The *Pavonia xanthogloea* (Ekman, Malvaceae): Phenolic compounds quantification, anti-oxidant and cytotoxic effect on human lymphocytes cells

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ABSTRACT

Introduction: *Pavonia xanthogloea* is traditionally used as an antimicrobial and anti-tumour medicine in Southern Brazilian region. However, investigations about this species are still incipient. **Hypothesis Tested:** The study postulated that *P. xanthologea* specie present some phenolic compound and present some biological properties as anti-oxidant and cytoprotective effect against oxidative stress. **Materials and Methods:** The content of eight phenolic molecules in the crude ethanolic extract of the aerial part of *P. xanthogloea* and its five fractions (hexane, dichloromethane, ethyl-acetate, n-butanol, and water) was determined by heterotrophic plate count method. The anti-oxidant capacity of the extract and the fractions was determined by 1,1-diphenyl-2-picryl-hydrazyl assay. The potential anti-oxidant and cytoprotective effect was also analyzed in human lymphocyte culture treated with extract/fractions at different concentrations with and without oxidative stress generated by hydrogen peroxide (H_2O_2) and sodium nitroprusside (SNP) exposition. **Results:** Tiliroside was the molecule detected in all extract. Water and ethyl-acetate fractions showed the highest radical-scavenging activity. The crude extract, hexane, water, and n-butanol reversed the higher reactive oxygen specie levels generated by H_2O_2 and SNP to levels similar to those observed in the control group. In addition, crude extract, hexane, ethyl-acetate and n-butanol did not caused cytotoxicity, whereas water fraction was cytotoxic at higher concentration tested here (300 µg/mL). The cytotoxicity reversion caused by SNP exposition was concentration-dependent of the extract and fractions. However, dichloromethane fraction increased cell mortality in all concentrations investigated and was not able to decrease cell death in the lymphocytes exposed to SNP. **Conclusion:** The results suggest potential medicine use of this species.

Key words: Anti-oxidant, cytotoxicity, oxidative stress, *Pavonia*, phenolic compounds

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INTRODUCTION

Pavonia constitutes the largest genus of flowering plants in the mallow family, Malvaceae, which is represented by over 250 species, with 134 occurring in Brazil.[1] Several species are used in traditional medicine in several countries including India, Tanzania, and Brazil. Some traditional applications of *Pavonia* include the treatment of gastrointestinal problems such as dysentery and gut hemorrhage, antimicrobial applications, and treatment of cancer.[2-4] Previous studies of the biological activity of *Pavonia* species, described antimicrobial and larvicidal effect,[2,4,5] antispasmodic activity,[3] anti-hypertensive and antinociceptive properties,[6,7] analgesic, and anti-inflammatory effects,[8] as well as hepatoprotective and anti-oxidant properties.[9]

However, studies of the bioactive properties of *Pavonia* species used in popular medicine in southern Brazilian region are still incipient such as *Pavonia xanthogloea* popularly known as "erva-de-ovelha" (herb sheep). Traditionally, this specie is used as antimicrobial and anti-tumor medicines.

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However, scientific evidences for existence of chemical molecules that presented these properties biological properties[10-12] have not yet been reported. Therefore, in this study, the presence and the concentration of eight phenolic molecules were determined in the crude extract and five fractions of the aerial parts of *P. xanthogloea*. Their anti-oxidant effect of all extract/fractions was determined. In the cell assay, the cytoprotective effect of plant extract/fractions was evaluated by the capacity to reverse toxic effects produced in human lymphocytes exposition to sodium nitroprusside (SNP), a pro-oxidant molecule that produce high levels of hydrogen peroxide (H_2O_2) and nitric oxide (NO).[13,14]

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA), Gibco Life Technologies Inc. (Grand Island, NY, USA,), Invitrogen (USA), or Cultilab Co. (São Paulo, Brazil), unless otherwise stated.

Pavonia xanthogloea material

Samples of this species were collected from the Caçapava do Sul municipality in the Pampa biome, located in the southernmost state of Brazil, Rio Grande do Sul. The Brazilian Pampa lies within the South Temperate Zone where grasslands scattered with shrubs and trees are the dominant vegetation.[15] The species was identified by a botanical specialist and a sample of the collected material is archived as voucher specimen number SMDB 13.744 at the Herbarium of Universidade Federal de Santa Maria, Brasil.

Pavonia xanthogloea extract and fraction preparations

The aerial parts of *P. xanthogloea* (900 g) were initially macerated with ethanol (EtOH) (5 L × 1 L) and maintained at room temperature over a period of 48 h. The ethanolic extract was filtered and vacuum evaporated to dryness to obtain a crude extract (120 g). The crude extract (100 g) was suspended in water and repeatedly fractionated with n-hexane, dichloromethane, ethyl-acetate, and n-butanol by using a separator funnel. The fractions were centrifuged and filtered. The solvents were removed using a rotary evaporator under reduced pressure. The fractions were then lyophilized and stored at −20°C until analyzed.

