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In vitro biochemical characterization and genotoxicity assessment of Sapindus saponaria seed extract

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ABSTRACT

Ethnopharmacological relevance: *Sapindus saponaria*, also popularly known as soapberry, has been used in folk medicinal values because of its therapeutic properties and several compounds in its composition, which represent a target in potential for drug discovery. However, few data about its potential toxicity has been reported.
Aim of the study: Plant proteins can perform essential roles in survival, acting as defense mechanism, as well functioning as important molecular reserves for its natural metabolism. The aim of the current study was to investigate the *in vitro* toxicity profile of protein extract of *S. saponaria* and detect protein potentially involved in biological effects such as collagen hydrolysis and inhibition of viral proteases.
Materials and methods: Protein extract of soapberry seeds was investigated for its cytotoxic and genotoxic action using the Ames test. The protein extract was also subjected to a partial purification process of a protease and a protease inhibitor by gel chromatography filtration techniques and the partially isolated proteins were characterized biochemically.
Results: Seed proteins extract of *S. saponaria* was evaluated until 100 μg/mL concentration, presenting cytotoxicity and mutagenicity in bacterial model mostly when exposed to exogenous metabolic system and causing cytotoxic and genotoxic effects in HepG2 cells. The purification and partial characterization of a serine protease (43 kDa) and a cysteine protease inhibitor (32.8 kDa) from protein extract of *S. Saponaria*, corroborate the idea of the biological use of the plant as an insecticide and larvicide. Although it shows cytotoxic, mutagenic and genotoxic effects.
Conclusion: The overall results of the present study provide supportive data on the potential use of proteins produced in *S. saponaria* seeds as pharmacological and biotechnological agents that can be further explored for the development of new drugs.

1. Introduction

The species *Sapindus saponaria* L., called as soapberry but popularly known in Brazil by many names, as sabão-de-macaco, saboeiro, saboneteiro, fruta de sabão e sabão-de-soldado, belonging to the family Sapindaceae Juss., is a medium-sized tree that has a wide distribution in Brazil, being native and present in all regions of the country. This species is also widely found in other countries of the Americas, such as Mexico and the United States (Neto et al., 2000; Somner et al., 2015).

The plant has been widely used by the inhabitants of the north and northeast regions of Brazil for the treatment of inflammations, injuries and even cancer (Albiero et al., 2001) In these places, the part of plant most used is fruit, but there are reports of use of seeds as an antibacterial and anti-oxidant agent in other countries, such as India (Niloufer and Lakshmi, 2021). The species has been studied due to biological actions such as diuretics and digestive tonic (DeFilipps et al., 2004), antiofidic

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action (Da Silva et al., 2012), fungicidal (Damke et al., 2011; Fiorini et al., 2016; Marinho et al., 2018), antitumor (Rashed et al., 2013), leishmanicidal and trypanocidal (Correa et al., 2014) and larvicidal potential (Santos et al., 2008). Thus, studies with this species contributed to the identification and isolation of various bioactive chemical compounds (Sachin et al., 2011).

Among these bioactive compounds studied, there are proteins due to their involvement in natural metabolism acting as a metabolic regulation protein in plant seeds, (Pesquet, 2012; Santamaría et al., 2014), or even being involved in defense mechanism of these plants against the attack of herbivorous insects (Abd El-latif, 2015; Macedo et al., 2011; Siqueira-Júnior et al., 2002). Verma et al. (2016) described the possibility of using inactive precursors (zymogens) of cysteine proteases as a potential tool in the treatment of diseases. These zymogens, when bound to the substrate, block the access of the substrate by the active site of the protease, inhibiting the activity of the active protease, preventing the development of the infectious agents in the human organism. In addition, an important step to use of plant extracts in production of new molecules or use as a new product is the performance of tests that assess the toxicological risks of these extracts. There are still no data on cytotoxicity or even on the mutagenicity of protein extract from *S. saponaria*, which makes this type of study even more relevant. In this work, we aimed to evaluate the cytotoxic and mutagenic activity of the protein extract of *S. saponaria* seeds and to describe the partial purification and biochemical characterization of the serine protease (*SS*T) and the phytocystatin (*Ss*Cyst) produced.

2. Material and methods

2.1. Plant materials

Seeds from *S. saponaria* were harvested from wild trees in the city of São João da Barra, RJ, Brazil and were authenticated by Dr. Laura J. M. Santiago, Department of Botany, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. A voucher specimen of the plant was deposited at UNIRIO herbarium (HUNI 1375). The plant material was first washed in 10% sodium hypochlorite solution and followed by washes with distilled water and then air dried and ground to powder using a Willye knife mill (Fortinox). Powdered material was then stored at −10 °C until its use.

2.2. Extraction and recovery of seed proteins

Proteins extraction was carried out by homogenizing 20 g of sample in 10% (w/w) insoluble polyvinylpyrolidone (PVP) added to 80 mL of ice-cold extraction buffer (50 mM Tris-HCl, pH 6.0). The prepared homogenate was incubated at 4 °C on a rotary shaker (Fisaton) at 400 RPM for 30 min. The homogenate was then filtered through a qualitative paper filter (80g, 44 μm), and then it was centrifuged at 15000×*g* for 30 min at 4 °C. The recovered crude supernatant was again filtered through a qualitative paper filter (80g, 44 μm) and used for the following experiments.

2.3. Protein determination

At all stages, protein concentration on protein crude extract from *S. saponaria* seeds (CESS) was determined according to the method of Bradford (Bradford, 1976) using the Bradford assay kit and bovine serum albumin (BSA) as standard protein according to the manufacturer's instructions (BioRad).

2.4. Salmonella mutagenicity assay (Ames test)

The mutagenic evaluation of CESS was performed using the Ames *Salmonella*/microsome assay as described by Mortelmans and Zeiger (2000) with slight modifications. Briefly, 100 μL of stationary growth cultures of *Salmonella enterica* Serovar Typhimurium strains TA97, TA98, TA100, TA 102 and TA104 (1–2 x 10^9 cells/mL) were pre-incubated with 100 μL of CESS at different concentrations (from 0.0 to 100 μg/μL) diluted in 0.9% NaCl, or positive and negative controls, and 500 μL of 0.2 M sodium phosphate buffer (pH 7.4) or metabolic activation system (4% S9 mix, Aroclor-preinduced, from Moltox Inc., USA) at 60 rpm, at 37 °C. After 20 min, 2 mL top agar (0.7% agar, 0.5% NaCl, 0.05 mmol/L histidine and biotin) at 40 °C were added to the test tubes and the final mixtures were poured into Petri's plate containing Vogel-Bonner agar medium E 10x (1.5% agar, 10 g/L $MgSO_4 \cdot 7H_2O$; 100 g/L $C_6H_8O_7 \cdot H2O$; 500 g/L K_2HPO_4; 175 g/L $Na(NH_4)HPO_4 \cdot 4H_2O$), 2% glucose). These final mixtures were incubated at 37 °C for 72 h. Each tester strain was assayed in triplicate, and the number of *His*$^+$ revertant colonies was counted for each tester strain and treatment group. The positive controls used in the Ames assay were 4- nitroquinoline 1-oxide (CAS: 56–57-5) at 0.01 μg/mL for TA97, TA98 and T100; and methyl methanesulfonate (MMS) at 0.25 μg/μL for TA102 and 2.5 μg/μL for TA104. According to OECD, Guidelines 471 (OECD, 2020a), the mutagenic response of CESS was considered positive when the number of revertant colonies in the test was at least twice the number of spontaneous revertants, when a significant response to the analysis of variance (ANOVA) ($p \leq 0.05$) followed by Tukey's post hoc test and reproducible positive dose-response curve were found. Tests were carried out in triplicates and repeated three times. The results were calculated as mean along with standard error values. In positive outcomes for mutagenicity, the mutagenic potency was calculated by linear regression (Pearson's Correlative Test), according to Claxton et al. (1991a).

