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Original Article

Phytochemical Analysis, Antioxidant Properties and Brine Shrimp Lethality of Unripe Fruits of *Solanum viarum*

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ABSTRACT

Objective: The aim of the study was evaluate the tannin content, antioxidant activity and the lethality of unripe fruits extract of *Solanum viarum* Dunal (Solanaceae) against brine shrimps. **Methods:** The tested samples for tannins content were water, ethanol:water (1:3) and acetone:water (1:3) extract. The unripe fruits powder of *S. viarum* was used to extraction in a Soxhlet apparatus for 2 hr. Cytotoxicity was screened using Brine Shrimp Lethality Test and aqueous extract. **Results:** The results demonstrated that the yield of tannins for the acetone:water extraction was substantially higher than that typical yields obtained by ethanol:water or water extraction [31.10% for Total Solids (%), 14.99% for Stiasny's Index (%), 3.99% for Condensed Tannins in the Extract (%) and 1.09% for Condensed Tannins in the Unripe Fruits (%). The antioxidant activity was 61.12±12.42 % compared to ascorbic acid (100%) and polyphenol concentration was 39.33±4.45 mg of tannic acid/g of extract obtained from the tannic acid calibration curve y = 0.0128x - 0.0015 (R^2 = 0.9988). Flavonoid concentration was 89.36 ± 11.6 mg of quercetin/g of extract obtained by quercetin calibration curve y=0.0153x+0.0109, R^2=0.9827. The results demonstrated that the positive control (potassium dichromate) hatching success was significantly higher than the *S. viarum* extract (P<0.05). LC_{50} was 66.01 µg/ml for the water extract of unripe fruit of *S. viarum* and in accord to Clarkson's toxicity criterion, LC_{50}< 100 µg/mL are considered highly toxic for aqueous extract. **Conclusion:** The unripe fruit extract of *S. viarum* demonstrated low percentage in phenolic content, but significant antioxidant activity and phenolic and flavonoid concentrations. *S. viarum* unripe fruits extract is toxic to *Artemia salina*.

Key words: *Solanum viarum*, Toxicity, Antioxidant, Flavonoid, Phenolic, LC_{50}, *Artemia salina*.

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INTRODUCTION

Solanum viarum Dunal also known as "juá, joá-bravo" in Brazil and is a common plant in the Latin America. It is a toxic plant, and have medicinal properties.[1] It can be found as an encroachment of pastures, along roadsides and crops, and is considered a problem for livestock farmers due to their toxicity around the world, principally in Brazil and USA.[2]

Because *S. viarum* is a plant full of thorns, including thorns in the leaves, it is not eaten by many animals. However, the cattle, using its tongue, reach the fruits of *S. viarum*, and in this way it is an excellent disseminator of seeds through feces. Others animals also spread the seeds through the feces.[3] However, some cases reported in the literature demonstrated that the fruits are toxic to cattle and to other types of herds.[4] Mentz *et al.*[5] explained that the fruits of *S. viarum* when ingested by cattle produce intense and lethal tympani and the leaves exhibit narcotic properties.

Genus *Solanum* is rich in steroidal glycoalkaloids and solasodine is a nitrogen analogue of sapogenins found in this genus. This compound (C27 cholestane skeleton) can be converted in a key intermediate in the synthesis of steroidal drugs and it is potential moiety to be used in the production of steroidal hormones in pharmaceuticals.[6] Glycoalkaloids are toxic for living organisms due to their anticholinesterase activities and rupture of cell membranes related to effects including teratogenicity, embryotoxicity and genotoxicity.[7] Among the medicinal properties of species of genus *Solanum* is the use of roots as diuretic, they improve the functions of the liver and bladder and its plaster eliminates skin boils.[8] Some glycoalkaloids are beneficial for the growth of plants and have medicinal effects on humans. They can protect against damage caused by insects and fungi, and may have anti-inflammatory[9] and anticancer activity in humans.[10] Biological investigations of glycoalkaloids of *Solanum* showed significant cytotoxicity against human cancer cell lines and skin tumours.[11]