High performance liquid chromatography analysis

High performance liquid chromatography analysis was performed on Agilent 1200 liquid chromatograph system (Agilent technologies, CA, USA) consisting of quaternary pump, an auto-sampler and diode-array detector. The mobile phase was A (formic acid 0.1%) and B (CH_3CN) at a flow rate of 1.0 mL/min following a linear gradient: 0-7 min (85% A), 7-30 min (50% A), 30-40 min (100% B) over 50 min. A reversed-phase analytical Zorbax Eclipse C18 column (250 × 4.6 × 5 μm; Agilent technologies) was used at 25°C temperature. The calibration curve and detection wavelength for the molecules analyzed were: Gallic acid y = 65.629x + 95.158 (r = 0,9998); catechin, y = 14.651x − 6.6277 (r = 0.9999) at 278 nm; chlorogenic acid, y = 66.768x − 147.92 (r = 0.9994); caffeic acid, y = 128.33x + 259.86 (r = 0.9996) at 314 nm; rutin, y = 40.667x + 91.863 (r = 0.9994); quercitrin, y = 21.321x + 24.133 (r = 0.9998) at 356 nm; quercetin, y = 94.731x + 111.49 (r = 0.9996), tiliroside, y = 34.755x + 62.671 (r = 0.9994) and Kempferol, y = 82.757x − 150.75 (r = 0.9997) at 366 nm. The injection volume was 20 μL.

Anti-oxidant capacity measurements

The anti-oxidant capacity of *P. xanthogloea* extract and their fractions was evaluated by a 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay comparing samples with three pure anti-oxidant molecules (ascorbic acid, rutin, and gallic acid) according to a previously published method.[16] All tests were performed in triplicate. The anti-oxidant capacities were described in terms of IC_{50} (concentration of sample required to scavenge 50% of the DPPH free radicals). The extract concentrations used in each reaction to calculate the IC_{50} were 1, 3, 10, 30, 100, 300, and 1000 μg/mL.

Redox effect of *Pavonia xanthogloea* extract/fractions

To test if *P. xanthogloea* was able to modulate redox states in human lymphocytes, cells were treated with extracts/fractions with or without concomitant exposure to H_2O_2 (100 mM) and SNP (5 μM). The SNP is used to generate NO over the course of 5 h. Both molecules (H_2O_2 and NO) are known to play a key role in many cellular processes, but at high levels can cause cellular redox imbalance,[17] resulting in oxidative stress.

The analyses were performed using lymphocytes from 3 to 4 healthy human donors, obtained from peripheral blood samples collected by venipuncture after overnight fasting for 12 h as previously described in Bittencourt e*t al.* and dos Santos Montagner e*t al.*[11,26] Blood specimens (5 mL) were routinely centrifuged for 15 min at 2500 rpm within 1 h of collection, and lymphocytes were transferred to solutions containing different concentrations of *P. xanthogloea* extract/fractions with or without H_2O_2 and SNP dissolved in Roswell Park Memorial Institute (RPMI) 1640 culture medium. The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere over a period of 5 h. Thereafter, reactive oxygen specie (ROS) levels were evaluated using the cell membrane permeable fluorescent compound,

2.7-dichlorofluorescein diacetate (DCFH-DA). ROS levels were evaluated after 5 h of SNP exposure in 20 μL samples of cells treated with DCFH-DA (10 mmol/L) for 60 min at 37°C. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.[18]

Similar experiments were performed to evaluate the effect of *P. xanthogloea* extracts on lymphocyte viability with or without 5 h of SNP exposure.[19] Cell viability was determined by measuring lactate dehydrogenase (LDH) activity. The LDH analysis was performed using a