In order to determine the cytotoxic effects of CESS, 100 μL of solution containing bacteria in stationary phase (1–2 x 10^9 cells/mL) were incubated at 37 °C, in contact with the samples, or positive and negative controls, under the same conditions described for the test of Ames. After 20 min pre-incubation the assay mixtures were diluted in 0.9% NaCl (w/v) to a final concentration of 1–2 x 10^2 cells/plate and plated on Louria Bertani agar medium (1% tryptone; 0.5% yeast extract; 1% NaCl; 1.5% bacteriological agar). The plates were incubated for 24 h at 37 °C, and the colonies were counted, and the percentage of survival was calculated in relation to the negative control. Samples were considered a toxic concentration when survival rates were less than 70% (Aiub et al., 2003). The Survival test in the context of mutagenicity testing is made using a proportion of living cells among dead cells, usually determined by colony counting methods after a treatment interval (20 min pre-incubation test). Toxicity might be detected by a reduction in the number of revertants, and/or clearing or diminution of the background lawn. The results were expressed as a % of survival (average of survival cells in the negative control * 100/average of survival cells induced in the sample) (Aiub et al., 2004).

2.5. Eukaryotic cytotoxicity determination

HepG2 cells were seeded at a density of 1 × 10^4 cells/well. The water-soluble tetrazolium salt assay (WST-1, Roche) (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche Co., South San Francisco, CA) was used to determine the number of viable cells after 24, 48 and 72 h of exposure to CESS (0–300 μg/mL). This salt is reduced by mitochondrial dehydrogenases in living cells, yielding a yellow product that is soluble in the cell culture medium. Briefly, after treatment, the culture medium was replaced by 90 μL fresh culture medium and 10 μL WST-1 reagent and incubated at 37 °C with 5% CO_2 for 3 h. The absorbance was then measured at 440 nm according to Pinto et al. (2018). The intensity of the yellow color in the negative control (0.9% NaCl) wells was designated as 100% viability, and all further comparisons were based on this reference level to determine the lethal concentration (LC_{50}) for 50% of cultured cells.

The LDH activity cytotoxicity assay was carried out according to the manufacturer's instructions. Briefly, 100 μL supernatant was transferred from each well to a 96-well flat-bottomed plate and 100 μL reaction mixture freshly prepared were added to each well. The plates were

incubated for 30 min at 20 °C in the dark and absorbance was measured at 492 nm using a Polaris Microplate Reader. Blank values indicating the absorbance of the LDH were subtracted from all samples. The percentage of cytotoxicity was calculated according to the kit protocol. Sterile 0.9% NaCl was used as negative control and Triton X-100 as the positive control (Pinto et al., 2018).

2.6. In vitro cytokinesis-block micronucleus assay

In vitro mammalian cell micronucleus test was performed following OECD test guideline No. 487 for the testing of chemicals (OECD, 2016). Fresh HepG2 cells were seeded at a density of 2–3 × 10^4 cells/mL in 24-well plates (1 mL per well). CESS was then added to the medium to final concentrations from 3.7 to 300 μg/mL diluted in 0.9% NaCl, and the incubation continued for 24 h. 0.9% NaCl was used as the negative control, and benzo-α-pyrene (BaP), 0,1 mg/mL was the positive control. After exposure to the extract, the cells were incubated for a further 24 h under growth conditions before quantification of the micronuclei. The cytogenetic studies were carried out in triplicate and repeated three times (n = 3). In order to determine the nuclear division index (NDI) and the number of cells with micronuclei, the medium was replaced by a cold methanol-glacial acetic acid (3:1) fixative for 30 min, and the cells were then rinsed with distilled water for 2 min and air-dried. The fixed cells were stained with Giemsa (1:20) from a stock solution (0.6%) for 60 min, washed with PBS 1X (phosphate-buffered saline) for 5 min, briefly rinsed with distilled water, and Entellan (Merck). To determine the mitotic index and the number of cells with micronuclei, 1000 cells per coverslip (3000 cells per concentration) were analyzed under a light microscope in each experiment, after coding the slides in order to reduce analytical bias. The percentage of viable cells was evaluated discounting apoptotic and necrotic cells.

2.7. Partial purification of seed proteins

The prepared CESS was brought to 30% saturation (w/v) with a gradual addition of solid ammonium sulfate [$(NH_4)_2SO_4$] saturation at 4 °C with vigorous stirring for 2h. The resulting precipitate (F30) was recovered by centrifugation at 10,000×*g* for 20min at 4 °C and resuspended in 50 mM Tris-HCl (pH 6.0). This step was repeated twice to obtain 60% (F60) and 90% (F90) saturation, respectively. The protein preparations F30, F60 were applied onto a gel filtration chromatography columm (Bio-Gel P-100 resin, BioRad). The proteins were eluted using 20 mM Tris-HCl (pH 6.0). Fractions (1 mL/fraction) were collected at a flow rate of 4 ml/h and concentrated by ultrafiltration using ultra centrifugal filters (Amicon). Proteins were detected by monitoring absorbance at 280 nm and active fractions containing the protease (*SS*T) and the phytocystatin (*Ss*Cyst) were resuspended in sample buffer (0.0625 M Tris–HCl, 10% sucrose, 2,5% sodium dodecyl sulfate, 5% β-mercaptoethanol 0.002% bromophenol blue, pH 6.8). All samples were loaded on to a polyacrylamide gel and subjected to electrophoresis (Laemmli, 1970).

2.8. Protease inhibitor activity assay

Activity of protease inhibitor against papain was detected according to Siqueira-Júnior et al. (2002) by measuring the remaining hydrolytic activity towards synthetic substrate N-benzoyl-arginine-naphthylamide (BANA) after pre-incubation with CESS and fractions resulting from both ammonium sulfate precipitation and gel filtration chromatography, respectively, with one inhibitory unit calculated as described by Macedo et al. (2011). Papain was individually incubated with 40 μg of total proteins contained in the samples for 10 min at 37 °C in 250 mM sodium phosphate buffer, 2.5 mM EDTA, 25 mM β-mercaptoethanol (pH 6.0). The assay was started with the addition of 35 μL of 5 mM BANA followed by incubation for 30 min at 37 °C. The activity was terminated by adding 500 μL of 2% HCl solution in ethanol. Substrate hydrolysis was followed adding 500 μL 0.06% p-dimethylaminacinmaldehyde in ethanol by measuring the absorbance of released *p*-naphthylamine at 540 nm.