Cipollini and Levey[12] comment about the propensity for immature *Solanum* fruits to accumulate high levels of alkaloids (1-7% dry mass) and that these levels may or may not drop during fruit maturation. Glycoalkaloids can be highly toxic to vertebrates; in some species, their concentration in ripe fruit (up to 7% of dry mass) should be high enough to cause lethal effects in a 1-2 kg vertebrate after consumption of <10 fruits (based on an estimate of a 100-1000 mg/kg lethal dose in clinical and case studies with domestic animals and humans.[13,14]

Considering the results from studies on *S. viarum* and the existence of few toxicological and biochemical studies regarding unripe fruits of *S. viarum*, we evaluated the tannins content, polyphenols and flavonoid concentration, antioxidant activity of unripe fruits of *S. viarum* and the toxicity of extract using brine shrimp lethality bioassay.

MATERIALS AND METHODS

Plant extract

The unripe fruits of *Solanum viarum* were collected in November 2016 in a farm in Pinhão District Municipality, Paraná State, Brazil. This area lies at Latitude 25°42'06.7" and Longitude 51°38'20.6". The plant was authenticated by Dr. Eneida Martins Miskalo of Universidade Estadual do Centro Oeste – UNICENTRO, HUCO – Herbarium, Campus de Irati

District, Paraná State, Brazil, and a voucher specimen (HUCO-7408) was prepared and deposited in the HUCO Herbarium. The unripe fruits was rinsed with deionized water and gently blotted with paper towel to remove the water, chopped into smaller bits and subsequently oven-dried at 55 °C for 72 hr until constant weight was achieved, then ground into powder. Tannins were extracted from samples (10 g) with water, or ethanol:water (1:3) or acetone:water (1:3) using a Soxhlet apparatus for 2 hr.

Determination of tannins

The yield of the extract using water, ethanol:water and acetone:water was determined according to Guangcheng *et al.*[15] By gravimetric method with adaptations. Stiasny's index [SI (%)] was determined by Equation (1): SI(%)= (m_1/m_2)* 100 (1) where m_1= dry mass of 50 ml of extract after 24 hr in the oven at 100 °C, m_2= dry mass of formaldehyde and HCl reaction with tannins of 50 ml of extract on the filter paper after 24 hr in the oven at 100 °C. Solid total (%) was obtained by Equation (2): ST(%)=[(M_1-M_2)]/M_1]*100 (2) where M_1 = plant mass for extraction, M_2 = plant mass after extraction, drying in the oven at 100 °C for 24 hr Condensed tannins content in the extract and Condensed tannins content in the unripe fruit were obtained by Equation (3) and (4): CTE(%)=[ST(%) * SI(%)]/100 (3) and CTF(%)=[CTE(%) * ST(%)]/100 (4).

Polyphenol concentration

Polyphenol concentration in the aqueous extract was evaluated by Folin-Ciocalteu method as reported by Singleton *et al.*,[16] The absorption was measured at 726 nm wavelength (Gehaka UV-340-G spectrophotometer) and the phenol concentration was expressed as tannic acid in $mg.g^{-1}$ of dry weight extract by a linear equation based on the calibration curve for the tannic acid $y = 0.0128x - 0.0015$, $R^2=0.9988$.

Total flavonoid concentration

A known volume of aqueous extract was placed in a glass tube. Distilled water was added to make 2 ml, and 0.15 ml $NaNO_2$ (1:20) were added. 0.15 ml $AlCl_3$ (1:10) were added 6 min later. After 6 min, 2 ml of 4% NaOH was added and the total was made up to 5 ml with distilled water. The solution was mixed well again and the absorbance was measured against a blank at 510 nm with a UV-visible spectrophotometer.[17] Calibration curve for the quercetin was prepared (0-0.2 mg/ml) and the flavonoid content was calculated using the following linear equation based on the calibration curve for the quercetin: $y=0.0153x+0.0109$, $R^2=0.9827$.