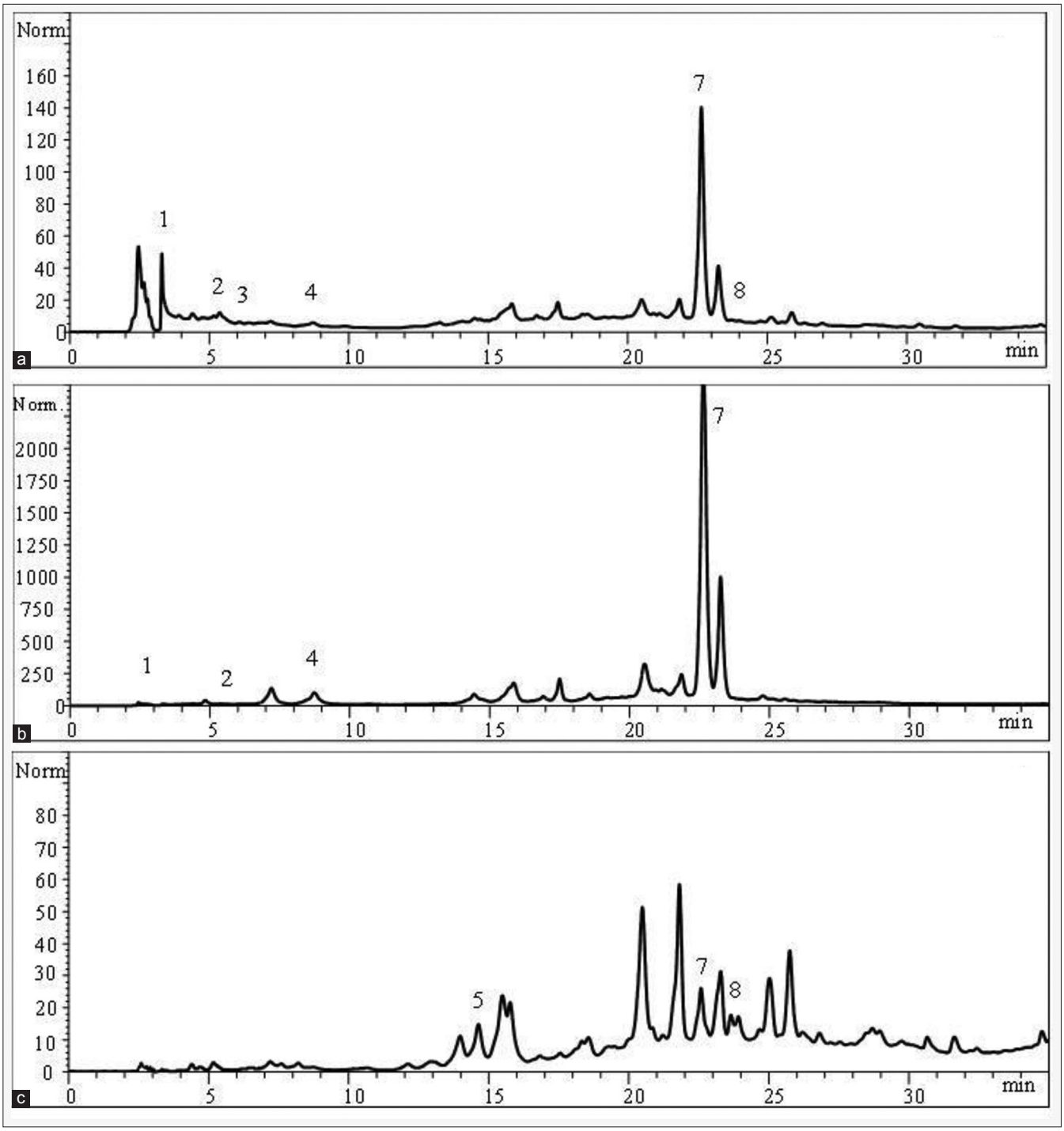

Figure 1: High performance liquid chromatography prolife of *Pavonia xanthologea*, crude extract (a), ethyl-acetate (b) and dichloromethane (c) at concentration 8 mg/mL, detection ultraviolet was at 314 nm. Gallic acid (peak 1, tr = 3.34), caffeic acid (peak 2, tr = 5.36), catechin (peak 3, tr = 6.07), chlorogenic acid (peak 4, tr = 8.71), rutin (peak 5, tr = 14.69), tiliroside (peak 6, tr = 22.64), quercetin (peak 7, tr = 23.74)