2.9. Protease activity assay

The proteolytic activity of CESS and fractions of proteins extracted from soapberry seeds was determined using an adaptation of methodology described by Siqueira-Júnior et al. (2002). Samples were incubated in 250 mM sodium phosphate buffer, 2.5 mM EDTA, 25 mM β-mercaptoethanol (pH 6.0), using BANA as a substrate. The assay was started with the addition of 35 μL of 5 mM BANA followed by incubation for 30 min at 37 °C. The activity was terminated by adding 500 μL of 2% HCl solution in ethanol. Then, the enzyme activity was measured as described above. To determine the specificity of *SS*T with other substrate the proteolytic activity of the samples was also determined in the presence of BApNA (benzoyl-arginine-p-nitroanilide) according an adaptation of methodology described by Botelho-Junior et al. (2008). Briefly, samples were incubated with 5 mM BApNA added to 50 mM Tris-HCl buffer, $CaCl_2$ 20 mM, pH 8.0, for 30 min at 37 °C in a 200 μL final volume. After incubation, the reaction was interrupted by adding 200 μL of 30% (v/v) acetic acid. Substrate hydrolysis was followed by measuring the liberated *p*-nitroaniline at 405 nm. One unit of proteolytic activity (UPA) was defined as the amount of protein causing change in optical density (O.D) of 0.01.

2.10. Effects of protease inhibitors on SST

To investigated the *SS*T belonging to which type of protease, selective inhibitors of protease activity were used: 1 μM L-trans- epoxysuccinylleucylamide-(4-guanidino)-butane (E−64; inhibition for cysteine protease), 10 mM ethylenediaminetetraacetic acid (EDTA; inhibition for metallo-protease), and 1 μM trypsin inhibitor (IT; CAS: 9035-81-8; inhibition for serine protease). Prior to the addition of substrate to the reaction mixture, the enzyme solutions were incubated with each protease inhibitor for 5 min and the enzyme activity was subsequently determined as described above.

2.11. SDS-polyacrylamide gel electrophoresis and zymogram analysis

Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% (w/v) acrylamide, according to Laemmli (1970) The protease activity on gel was detected by adding heat gelatin (0.1% (w/v), final concentration) to acrylamide solution prior to polymerization of the gel mixture (Egito et al., 2007). Samples were incubated in sample buffer as described above but were not subjected to thermal denaturation (SDN) while a replica of each sample was subjected to heat denaturation for 3 min. After electrophoresis, the gel was washed twice, during 30 min in 2.5% (v/v) Triton 100 X, and incubated for 1 h, at 37 °C in 250 mM sodium phosphate buffer, 2.5 mM EDTA (pH6.0) to hydrolysis reaction. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R and destained in 40% (v/v) methanol/10% (v/v) acetic acid for 60 min and then destained after several washings in solution containing 30% (w/v) ethanol and 7.5% (v/v) acetic acid. The active enzymes were revealed as translucent bands.

3. Results

3.1. Bacterial mutagenicity

The mutagenic index (MI) was calculated observing the number of His^+ revertant colonies related to control group, for each tester strain. Table 1 show the results of the cytotoxicity and mutagenicity assays, with and without S9 mix. In absence of S9 mix, CESS did not induce mutagenic (MI > 2) or cytotoxic (survival rates < 70%) responses for

Table 1
Induction of *His*$^+$ revertants in *S.enterica* Typhimurium strains by CESS in reverse mutation assay (Ames test).

		Without S9			With S9		
Strain	µg/plate	M. I.[a]	*His*$^+$ ± SD[b]	% Survival[c]	M. I.[a]	*His*$^+$ ± SD[b]	% Survival[c]
	0	1.0	167 ±13	100.0	1.0	191 ± 4	100.0
	0.1	1.0	165 ±16	96.6	1.1	212 ± 14	96.7
TA97	1	1.0	169 ±11	92.4	1.3	242 ± 14	100.0
	10	1.0	160 ± 11	91.5	1.2	225 ± 10	100.0
	100	1.0	164 ± 10	92.4	1.0	183 ± 13	79.9
	0	1.0	36 ± 2	100.0	1.0	37 ± 9	100.0
	0.1	0.9	32 ± 3	81.6	2.2*	81 ± 9	100.0
TA98	1	0.9	34 ± 3	84.4	2.6*	96 ± 8	95.0
	10	1.0	37 ± 4	80.9	2.8*	103 ± 4	89.1
	100	1.0	37 ± 1	81.5	2.0*	76 ± 5	59.6
	0	1.0	115 ± 4	100.0	1.0	171 ± 4	100.0
	0.1	1.1	125 ±11	100.0	1.1	190 ± 15	100.0
TA100	1	1.3	153 ± 12	100.0	1.2	211 ± 5	100.0
	10	1.1	127 ±13	100.0	1.3	214 ± 10	100.0
	100	0.9	103 ± 13	99.6	1.3	220 ± 16	100.0
	0	1.0	206 ± 8	100.0	1.0	388 ± 18	100.0
	0.1	1.7	342 ± 6	79.6	1.1	440 ± 18	99.4
TA102	1	1.6	322 ± 1	80.9	1.2	461 ± 20	98.2
	10	1.6	333 ± 19	72.9	1.0	396 ± 23	97.0
	100	1.1	219 ± 16	71.4	0.8	312 ± 49	86.9
	0	1.0	479 ± 24	100.0	1.0	307 ± 4	100.0
	0.1	0.9	421 ± 22	90.5	1.0	312 ± 30	100.0
TA104	1	1.0	469 ± 22	87.2	1.0	320 ± 7	100.0
	10	1.0	494 ± 25	87.9	1.0	302 ± 16	100.0
	100	1.2	551 ± 22	81.2	0.9	290 ± 10	100.0

*Different of negative control, One-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).
[a] Mutagenicity index: No. of *His* $^+$ induced revertants in the sample/number of spontaneous *His*$^+$ revertants in the negative control (0.9% NaCl).
[b] *His*$^+$/plate: mean values of 3 replicate plates, repeated at least twice.
[c] Percentage of survival relative to the negative control: cytotoxicity is considered when percent survival <70% related to the control group.

any tested concentration and the *Salmonella* strains used. However, significant differences ($P < 0.05$) were observed in the MI of the T102 strain, when treated with concentrations 0.1 µg/plate and compared to control. A possible cytotoxicity was detected with a reduction ($P < 0.05$) for TA 102 in the presence of 10 and 100µg/plate, respectively, when compared to controls.

