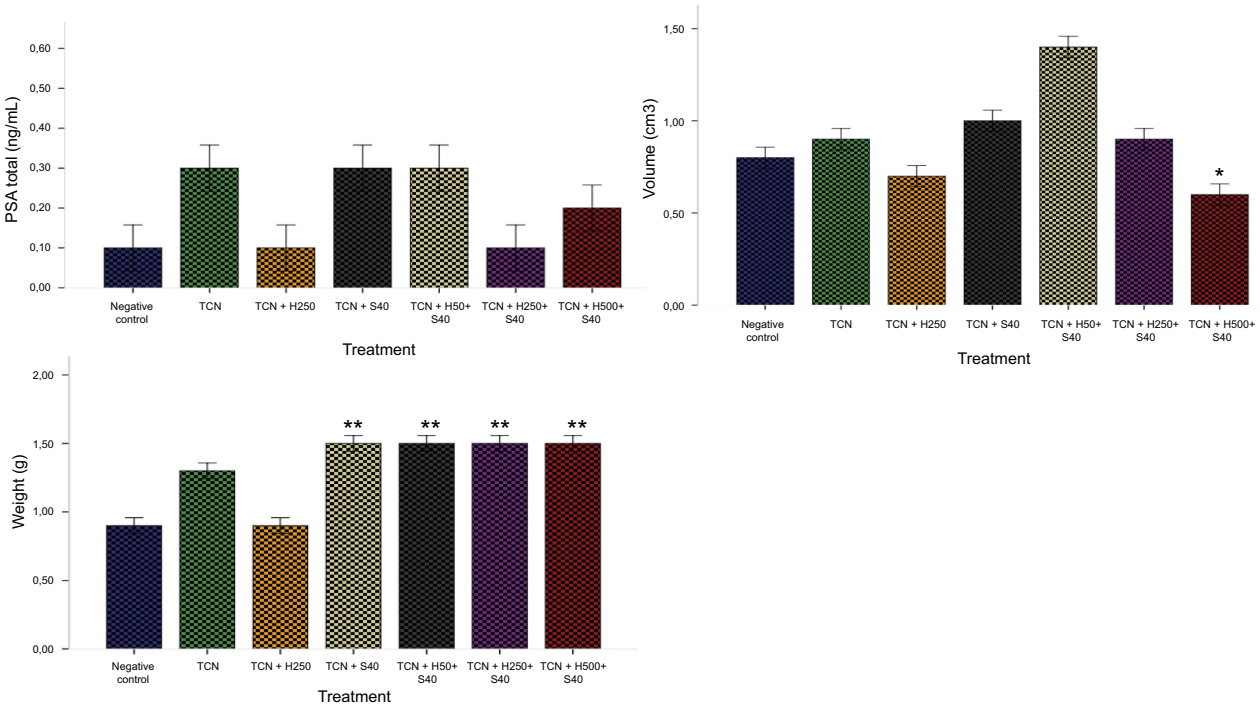


Figure 2 Levels of PSA and prostate size in rats treated.
Notes: Data were expressed as mean ± SD values (n=6) Test ANOVA. Tukey test * ($p<0.05$) vs group TCN + ChS50+ S40. ** ($p<0.05$) vs negative control group.
Abbreviations: ChS, *Chuquiraga spinosa* Lessing; PSA, prostate-specific antigen; TCN, TCN (Inductor cancer): Testosterone 100 mg/kg + cyproterone 50 mg/kg + NMU 50 mg/kg; ChS50, ChS 50 mg/kg; H250, ChS 250 mg/kg; ChS500, ChS 500 mg/kg; S40, Simvastatin 40 mg/kg.