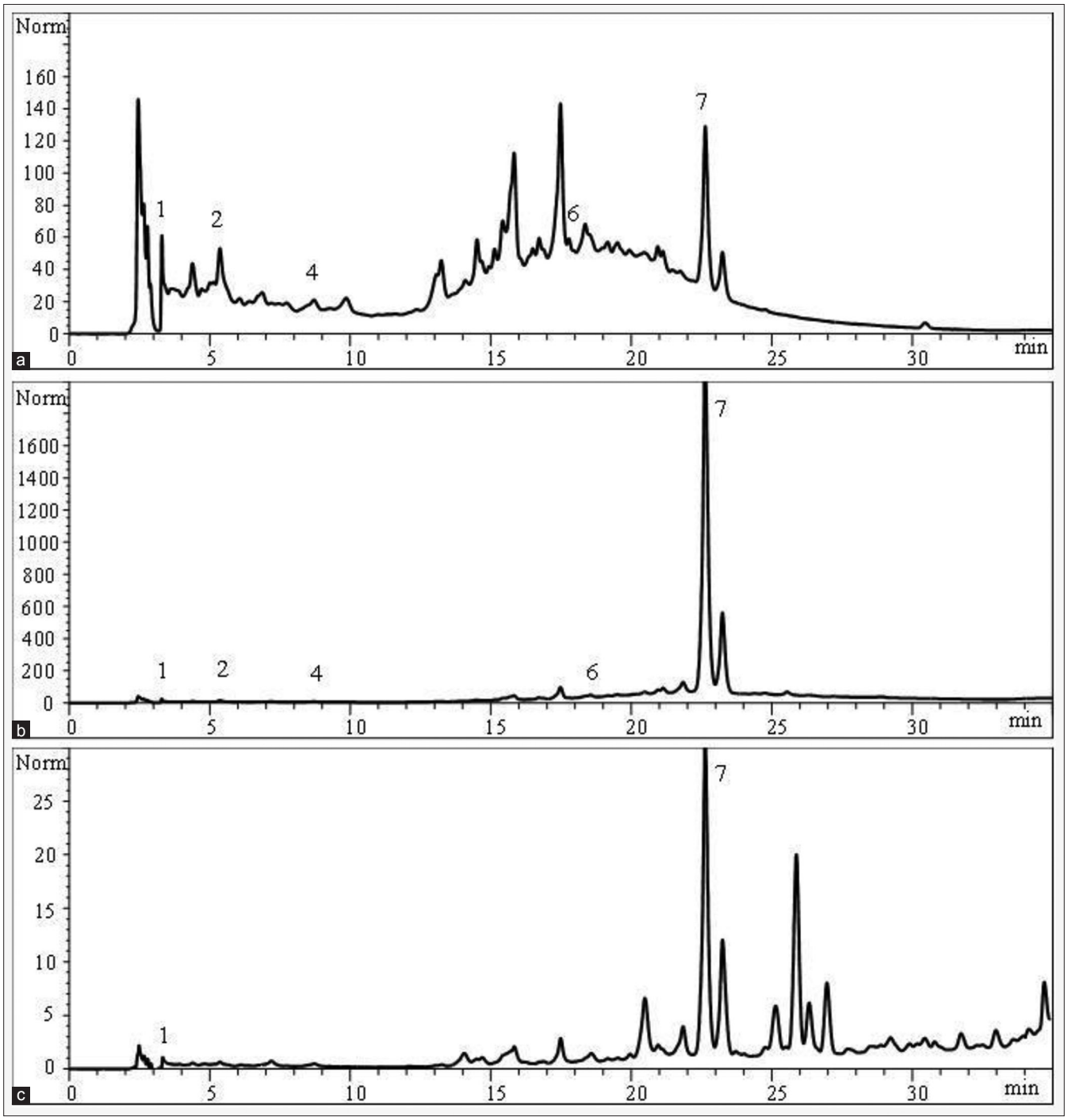

Figure 2: High performance liquid chromatography prolife of *Pavonia xanthologea*,n-butanol (a), water (b) and hexane (c) at concentration 8 mg/mL, detection ultraviolet was at 314 nm. Gallic acid (peak 1, tr = 3.34), caffeic acid (peak 2, tr = 5.36), chlorogenic acid (peak 3, tr = 8.71), quercitrine (peak 4, tr = 17.69), tiliroside (peak 6, tr = 22.64)

kit (Labtest Co., São Paulo, Brazil) following the manufacture instructions. The enzyme activity was read at 340 nm by using a spectrophotometer. To analyze the ROS levels and LDH activity data, the absorbance values obtained for all treatments were calculated as a percent of those obtained for the untreated control group by using the follow equation: ([Absorbance of sample treatment/ absorbance of untreated sample] ×100). Each treatment was analyzed in triplicate.

Statistical analysis

Data are presented as means and standard errors of the means (±SE). The anti-oxidant effect of *P. xanthogloea* crude extracts and their five fractions were compared

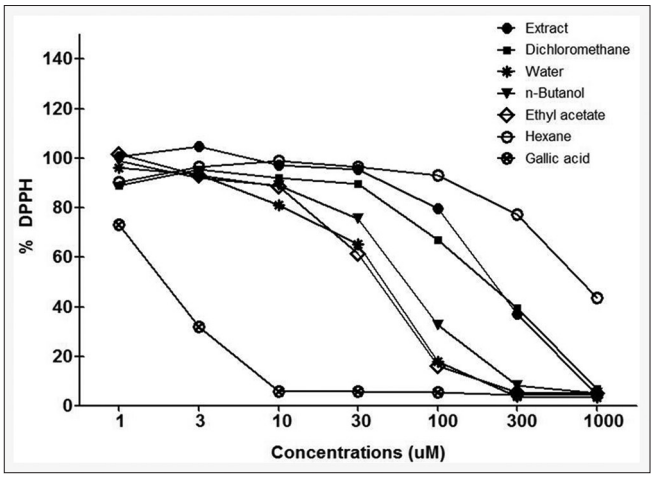

Figure 3: Anti-oxidant capacity of *Pavonia xanthogloea* crude extract and five fractions evaluated by 1,1-diphenyl-2-picryl-hydrazyl assay. Vitamin C, rutin and gallic acid were used as reference of anti-oxidant molecule

using one-way analysis of variance followed by Dunnet and/or Tukey *post-hoc* tests. All statistical analyses were performed, where all *P* values were two-tailed, and $P \leq 0.05$ was considered as statistically significant.