On the other hand, in presence of S9 mix, CESS did induce mutagenic and cytotoxic effects, presents in TA98. The mutagenicity was detected as from 0.1 µg/mL and cytotoxicity just at 100 µg/mL. So, we decided to investigate the mutagenicity of CESS in lower concentrations, to determine a dose-response curve (Fig. 1), only in the strain which mutagenicity was detected (TA98). With these results, it was also determined the mutagenic potency of CESS. By linear regression, the extract

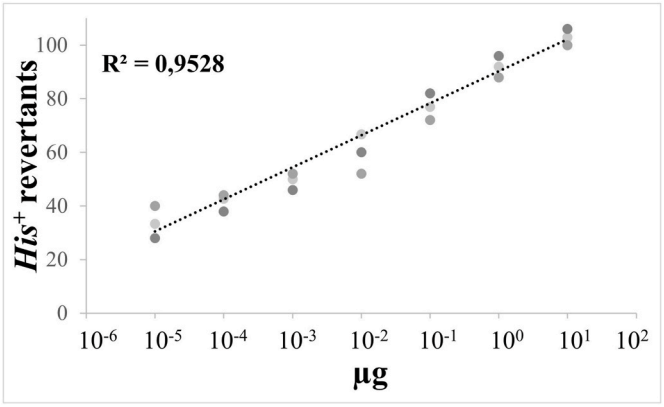


Fig. 1. Dose-response curve of Salmonella enterica serovar Typhimurium TA98 strain His + revertants after exposure to protein crude extract of Sapindus saponaria seeds (CESS). It is possible to observe, by linear regression (Pearson's Correlative Test), a clear positive dose-response fit (r2 = 0.9528), pointing a mutagenic potency slope of 5.3 ± 0.4 revertants/µg of CESS.

presented a mutagenic potency slope of 5.3 ± 0.4 revertants/µg.

Positive controls without S9: 4NQO (1.0 µg/plate) for TA97, 607 ± 40 revertants, TA98, 665 ± 83 revertants, TA100, 266 ± 46 revertants; MMS (25 µg/plate) for TA102, 1337 ± 16 reverntants, for TA104, 969 ± 25 reventants. With S9: 2AA (5 µg/plate) for TA97, 3328 ± 45 revertants, for TA98, 246 ± 37 revertants, for TA100, 1808 ± 135 reverants, for TA102, 1379 ± 67 revertants and for TA104, 2053 ± 56 revertants.

3.2. Eukaryotic cytotoxicity

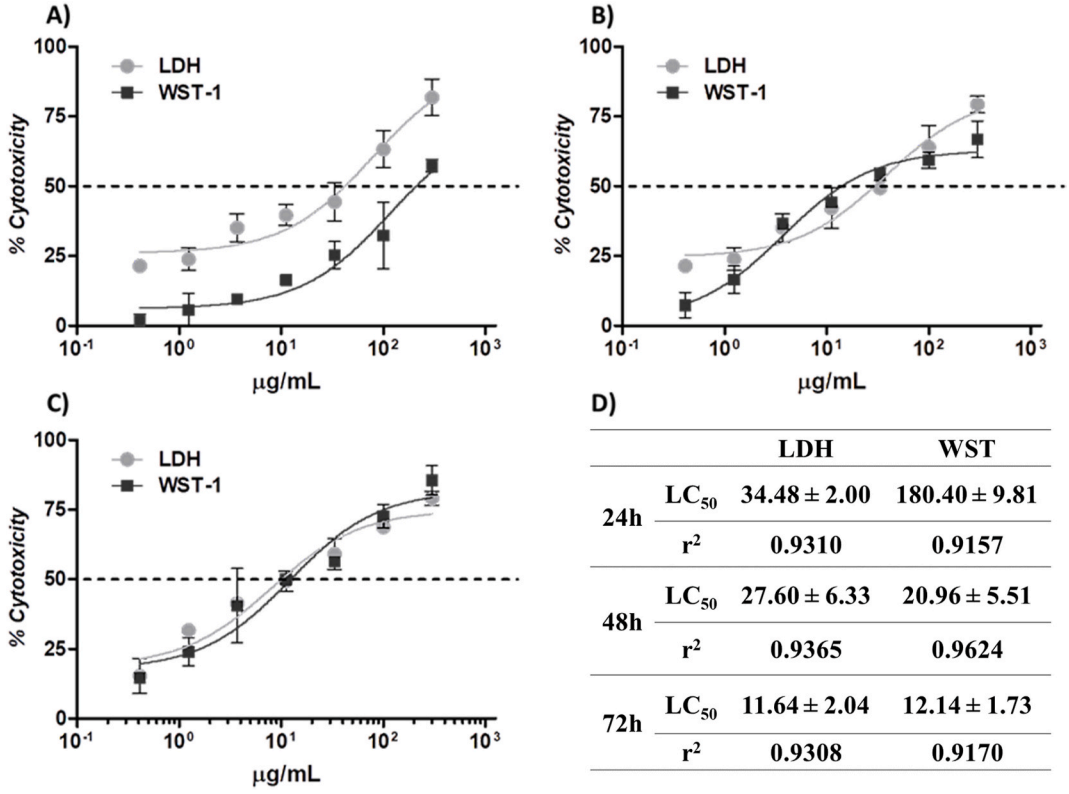
Eukaryotic cytotoxicity assays show that the exposure to CESS induced dose-related and time-related cytotoxicity responses on HepG2 cells. After 24 h of exposure (Fig. 2A), cell membrane damage detected by LDH assay was five times more prominent than mitochondrial dysfunction, detected by WST-1 assay. At this time point, LC_{50} were 34.48 ± 2.00 on LDH and 180.40 ± 9.81 on WST-1 endpoints (Fig. 2D). At the time of 48 h of exposure (Fig. 2B), occurred a shift between the LDH and WST-1 curves, with intercrossing between them at intermediate concentrations but still with higher values for LDH at lower and higher concentrations, presenting LC_{50} = 27.60 ± 6.33 on LDH and LC_{50} = 20.96 ± 5.51 on WST-1 endpoints (Fig. 2D). After 72 h of exposure (Fig. 2C), both curves presented the same behavior, with an overlap between them. At this time, the LC_{50} were 11.64 ± 2.04 and 12.14 ± 1.73 ond LDH and WST-1, respectively (Fig. 2D). All the curves presented acceptable r^2 values (>0.9) and can be considered dose-response Q-curves for cytotoxicity.

3.3. Eukaryotic genotoxicity

After 24 h of exposure, CESS induced significant increase in micronuclei frequencies (Fig. 3A) on HepG2 cells. Despite there was a genotoxic effect at all tested concentrations, this effect manifested itself as a dose-response curve. At the concentration of 3.7 µg/mL, the frequency of MN was 5 times higher than the negative control, 9 times at the concentration of 11.1 µg/mL, 10 times at 33.3 µg/mL and 13 times higher at 100 µg/mL and 300 µg/mL. Complementarily, the NDIs also behaved dose-dependently, showing significant reduction on HepG2 cell division (Fig. 3B) at all tested concentrations. These decreases on NDI may indicate cytotoxic or cytostatic effects on eukaryotic cells.

3.4. Detection of protease inhibitors and protases in the plant seed extract

After the extraction process, both CESS and the fractions obtained



D)

		LDH	WST
24h	LC_{50}	34.48 ± 2.00	180.40 ± 9.81
	r^2	0.9310	0.9157
48h	LC_{50}	27.60 ± 6.33	20.96 ± 5.51
	r^2	0.9365	0.9624
72h	LC_{50}	11.64 ± 2.04	12.14 ± 1.73
	r^2	0.9308	0.9170

Fig. 2. Eukaryotic cytotoxicity of protein crude extract of *Sapindus saponaria* seeds (CESS) HepG2 cells were incubated with CESS during (**A**) 24 h, (**B**) 48 h and (**C**) 72 h. After the incubations, cell membrane damage (LDH assay) and mitochondrial function (WST-1 assay) were measured. The lethal concentration of 50% of cell population (LC_{50}) values (**D**) were calculated by nonlinear regression fit of dose–response (agonist behavior) curves with $R^2 > 0.9$.

after saturation with $(NH_4)_2SO_4$ were subjected to the inhibitory activity test, according to the methodology proposed by Siqueira-Júnior et al. (Siqueira-Júnior et al., 2002). Fig. 4 show the residual activity of papain when incubated with CESS, F30, F60 and F90, respectively. Among the different tested samples screened, CESS (63%) and F30 (78%) recorded high levels of inhibition against papain activity reducing its proteolytic activity ($P < 0.05$). Fractions F60 and F90 however recorded very less amount of papain inhibitor activity. A slight difference was observed in the reduction at proteolytic activity of papain caused by fractions F60 and F90 ($P > 0.05$), respectively.