Evaluation of Total Antioxidant Activity

A 0.3 ml aliquot of an aqueous diluted extract solution to 200 µg/ml was combined in a tube with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes containing the reaction solution were capped and incubated in a boiling water bath at 95°C for 90 min. After cooling to room temperature, the absorbance of the solution was measured at 695 nm using a spectrophotometer. The reference substance used was ascorbic acid 200 µg/mL. Ascorbic acid was considered as 100% activity antioxidant.[18]

Artemia salina Hatching Assay

This assay was evaluated as described by Otang *et al.*[19] with little modifications. A density of ten *A. salina* cysts was stocked in each of tube containing 5 ml of the prepared concentrations of the unripe fruits aqueous extract of *S. viarum* and positive control (potassium dichromate). The unripe fruits extract of *S. viarum* was dissolved in sea water to complete 10 ml of total volume in concentration of 0.005, 0.05, 0.5, 2.5, 5.0, 7.5 and 10 mg/ml. One ml of each dilution was added to tubes containing 4 ml sea water and 10 cysts each to afford the final sample concentration. The final concentration was 1, 10, 100, 500, 1000, 1500 and 2000 µg/ml. A positive control group was also prepared by dissolving the potassium dichromate in water at the same concentrations as the plant extract. The tubes were partly covered, incubated at 30 °C and under constant illumination for 72 hr the percentage of hatchability was assessed by comparing the number of hatched nauplii with the total number of cysts stocked.

Brine Shrimp Lethality Assay

The brine shrimp lethality assay was performed following the reported procedure by Meyer *et al.*[20] and McLaughlin *et al.*[21] with some modifications. The growth medium was prepared with sea water in a small tank divided into two compartments. The shrimp eggs were added to the covered compartment. After 48 hr, the shrimps mature as nauplii (*A. salina*) and are ready for the assay. The final concentration tested of unripe fruit extract of *S. viarum* was 1, 10, 100, 500, 1000, 1500 and 2000 µg/ml. A positive control group was also prepared by dissolving the potassium dichromate in water at the same concentrations as the plant extract. After 24 hr incubation under light, the number of dead and survivor brine shrimps in each tube was counted. The percentage of mortality was calculated as Equation (5): Mortality (%) = [(Total nauplii – Alive nauplii) * 100 / Total nauplii] (5)[5]

Data Analysis

Antioxidant activity, polyphenols and flavonoid concentration values were determined from the best-fit line obtained by regression analysis. The median lethal concentration (LC_{50}) and 95% confidence intervals of the test samples were calculated using the probity analysis method described by Finney,[22] as the measure of toxicity of the plant extract. For statistical significance, the study was carried out in 5 experiments in triplicate. The differences between the groups were determined by Analysis of Variance (ANOVA) followed, when detected, by Tukey's multiple comparisons test. The analysis was done using GraphPad Prism® version 5.01 for Windows®, Graph Pad Software®, and San Diego, California, USA. The results were considered statistically significant when $P < 0.05$.