RESULTS

The chemical compounds present in the *P. xanthogloea* crude extract and its fractions are shown in Table 1. Tiliroside was detected in all extract/fractions of *P. xanthogloea,* and ethyl-acetate and water fractions presented highest content of this molecule [Figures 1 and 2]. Gallic and chlorogenic acid were quantified in crude extract and ethyl-acetate [Figure 1a], n-butanol [Figure 2a] and water [Figure 2b] fractions whereas quercitrin was quantified just in n-butanol [Figure 2a] fraction. On the other hand, catechin, rutin, quercetin, caffeic acid and kempferol were

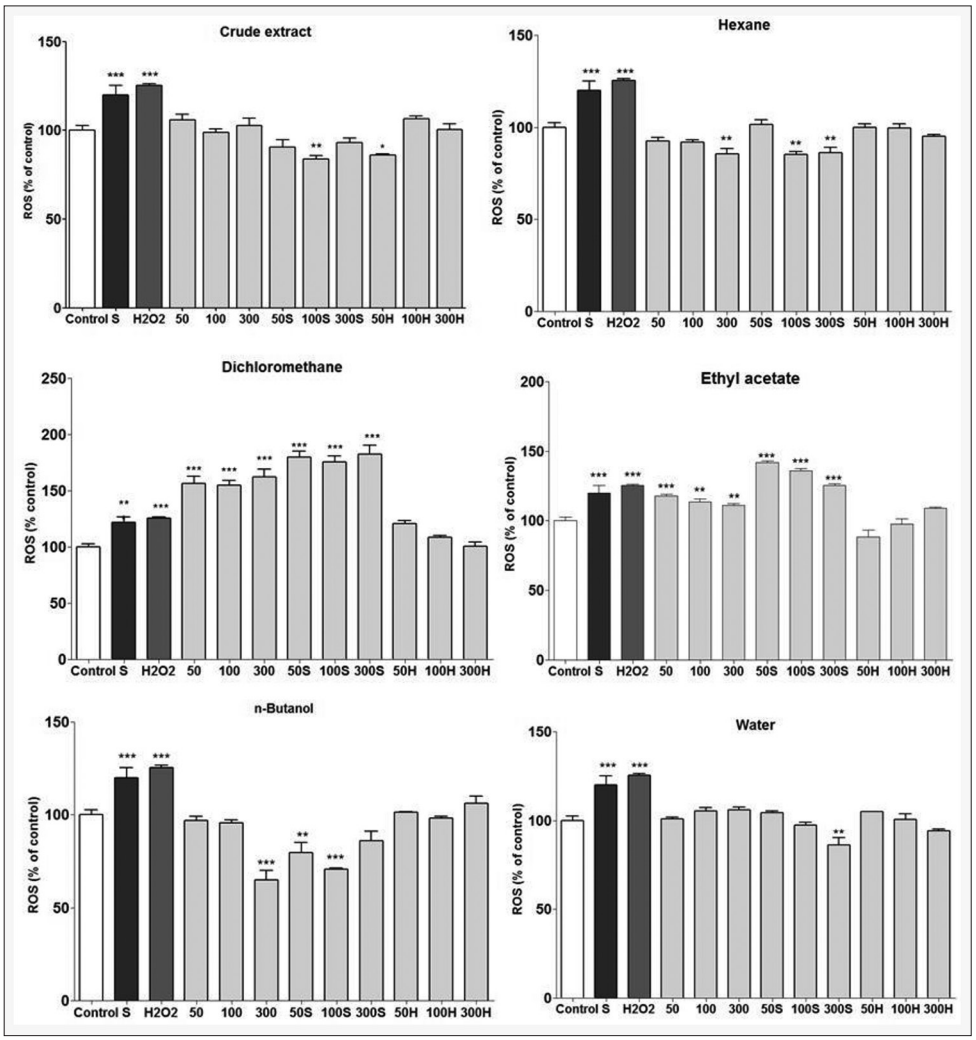

Figure 4: Effect on human lymphocytes reactive oxygen species production of *Pavonia xanthogloea* extract/fractions (μg/mL) with and without sodium nitroprusside (S) exposition a pro-oxidant molecule that generates higher nitric oxide levels. The different concentrations of each extract/fractions were statistically compared by one-way analysis of variance followed by Dunnet *post-hoc* test. *$P \leq 0.05$; **$P \leq 0.01$; $P \leq 0.001$