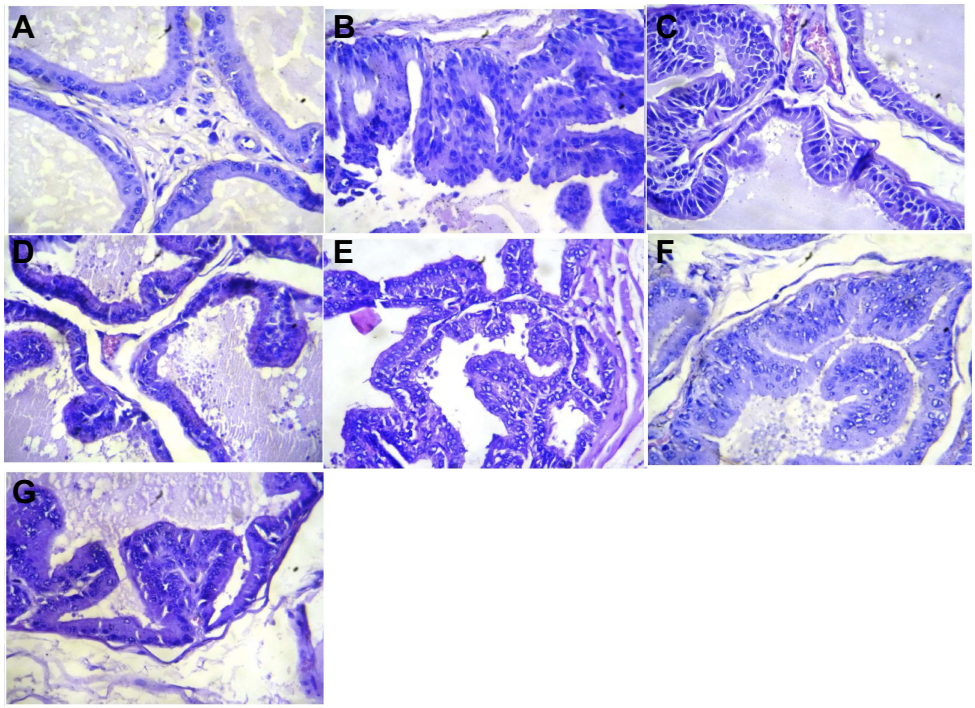


Figure 3 Prostate histological sections taken with hematoxylin & eosin (400x): (**A**) Negative control: Normal. Simple cuboidal epithelium. (**B**) TCN: High-grade prostatic intraepithelial neoplasia (HG-PIN), with cells in mitosis. (**C**) TCN + H250: columnar epithelia with papilla formation. (**D**) TCN + S40: Isolated foci of low grade prostatic intraepithelial neoplasia (LG-PIN). (**E**) TCN + ChS50+ S40: LG-PIN with few bulbs (**F**) TCN + ChS250+ S40: LG-PIN and some HG-PIN bulbs. (**G**) TCN + ChS500+ S40: LG-PIN with several spotlights of HG-PIN.
Abbreviations: TCN, TCN (Inductor cancer): Testosterone 100 mg/kg + cyproterone 50 mg/kg + NMU 50 mg/kg; ChS50, ChS 50 mg/kg; ChS250, ChS 250 mg/kg; ChS500, ChS500 mg/kg; S40, Simvastatin 40 mg/kg.

Table 3 Histological parameters of *Chuquiraga spinosa* Lessing associated with simvastatin

Experimental group	Lumen	Epithelium	Layers	LG-PIN	HG-PIN
Negative control	Regular	Cubic	1	–	–
TCN	Papillary	Columnar	>5	+++	+++
TCN + ChS250	Papillary	Columnar	2. 3	+	–
TCN + S40	Papillary	Columnar	>5	++	+
TCN + ChS50+ S40	Papillary	Columnar	2. 3	+	–
TCN + ChS250+ S40	Papillary	Columnar	2. 3	+	–
TCN + ChS500+ S40	Papillary	Columnar	>5	++	+

Notes: (–) = negative; (+) = Slight amount; (++) = moderate; (+++) = Abundant.
Abbreviations: LG-PIN, low grade-prostatic intraepithelial neoplasia; HG-PIN, high grade-prostatic intraepithelial neoplasia; TCN, TCN (Inductor cancer): Testosterone 100 mg/kg + cyproterone 50 mg/kg + NMU 50 mg/kg; ChS250, ChS 250 mg/kg; S40, Simvastatin 40 mg/kg; ChS50, ChS 50 mg/kg; ChS500, ChS 500 mg/kg.

which the flavonoid isorhamnetin-3-O-glucuronide has anti-inflammatory activity in vitro and the flavonoids kaempferol and quercetin are inversely associated with lung cancer.[29,30] Finally, experimental trials have shown antioxidant, anti-inflammatory, and antifungal activity of the methanolic extract. Limitations of this study included the lack of determination of metastasis and vascular flow prostate also increased induction time of prostate cancer and dysplasia may show elevated levels of PSA.

In conclusion experimental conditions, the extract *C. spinosa* associated with simvastatin has a chemopreventive effect on prostate cancer through the hypolipidemic and antioxidant activity.

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Disclosure

The authors report no conflicts of interest in this work.

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