RESULTS

Figure 1(a-d) represent the mean values of total solids content [TS (%)], Stiasny's index [SI (%)], condensed tannins content in the extract [CTE (%)] and condensed tannins content in the unripe fruits [CTF (%)]. The solvents water, ethanol:water (1:3) and acetone:water (1:3) were used to prepare unripe fruits extract of *S. viarum* using a Soxhlet apparatus for 2 hr TS (%) express the sum of tannic and non-tannic substances contained in the extracts. When the extraction was made with water, ethanol:water and acetone:water the TS (%) value was 16.87 ± 1.69%, 13.35 ± 2.50% and 31.10 ± 0.32%, respectively, and the comparison between water × acetone:water and ethanol:water × acetone:water were statistically different (P=0.0143, P=0.0196, respectively), but water × ethanol:water was not statistically different (P=0.3637) (Figure 1a). The Stiasny's Index (SI %) represents the percentage of polyphenols (condensed tannins) in the extract. Using water, ethanol:water or acetone:water as solvent the SI (%) was 4.85 ± 0.68%, 4.43 ± 0.15% and 14.99 ± 2.82% (Figure 1b), respectively. Comparison between water × acetone: water and ethanol: water × acetone:water were statistically different (P=0.0129 and P=0.0015, respectively). The CTE (%) was 0.74 ± 0.14%, 0.57 ± 0.39% and 3.99 ± 0.51% for water, ethanol:water and acetone:water (Figure 1c), respectively. The CTE (%) was statistically different between water × acetone: water (P=0.0002) and ethanol: water × acetone: water (P=0.0005). The CTF (%) was 0.234 ± 0.05%, 0.173 ± 0.04% and 1.09 ± 0.08% for water, ethanol:water and acetone:water (Figure 1d), respectively. The CTF (%) was statistically different between

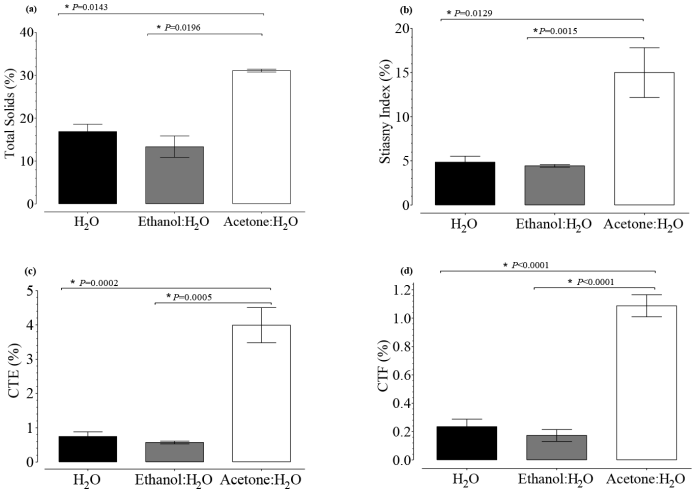

Figure 1: Percentage of total solids [TS (%)] (**a**), Stiasny's index [SI (%)] (**b**), condensed tannins content in the extract [CTE (%)] (**c**) and the condensed tannins content in the unripe fruits [CTF (%)] (**d**) for the solvents water, ethanol:water (1:3) and acetone:water (1:3) present in the unripe fruits extract of *S. viarum* obtained in Soxhlet extraction for 2 hr * statistically significant ($P < 0.05$).

Table 1: Total Polyphenolic concentration, Flavonoid concentration and Antioxidant Activity of unripe fruit extract of *S. viarum*.

	Polyphenolic concentration	Flavonoid concentration	Antioxidant Activity
	(mg tannic acid/g of extract)	(mg quercetin/g of extract)	%
Solanun viarum unripe fruit extract	39.33 ± 4.45	89.36 ± 11.60	61.12 ± 12.42

water × acetone: water (P<0.0001) and acetone: water × ethanol:water (P<0.0001).

Table 1 shows the results for total phenols and flavonoid concentration, and antioxidant activity (%AA) of the unripe fruit extract of *S. viarum*. The results demonstrated that antioxidant activity was 61.12 ± 12.42 % compared with the antioxidant activity of ascorbic acid. The antioxidant capacity of the samples was expressed in relation to ascorbic acid, considering its absorbance corresponding to 100% of antioxidant activity.

The flavonoid concentration was 89.36 ± 11.6 mg of quercetin/g extract. They can act as antioxidant, anti-inflammatory, antiviral, antimicrobial among others. Polyphenol concentration was 39.33 ± 4.45 mg tannic acid/g extract. The extract contains polyphenolics with appreciable amounts of condensed tannins and flavonoid.