In order to understand this difference, the presence of proteases in F60 fraction was evaluated. As a result, the hydrolysis of BANA substrate was detected, indicating the presence of a protease in *S. saponaria* seeds. Proteases can be classified based on their sensitivity to various inhibitors (Rao et al., 1998). Totally inhibition of proteolytic activity was observed in the presence of trypsin inhibitor, whereas the maintenance of activity in the presence of other inhibitors such as E−64 (95%) and EDTA (91.5%) indicated that *SST* belongs to the serine group (Fig. 5).

3.5. SDS–PAGE and activity staining (zymography) of detected proteins

The extracted protein precipitation process from *S. saponaria* seeds in $(NH_4)_2SO_4$ was monitored via SDS-PAGE. As a result, only two proteins were precipitated in the F30 fraction (0–30%). In Fig. 6A (lane 1) a major protein band of 32.8 kDa was observed compared to the proteins present in the protein extract (Fig. 6A – lane CESS), indicating that the inhibitory activity of papain analyzed in F30 may be associated with this protein. In contrast, several proteins are precipitated by increasing the percentage of saturation (30–60%) (Fig. 6A – lane 2) indicating that there are other proteins not yet removed in process. The protease (*SST*) appeared as a single band with estimated molecular mass of 43 kDa in SDS–PAGE (Fig. 6B – lane 4). By zymography, the F60 fraction showed one distinct clear zone confirming the proteolytic activity of the *SST* (Fig. 6B, lane 5), eliminated when the sample was subjected to heat treatment (Fig. 6B, zymogram - lane 5). The molecular mass of the enzyme was determined comparing the migration distance of the protease to the standard marker proteins.

3.6. Partial purification of proteins

Fraction F30 (Fig. 6 – lane 1), containing protease inhibitory activity, was separated in a third step with a gel filtration column. The chromatogram of the partial purification process is shown in Fig. 7A. The partial separation resulted in 2 peaks which were analyzed. Peak 1 (*Ss*Cyst) showed high papain inhibitory activity (85%), corroborating the idea that the 32,8 kDa protein present at fraction F30 could be responsible for this activity (Fig. 7B). The specific activity of the partially purified PI is 1359.48 UI/mg of protein as compared to the inhibitory activity in the crude extract, which was 900,78 UI/mg of protein using BANA as substrate and papain as enzyme target. The total recovery of the PI activity was almost 51% (Table 2).

Whereas, the chromatogram of the F60 P-100 gel filtration was obtained (Fig. 8A). The enzyme was eluted with fractions 16–20 as a single peak with protease activity. All the fractions (16–20) with protease activity were pooled and concentrated by ultrafiltration. Table 3 shows that 11.43%, from 925,89 units/mg of the F60 were recovered in the gel filtration chromatography. This yield was confirmed by the evaluation of the proteolytic activity of partially purified *SST* against substrate BApNA, compared to the CESS. Fig. 8B showed an increase of 5.08 fold in the specific proteolytic activity of *SST*, compared to the activity of this enzyme in the crude extract.

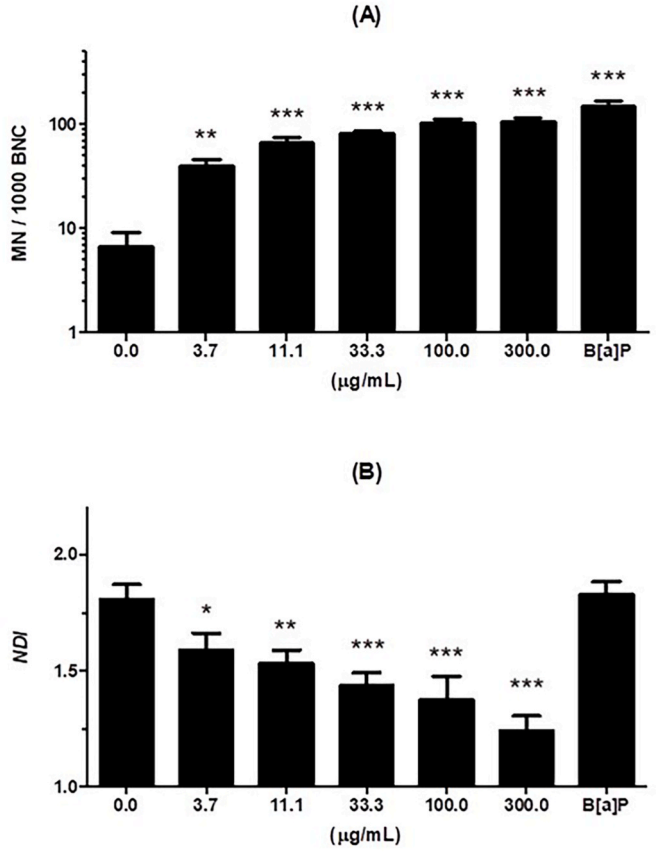


Fig. 3. Protein crude extract of *Sapindus saponaria* seeds (CESS) micronuclei induction in HepG2 cells. After 24 h of exposure, CESS induced (**A**) micronuclei formation (upon 3.7 µg/mL) and (**B**) also at the same concentrations, reduced the Nuclear Division Indexes (NDI) of human hepatocellular carcinoma cells of HepG2 lineage. 3000 cells were scored per treatment for each experiment (*p > 0 05; **p > 0 01; ***p > 0001 versus negative control; n = 3 in triplicate; one-way ANOVA followed by a Tukey's post hoc test).

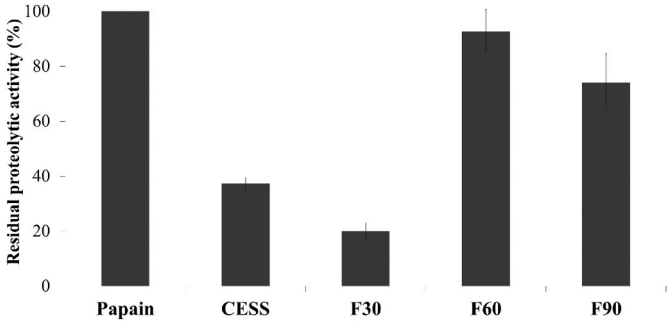


Fig. 4. Papain inhibitory activity (residual proteolytic activity) in CESS, and protein fractions of *S. Saponaria* seeds. F30, F60 and F90 represent the fractions obtained by precipitation with $(NH_4)_2SO_4$ at respective saturation ranges of 0–30%, 30–60% and 60–90%. Papain (2 µg) without the addition of plant extract, was used as an experimental control. Data with the same letters are not significantly different according to student *t*-test ($P \leq 0.05$).