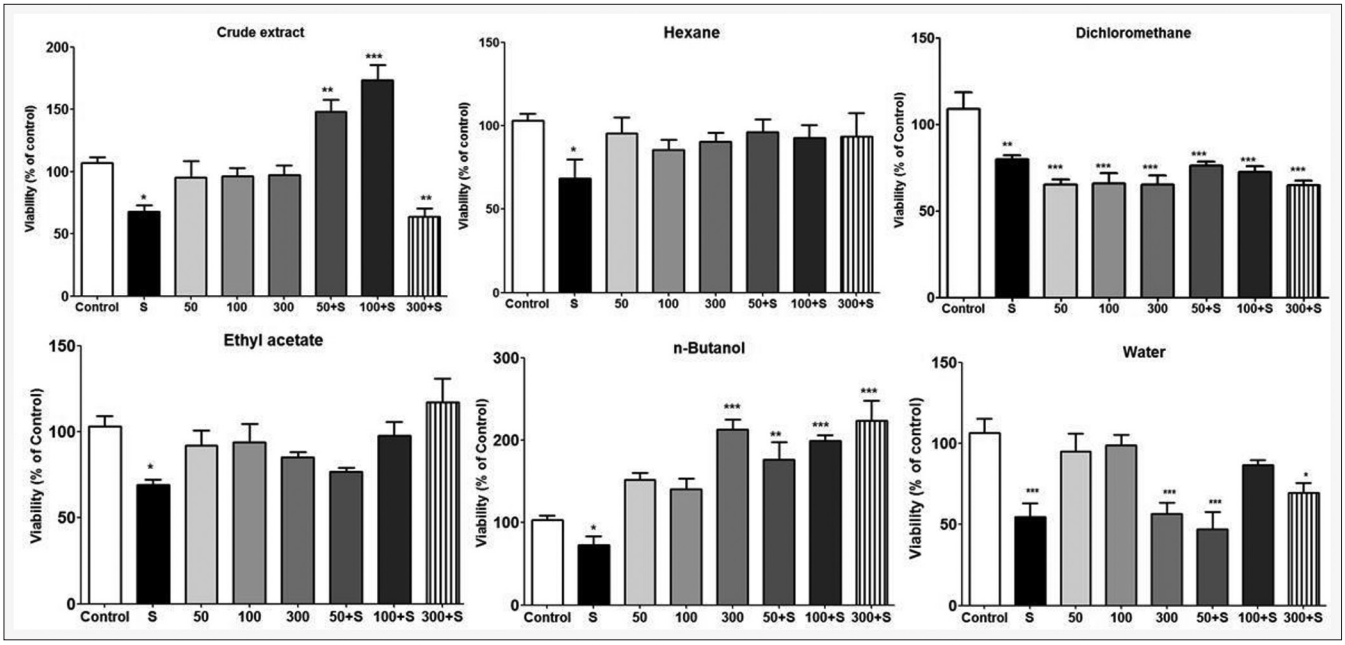

Figure 5: Citoprotective effect of *Pavonia xanthogloea* extract/fractions (µg/mL) on human lymphocytes exposed to sodium nitroprusside (S). The different concentrations of each extract/fractions were statistically compared by one-way analysis of variance followed by Dunnet *post-hoc* test. *$P \leq 0.05$; **$P \leq 0.01$; $P \leq 0.001$

Table 1: Chemical composition of *P. xanthogloea* from crude extract and fractions

Compounds	*P. xanthogloea* (mg/g±SD)					
	Ethanolcrude extract	Fractions				
		Hexane	Dichloromethane	Ethyl acetate	n-butanol	Water
Gallic acid	2.17±0.04[b]	d	d	1.81±0.16[c]	2.70±0.25[a]	1.05±0.02[d]
Catechin	d	nd	nd	nd	nd	nd
Chlorogenic acid	0.48±0.0[c]	nd	nd	6.47±0.03[a]	0.79±0.02[b]	0.57±0.02[c]
Caffeic acid	d	nd	nd	d	d	d
Rutin	nd	nd	d	nd	nd	nd
Quercitrin	nd	nd	nd	nd	11.09±0.05	d
Quercetin	nd	nd	d	n	n	n
Tiliroside	3.05±0.02[c]	0.42±0.04[e]	0.26±0.05[f]	81.67±0.80[a]	2.77±0.02[d]	55.95±0.20[b]

Results are expressed as mean±SD of three determinations. Averages followed by different letters in each column differ by Tukey test at *P*<o.oo5. nd: Not detected, SD: Standard deviation, *P. xanthogloea: Pavonia xanthogloea*

not detected or detected in very low concentrations in the *P. xanthogloea* crude extract and fractions analyzed here.

Results are expressed as mean ± standard deviation of three determinations. Averages followed by different letters in each column differ by Tukey test at $P < 0.005$ d, detected.

The *P. xanthogloea* anti-oxidant capacity was assessed in on the basis of DPPH reduction after 30 min [Figure 3]. As expected, varying anti-oxidant capacity was observed in the *P. xanthogloea* extract/fractions. Both the water (IC_{50} = 43.4 ± 1.7 µg/mL) and ethyl-acetate (IC_{50} = 55.8 ± 2.0 µg/mL) fractions showed higher radical-scavenging activity than did n-butanol (IC_{50} = 72.8 µg/mL), dichloromethane extract (IC_{50} = 158.9 µg/mL), and crude extract (IC_{50} = 241.6 ± 16.1 µg/mL).