Figure 2 (a and b) shows the activities of the extract and positive control at varying concentrations to the hatching success of the *A. salina* cysts. The hatching success of *A. salina* cysts significantly decreased with increasing concentrations of the *S. viarum* extract and the positive control (potassium dichromate) while sea water was the highest hatching potential being observed (Figure 2). The percentage hatching success of cysts incubated with the *S. viarum* extract demonstrated significant differences at varying concentrations (Figure 2 a). The lowest concentration (1 µg/ml) had the highest hatching percentage (85.3±2.1%) and it was not significantly different from the cysts incubated at 10 µg/ml with a hatching success of 83 ± 10.1 % at 72 h, respectively. With the sea water having a significantly higher hatching success (93.3 ± 6.1 %) than the *S. viarum* extract (46.3 ± 8.1 %; 26.7 ± 7.6 %; 13.3 ± 1.5; 10.7 ± 1.2 %; 6.3 ± 3.1 % at 100, 500, 1000, 1500 and 2000 µg/ml at 72 h, respectively) ($P < 0.05$), and the positive control (potassium dichromate) presented 65.3 ± 9.5 %; 16 ± 4.2 %; 18.3 ± 2.9 %; 14.5 ± 2.1 % and 7.5 ± 3.5 % at 1, 10, 100, 500 and 1000 µg/ml at 72h, respectively) ($P < 0.05$). There was zero percent hatchability observed from 1500 µg/ml – 2000 µg/ml for potassium dichromate (Figure 2 b). The results demonstrated that the positive control (potassium dichromate) hatching success was significantly higher than the extract of *S. viarum* (P<0.05).

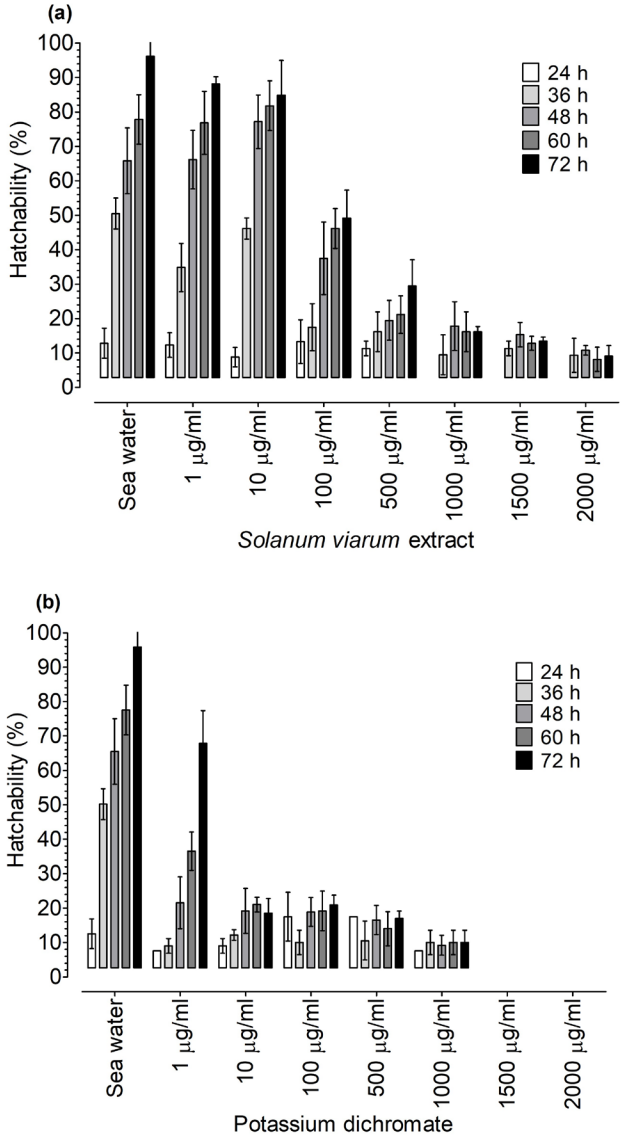

Figure 2: Percentage hatching success of *Artemia salina* cysts incubated in different concentrations of *S. viarum* extract (**a**) and potassium dichromate (positive control) (**b**). The values are means ± SD of three experiments in triplicate at different concentrations for the extract and controls.