4. Discussion

The use of medicinal plants has always been present and continue to increase in many different cultures. This result in a growth, not only in therapeutic solutions, but also in the number of reports of adverse reactions and other problems, mainly due to their indiscriminate use and the absence of studies proving their biological effects, dosage and

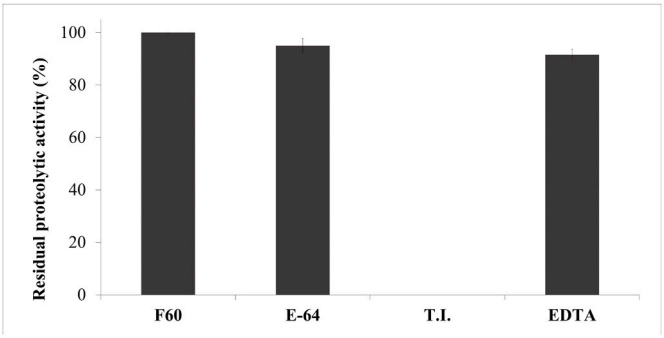


Fig. 5. Effect of specific inhibitors on the total proteolytic activity of *SST* in F60 fraction. The fraction was incubated with EDTA (10 mM), trypsin inhibitor (T. I.) (1 µM), or E−64 (1 µM) at 37 °C for 30 min. Proteolytic activity measured in the absence of any inhibitor was taken as 100%. Data are mean ± SD, n = 3. Same letters indicate the values did not present significant difference at the p < 0.0001 level by Student *t*-test ($P < 0.05$).

toxicity (Ekor, 2014; Junior et al., 2005). Among many traditional uses of the soapberry, therapeutic use of the plant has received most of the attention, resulting in studies that shown its efficiency in folk medicine, as well reveals its pharmaceutical potential in the treatment of ulcers, as hemostatic, antibacterial and antifungal agent (Albiero et al., 2002; Niloufer and Lakshmi, 2021; Tsuzuki et al., 2007).

Regarding the dose-concentration safe for the use of *S. saponaria* seed, a preliminary test was selected. The Ames Test was used to evaluate the toxicological activity using a very traditional screening towards cytotoxicity and mutagenicity analysis. Initially, mutagenicity and cytotoxicity were not detected within the established test limits (MI > 2.0 and survival rate <70%), 10 and 100µg/plate. However, the statistical analysis of these results, showed significant differences for the concentrations 0.1 and 1µg/plate to strain TA102. Despite reaching MI below the considered limit, the statistical difference found was significant, which can indicate damage to the cell. The strain used is effective in detecting oxidizing mutagens (Levin et al., 1982), which may indicate the presence of oxidizing agents in protein extract from *S. saponaria* able of mutation induced, by base pair substitution G:C-A:T in the histidine hot spot gene. Nevertheless, in this specific case, it is clear that the CESS, in cells with poor metabolic competence, such as epidermal cells, should be considered as a risk for concentrations above 10 µg/plate, specially once some studies have pointed out the pharmacological potential of plant proteins with anti-tumor and anti-inflammatory activity (Gomes et al., 2011; Srikanth and Chen, 2016).

The importance of risk assessment by simple toxicological tests as Ames Test, is the critical point. The test is used among the chemical, cosmetic industry, pharmaceutical and agro-industrial fields as part of the genetic toxicity testing battery required by regulatory agencies to enable marketing of the products. Regulatory acceptance of Ames test data often requires that the test be performed according to the Organisation for Economic Cooperation and Development (OECD) test guideline (TG)471 (OECD, 2020b) and/or ICH S2R1 (ICH, 2012). The study from Damke et al.(2013) showed that the use of *S. saponaria* extracts has been tested as a human potent sperm immobilization, including cytotoxicity effects at 2.5 mg/mL. In this way, the use for seeds from *S. saponaria* should be reviewed once are used in the cure of dental caries, arthritis, common colds, constipation and also in nausea by natives in Brazil.

Many substances, in their native forms, are promutagens. That means they are biologically inactive to interact with DNA and cause lesions. However, after biotransformation, their metabolites can be converted in potent mutagens. In humans and other mammals, the metabolic system of cytochrome P450, that is present mainly in the liver, is capable of metabolize many of these substances, making them more electrophilic and DNA reactive. Since bacteria do not have this metabolic capability,

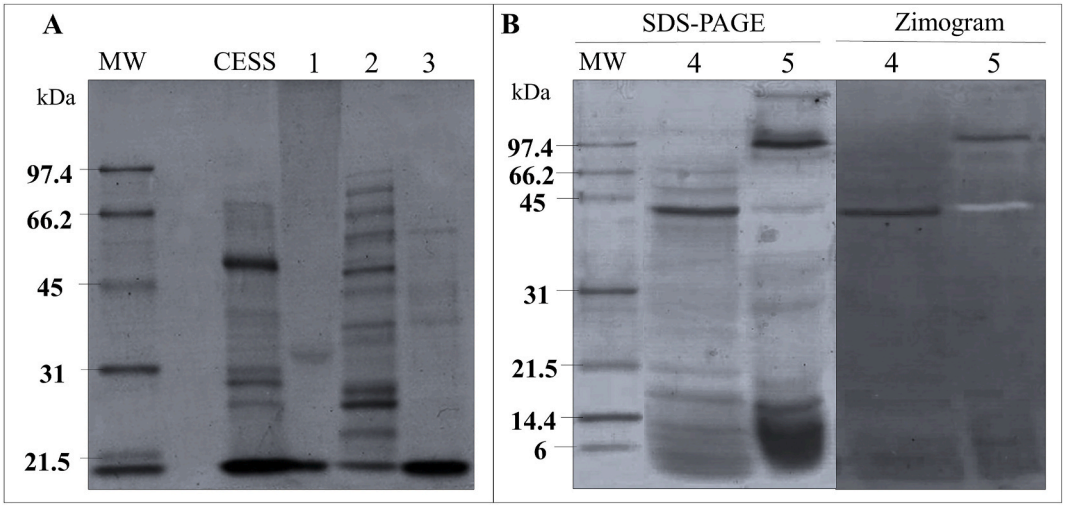


Fig. 6. SDS-PAGE 10% of the CESS and F30 (1), F60 (2), and F90 (3) fractions of *S. Saponaria* seeds (**A**). SDS-PAGE 12.5% of F60 under reducing and heat denaturing (4) and not heated conditions (5) and zymogram detection of proteolytic activity of the F60 fraction (**B**). In each well, 20 μg protein was applied. The gel was stained with Coomassie Brilliant Blue R 250. MW: standard protein markers.