The hexane extract showed a lower DPPH reducing activity (IC_{50} = 857.9 ± 11.6 µg/mL) when compared to other fractions. The anti-oxidant capacity of positive controls (IC_{50} values) was 5,7 ± 0.2, 2.6 ± 0.2, and 16,02 ± 0.1 µg/mL for ascorbic acid, gallic acid, and rutin, respectively.

Figure 4 shows the lymphocyte ROS levels after 5 h of *P. xanthogloea* treatment with or without exposure to SNP and H_2O_2, two important pro-oxidant molecules. In general, lymphocytes exposed to only crude *P. xanthogloea* extract, water, n-butanol and hexane at 50 or 100 µg/mL did not alter ROS levels when compared with the untreated control group.

Treatments with hexane and n-butanol at 300 µg/mL resulted in lower ROS concentrations than the control

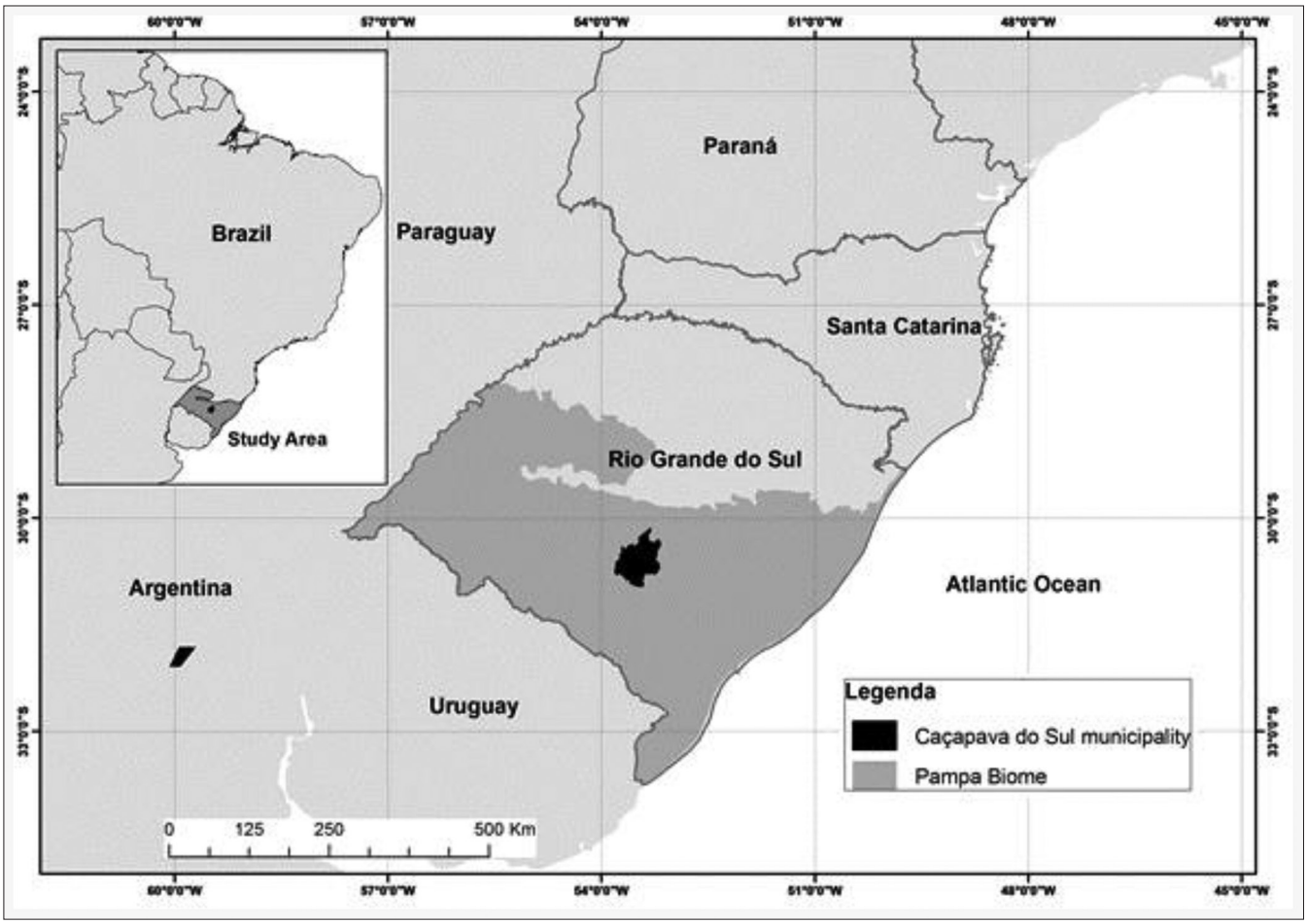

Figure 6: Map showing the local where the *P. xantogholea* was collect in Brazilian Bioma Pampa region (Rio Grande do Sul, Brazil)

group indicating an anti-oxidant effect. On the other hand, all concentrations of the dichloromethane and ethyl-acetate fractions significantly increased ROS levels when compared to the control group, indicating oxidative stress.