The results also demonstrated that after 36 h of exposure, hatching success of the cysts incubated in sea water was significantly increased by 3.1-fold having a 7.9-fold increase in cyst hatching at the end of 72 hr. For cysts incubated in aqueous extract of *S. viarum* only significantly increased by 1.7-fold after 36 hr and increased 4.6-fold after 72 hr. For cysts incubated in potassium dichromate, cysts hatching decreased significantly 0.2-fold after 36 h and 1-fold after 48 h ($P < 0.05$), followed by no hatching of cyst after 60 hr.

Table 2: The estimated LC_{50} results for aqueous extract of *S. viarum* and potassium dichromate in Brine Shrimp Lethality Bioassay.

Sample	Regression equation	LC_{50} (µg/ml)	Toxicity status	R^2
S. viarum aqueous extract	y = 0.7422x + 3.6495	66.01	Toxic	0.9756
Potassium Dichromate	y=1.208x + 3.2307	29.15	Toxic	0.9536

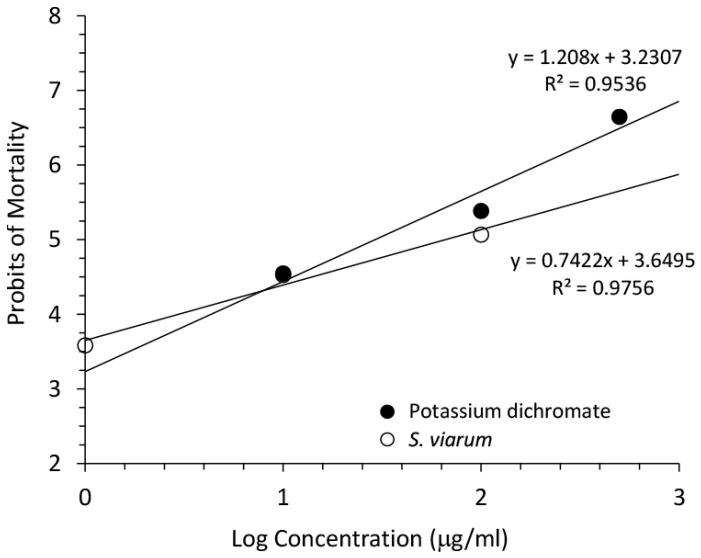

Figure 3: Toxicity effect of potassium dichromate and unripe fruit extract of *S. viarum* in Brine shrimp nauplii.

Brine shrimp lethality bioassay is a useful test for toxicity screening of plant extracts because it is rapid and also inexpensive. The unripe fruits extract of *S. viarum* have a high LC_{50} value of 66.01 µg/ml (Figure 3 and Table 2). Potassium dichromate is highly toxic even at very low concentrations. The sea water was observed 0% mortality through the duration of the experiment. The mortality of nauplii with unripe fruit extract of *S. viarum* was significantly higher than sea water ($P < 0.05$).

DISCUSSION

The results demonstrated that the distinct solvents promoted in an unequal manner in the solubility of polyphenolic compounds of extracts, being higher in the acetone: water extract. The aqueous extract presented the lowest value. Ethanol:water (1:3) solvent provided values close to that of aqueous extract, not statistically differing from this solvent ($P>0.05$). The aqueous extract reveals relative low phenolic contents evaluated by Stiasny's Index. The low value obtained with gravimetric method can be due to the fact that water dissolves preferentially low weight polyphenols. On the other hand, during extraction process in Soxhlet apparatus, the ebullition was violent, because the boiling point of pure water is greater, making the polymerization of the polyphenols possible, and diminishing their reactivity to formaldehyde. The extraction time in Soxhlet was 2 hr and the temperature was the boiling point of each solvent. Ethanol and acetone have boiling points of 78.37 ºC and 56 ºC, respectively. The Stiasny's Index (SI) gives an absolute measure for the condensable polyphenols in the extract, but does not represent itself the content of the phenolic material in the extract. The SI by gravimetric method is based on their ability to precipitate polyphenols with a molar excess of formaldehyde during 24 hr in the oven at 100 °C. Additionally, this method is a slow and messy procedure that demands large quantities of extract solution.