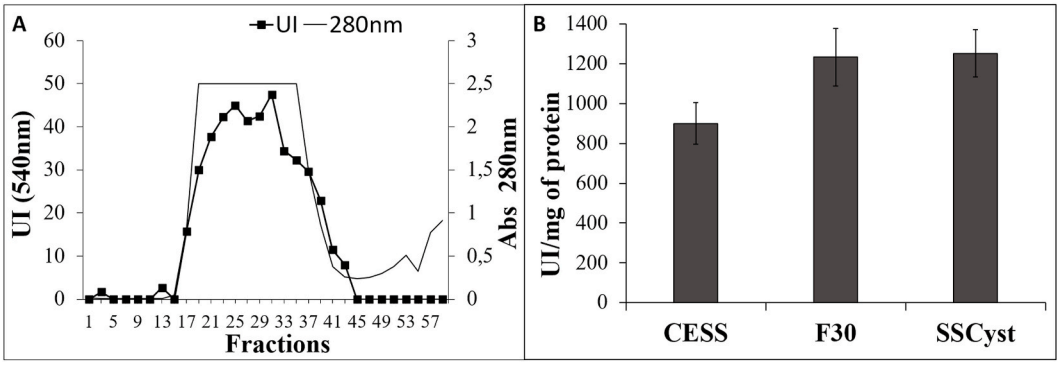


Fig. 7. Elution profile of P100 biogel (BioRad) gel filtration of $(NH_4)_2SO_4$ 0–30% fraction (F30) (A). The column was pre-equilibrated with 20 mM Tris-HCl pH 6.0. The flow rate was 4 ml/h, and fractions of 1 ml were collected. The protein concentration was monitored at 280 nm and the fractions were assayed against and papain (**A**). Evaluation of enrichment of purified protease inhibitor (*SS*Cyst) activity (**B**). One unit of inhibitory activity (UI) was defined as the amount of inhibitor that decreased absorbance by 0.01, under assay conditions.

Table 2
Purification steps of protease inhibitors from S. saponaria seeds.

Sample	Total protein (mg)	Protease Inhibitor Activity (UI[a])	Specific inhibitor activity (UI/mg)	Yield of activity (%)	Fold of purification
CESS	40	36.03	900.78	100	1
$(NH_4)_2SO_4$ fraction (0–30%)	11.5	49.35	1233.75	39.38	1.37
Gel filtration by P100 – Biogel (*SS*Cyst)	4	54.38	1359.48	15.09	1.51

[a] One unit of inhibitory activity (UI) was defined as the amount of inhibitor that decreased absorbance by 0.01, under assay conditions.

an exogenous metabolic activation system of mammals is needed to detect these mutagens (Barbezan et al., 2017; Mortelmans and Zeiger, 2000). In presence of metabolic activation, CESS induced a TA98 clear dose-response curve for mutagenicity, since 0.1 μg/mL, and cytotoxicity (survival rate lower than 70%), at 100 μg/mL, which indicates frame-shift mutations on G:C pair induction (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). Beyond that, it is important to measure the mutagen potency to aid in the assessment of human risk exposure (Ames, 1979). In Claxton et al. (1991a, 1991b), various substances, that have a known mutagenic activity, were classified in different potencies according to the number of revertants per concentration unit. It is used as parameter to compare the mutagenicity of other substances. CESS presented a mutagenic potency slope of 5.3 ± 0.4 μg/mL, which represents a low mutagenicity level (Claxton et al., 1991a, 1991b).

Hepatotoxicity is one of the most common adverse effects on drug therapeutics and for this reason we decided to investigate the effects of CESS against HepG2 cells, which represent a fast, easily executed and unexpensive model to predict this outcome (OECD, 2016). Cytotoxicity and Genotoxicity using HepG2 cells has been largely used through the last decade to detect synthetic and natural drugs genetic toxicity (Valentin-Severin et al., 2003) and predict liver injury (O'Brien et al., 2006). Drug-induced liver toxicity is devided into fatty liver disease (steatosis), cholestasis or acute liver injury with necrosis. In this sense, van Summeren et al. (2011) described, through proteomic analysis that HepG2 cells are a better model to predict drug-induced hepatotoxicity.

So, CESS induced dose-related HepG2 cell death after 24 h of exposure, with a prominent cell membrane damage, in comparison to mitochondrial dysfunction. After 48 h and 72 h of exposure, the curves tended to be closer, with similar values of LC_{50}. These results suggest that possibly there is a mechanism of death by necrosis involved, since

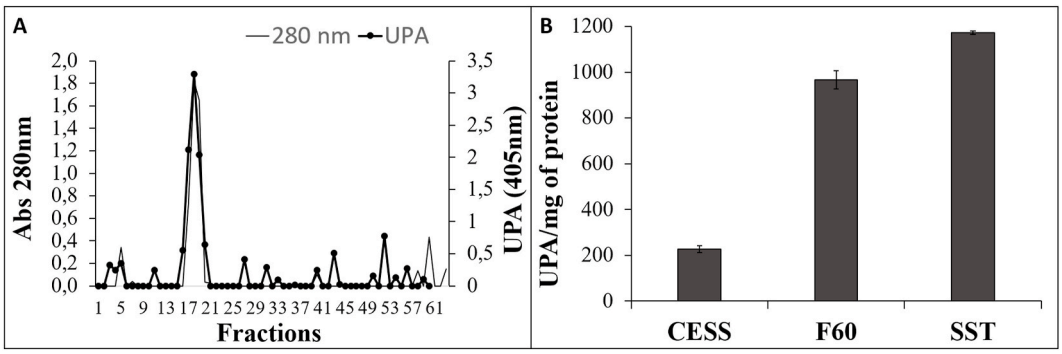


Fig. 8. Elution profile of P100 biogel (BioRad) gel filtration of $(NH_4)_2SO_4$ 30–60% fraction (F60). The column was pre-equilibrated with 20 mM Tris-HCl pH 6.0. The flow rate was 4 ml/h, and fractions of 1 ml were collected. The protein concentration was monitored at 280 nm and fractions were tested for proteolytic activity on the BApNA substrate (**A**). Evaluation of the enrichment of the enzymatic activity of purified *SST* (**B**). One unit of proteolytic activity (UPA) was defined as the amount of protease able to increase the absorbance by 0.01, under assay conditions.

Table 3
Purification steps of serine protease from S. saponaria seeds.

Sample	Total protein (mg)	Protease Activity (UPA[a])	Specific protease activity (UPA/mg)	Yield of activity (%)	Fold of purification
CESS	40	9.08	226.96	100	1
$(NH_4)_2SO_4$ fraction (0–30%)	7.5	37.04	925.89	76.49	4.08
Gel filtration by P100 – Biogel (*SST*)	0.9	46.14	1153.48	11.43	5.08

[a] One unit of proteolytic activity (UPA) was defined as the amount of protease able to increase the absorbance by 0.01, under assay conditions.

there is greater membrane damage and high mitochondrial activity early and, with the increase in the exposure time, there is cytotoxicity mediated by both membrane damage and mitochondrial dysfunction in HepG2 cells (Chan et al., 2013).