As expected, lymphocytes exposed to only SNP and H_2O_2 showed a ROS concentration approximately 25% higher than that in the untreated cells. The anti-oxidant effect of *P. xanthogloea* in the lymphocytes exposed to SNP and H_2O_2 was dependent each extract/fraction and their concentrations. When lymphocytes were exposed to SNP and H_2O_2, the crude extract, hexane, water, and n-butanol reversed the ROS levels to levels similar to those observed in the control group, or in some cases, lower than those in the control group. However, lymphocytes treated with dichloromethane or ethyl-acetate fractions and exposed to SNP continued to show high ROS concentrations. These fractions decreased the ROS levels generated by H_2O_2 exposure to levels similar to those observed in the control group. Therefore, these two fractions showed a specificity of their anti-oxidant activity since they decreased ROS levels in cells exposed to H_2O_2, but not in the cells exposed to SNP.

Considering the effect of *P. xanthogloea* extract/fractions on lymphocytes viability [Figure 5] neither the crude extract nor the n-butanol fraction altered cell viability when compared with the control group. In contrast, 300 μg/mL of the crude extract and n-butanol decreased the cell viability in the presence of SNP. The hexane and ethyl-acetate fractions did not change the viability pattern at any of the concentrations tested here. On the other hand, in the presence of SNP, these fractions significantly reversed the toxicity. The water fraction at 300 μg/mL showed cytotoxic effect when compared to the control group, and at 100 μg/mL concentration this fraction was able to reverse the SNP cytotoxic. However, the dichloromethane fraction caused cytotoxicity at all concentrations investigated and was not able to decrease cell death in the lymphocytes exposed to SNP.

DISCUSSION

The Brazil presents a mega biodiversity, mainly associated to different ecosystems including Pampa biome [Figure 6]. However, in this biome the last 20 years increased the number of species threatened with extinction by social factors as expansion of monocultures and construction of

Figure 7: *P. xanthogloea* and flower and leaf details that are used to specie identification when compared to other species at same genera (Source: Flora-RS, 2010)

dams [Figure 7].[1] Several species, including *P. xanthogloea* are used in popular medicine without scientific studies to confirm its potential effects. However, for our best knowledge, this is the first study involving identification and quantification of phenolic composition as well as H_2O_2 and NO modulation by *Pavonia* species. The analysis of phenolic compounds in *P. xanthogloea* extract and fractions detected three main molecules: Tiliroside, quercetrin, gallic and chlorogenic acids.

Despite the few number of phenolic molecules found in *P. xanthogloea* the extract and the most fractions presented some anti-oxidant activity. The high anti-oxidant capacity observed in ethyl-acetate and water fraction probably is due higher tileroside concentrations.

Tiliroside is a glycoside flavonoid [kaempferol 3-O-(6"-trans-p-coumaroyl)-β-D-glucopyranoside] and is present in several medicinal and dietary plants, such as rose hip, strawberry and linden.[20-22] Despite to be less studied than other polyphenols as catechin and quercetin, previous investigations of tiliroside biological activity have described anti-oxidant activity[23-25] as well as other biological properties including described anti-inflammatory, anticarcinogenic and anti-obesogenic tiliroside effects.[22,23,26,27] The n-butanol fraction also presented anti-oxidant activity probably due higher quercitrin concentration, a glycosylated form of quercetin.[28]

The NO and H_2O_2 are natural components of living cells and serve various biological functions. However, high concentrations of these molecules cause oxidative stress that can be reversed by chemicals with anti-oxidant properties.[17] Therefore, the identification of plant extract that are able to modulate the levels of these ROSs are pharmacologically relevant. The whole of results suggested that *P. xanthogloea* has an important modulatory anti-oxidant activity, as indicated by SNP- and H_2O_2 exposure-induced ROS levels. However, this effect was both fraction- and concentration-dependent. The differences among anti-oxidant effects observed in the *P. xanthogloea* extract and fractions could indicate potential differential biological properties as antimicrobial, antitumoral, anti-inflammatory etc., For these reason complementary studies exploring the *P. xanthogloea* effects are important to be performed.

CONCLUSION

Considering the presence of, at least of four important phenolic compounds are present in *P. xanthogloea* extract/fractions and probably contribute to anti-oxidant activity observed from no-cell and cell *in vitro* assays. These results suggest that this specie could to be medicinal properties.

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