Corroborating the results of cytotoxicity, in the Micronucleus assay it is possible to verify that all tested concentrations of CESS were able to induce the formation of MN and the reduction of NDI, causing nuclear breakages and cell cycle arrest. Therefore, there is evidence that even very low concentrations of the extract can cause genotoxic effects in liver cell line. The increase in cell death and nuclear damage seen in HepG2 cannot necessarily be associated with deleterious genotoxic effects, since this is a tumor cell line. Even so, due to the hepatotoxicity outcomes observed in our investigation, it is necessary to be aware of possible hepatic adverse effects. Other natural products induced cell death and micronuclei formation in HepG2 cells and were pointed as possible anti-tumor agents but, differently of CESS, they induced apoptosis and not necrosis of hepatocarcinoma cells (Melušová et al., 2014), a preferred mechanism of cell death induction for anticancer chemotherapeutics (Ocker and Höpfner, 2012). On the other hand, necrosis releases proliferative signals to liver tissue microenvironment (Galluzzi and Kroemer, 2008) and consequently, necrotic cells can recruit cellular and humoral immune response. Furthermore, necrotic cells frequently release chemokines and bioactive factors that can stimulate other viable cells to proliferate, with the potential, once again, to facilitate neoplastic progression (Hanahan and Weinberg, 2011).

Thus, the mutagenicity on TA98 after CYP metabolism in bacterial model and the genotoxicity observed on Eukaryotic cell model in a metabolic competent cell (HepG2) reinforce the possibility that CESS contains promutagenic substances, once there was no evidences of direct mutagenicity and just after biotransformation the extract caused DNA point mutations and chromatin breakages.

Therefore, comparing the mutagenic result, which the effect was more evident with presence of metabolic activation, and the hepatotoxic and genotoxic activity, it suggests that liver enzymes play a key role in toxicity potential of CESS extract. The liver has an important role in redox cycling and inflammatory and oxidative microenvironment in damaged liver can contribute for hepatotoxicity and genotoxicity (Cardoso et al., 2016; Lin et al., 2016). A variety of phytochemicals interact with liver enzymes, such as CYP450 and others, that are essential to mammalian metabolism. This can cause a bioactivation of these biochemicals that produce reactive alkylating metabolites able to induce DNA damage and cell death (Rietjens et al., 2005). Despite the popular belief that the use of plant is absent of danger and only brings benefits, the composition of vegetal extracts can generate the most diverse lesions to living matter, especially for DNA (Araújo-Lima et al., 2020; Spontchiado et al., 2016). For this reason, analyze the genotoxic profile is so important to any kind of natural product able to human consumption, in order to a better understanding for a safety use of these products (Araujo-Lima et al., 2018).

The decrease in papain activity when incubated with CESS suggests the presence of cysteine protease inhibitors in *S. saponaria* seeds. This activity was later detected in fractions F30. The partial CESS purification process resulted in separation of a 32.8 kDa protein in F30 related to inhibitory activity on papain, suggesting that this inhibitor belongs to the phytocystatin family. Although partially purified, Brito et al. (2016) demonstrated the antibacterial potential of inhibitors of serine and cysteine proteases contained in the same fraction of aqueous seed extract of *Hymenaea courbaril*. In addition to presenting the pharmacological potential of cysteine protease inhibitors, such studies are in agreement with the data obtained in the present study, and it is possible to generate the hypothesis of the existence of a relationship between some of the biological effects of traditional use of soapberry with the presence of this phytocystatin. The presence of cysteine protease inhibitors in seeds is widely described in the literature. Some of these inhibitors have been isolated and characterized in the seeds of *Pennisetum glaucum* and *Helianthus annuus* (Joshi et al., 1998; Kouzuma et al., 1996). Previously, other serine protease inhibitors have been described in *S. saponaria* seeds. Macedo et al. (2011) described the activity of an 18 kDa serine protease inhibitor, purified from soapberry seeds, against digestive enzymes present in midgut of species larvae from Lepidoptera order, all known as pests of agricultural relevance. Further, Lima (2012) detected, purified and characterized other 7.5 kDa serine protease inhibitor, also effective in inhibiting digestive enzymes from insect pests. These data reinforce the idea of the importance of these inhibitors in seeds, also suggesting the agricultural application of partially purified phytocystatin in the present work.

Biochemical analyzes carried out also indicated the presence of a 43 kDa protein, detected in the F60 fraction, with proteolytic activity

evidenced in the zymogram. Subsequently, enzyme activity assays were performed using specific protease inhibitors demonstrating that trypsin inhibitor was the only one able to inhibit the proteolytic activity present in the fraction, suggesting that this activity may be related to presence of a serine protease. The minimum proteolytic activity detected in the CESS which, after precipitation by ammonium sulfate, reaches a yield of 4.08 fold in the F60, can be explained not only by the low concentration of the protease, but also by the interaction with the serine protease inhibitors already described for the soapberry seed (Lima, 2012; Macedo et al., 2011), reinforcing the idea that the protease detected in the present study belongs to trypsin family.

5. Conclusions

Proteins of aqueous extract from *S. saponaria* seeds does not induce mutagenic response in any of the tested concentrations. However, the extract induced cytotoxic and genotoxic effects in hepatocarcinoma cell line (HepG2). Such results not only demonstrate the biotechnological potential to be studied in the plant, by the detection of new bioactive molecules. In addition, the detection and isolation of a serine protease and a cysteine protease inhibitor among the molecules detected in a plant such as soapberry, that is a Brazilian native plant, with wide distribution throughout the territory and not economically explored, can give this plant an important role as biotechnological resource.

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CRediT authorship contribution statement

Gustavo Duarte Bocayuva Tavares: Methodology, Formal analysis, Investigation, Validation. Claudia Alessandra Fortes Aiub: Project administration, Conceptualization, Resources, Methodology, Supervision, Writing - review & editing. Israel Felzenszwalb: Methodology, Formal analysis, Investigation, Validation. Eduardo Kennedy Carrão Dantas: Methodology, Formal analysis, Investigation, Validation. Carlos Fernando Araújo-Lima: Methodology, Formal analysis, Investigation, Validation, Writing - Review & Editing. César Luis Siqueira-Junior: Project administration, Conceptualization, Resources, Methodology, Supervision, Writing - Original Draft, Writing - review & editing.

Declaration of competing interest

The authors declare no conflicts of interest.

Abbreviations

ANOVA	analysis of variance
BANA	N-benzoyl-arginine-naphthylamide
BaP	benzo-α-pyrene
BApNA	benzoyl-arginine-p-nitroanilide
CESS	crude extract from *S. saponaria* seeds
CYP	cytochrome P450
E64	L-trans- epoxysuccinylleucylamide- (4-guanidino)- butane
EDTA	Ethylenediamine tetraacetic acid
F30	30% saturation fraction
F60	60% saturation fraction
F90	90% saturation fraction
HEPG2	human liver cancer cell line
LC	lethal concentration
LDH	Lactate dehydrogenase assay
MI	mutagenic index
MMS	methanesulfonate
MN	micronuclei
MW	molecular weitght
NDI	nuclear division index
NDI	Nuclear Division Indexes
OD	optical density
OECD	Organisation for Economic Co-operation
PBS	phosphate-buffered saline
PVP	polyvinylpyrolidone
S9 mix	tissue homogenate containing cytochrome P450 isoforms and other enzymes
SD	standard deviation
SDN	subjected to thermal denaturation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SsCyst	Sapindus saponaria cystatin
SST	Sapindus saponaria trypsin
TI	trypsin inhibitor
UI	unit of inhibitory activity
UPA	unit of proteolytic activity
WST-1	water-soluble tetrazolium salt assay

Appendix B. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jep.2021.114170.

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