On the other hand, we also used a method of polyphenol quantification based in redox chemistry. To quantify the phenolic material content of extract by the method of *Folin-ciocalteu*, a calibration curve absorbance-concentration was used. A known standard that could be considered to infer the concentration of the phenolic material, namely the oligomeric molecules of condensed tannins was selected. Tannic acid was used because it can be considered the basic unit for the most common molecules of condensed tannins. This standard provides good linear correlation, and it is frequently used standard for this method. The calibration curve was presented by the equation in materials and methods. We can conclude that the relation absorbance vs. concentration can be acceptably fitted by a linear model for tannic acid as standard. Unripe fruits of *S. viarum* contain a significant quantity of alkaloids, phenols and flavonoids which attribute to the antioxidant capability of this plant. The results suggest that *S. viarum* extract possess a relative free radical scavenging potential and could act and be exploited as a natural source of antioxidants.

The unripe fruit extract of *S. viarum* had significant lower hatching percentage of the cysts above 100 µg/ml, and potassium dichromate had significant lower hatching percentage of the cysts above 1 µg/ml (Figure 2 a and b, respectively) compared to the control (sea water). The hatching success significantly decreased with increasing concentrations of the unripe fruits extract, and potassium dichromate elicit 100% hatching inhibition at 1500-2000 µg/ml. *Artemia salina* has a resistant cyst stage where it can withstand pH variation (from fresh water to saturated saline), and when dormancy is not interrupted, incubation does not occur. However, 1-10 µg/ml of the unripe fruit extract showed an optimal breaking of dormancy of the cyst and an additional increase in concentration revealed an inhibitory action on the cyst. Evaluation of the hatching success of the cysts in response to exposure time revealed that the extract had significant hatching success after 36-48 hours which is known to be the best hatching time for brine shrimp according to Meyer *et al.*[20] However, the cysts incubated in the unripe fruit extract continued to hatch until the end of 72 hr at doses between 1 and 500 µg/ml. In incubation with potassium dichromate a low hatching rate of eggs was observed at all doses. The poor hatching success observed in potassium dichromate is due to its high toxicity on cysts of *Artemia salina*.

Sango *et al.*[23] demonstrated that aerial parts extract of *Solanum nigrum* showed LC_{50} values of 670 µg/ml and 1840 µg/ml for methanol and water solvents. The unripe fruits of *S. viarum* present higher toxicity in brine shrimp lethality bioassay. There was an increase in mortality with increasing extract concentrations. The lethality rates (probit) were strongly and positively correlated to the extract concentrations, as shown by the correlation coefficients (R^2). The respective regression line gave LC_{50} value of 29.15 µg/ml for potassium dichromate (positive control). We use the Clarkson's toxicity criterion [24] that for the toxicity assessment of plant extracts classifies extracts in the following order: extracts with LC_{50} above 1000 µg/ml are non-toxic, LC_{50} of 500 – 1000 µg/ml are low toxic, extracts with LC_{50} of 100 – 500 µg/ml are medium toxic, while extracts with LC_{50} of 0 - 100 µg/ml are highly toxic. The unripe fruit extract of *S. viarum* was considered highly toxic by the Clarkson's toxicity criterion (LC_{50} = 66.01 µg/ml).

CONCLUSION

The results of this study indicated that the unripe fruit extract of *S. viarum* reduces cysts hatching and is highly toxic to *A. salina* suggesting further toxicological studies *in vivo* and *in vitro*. It is relevant to know that *S. viarum* rarely involved in human intoxications due to the unique environments in which they are kept. The highest amount of phenolic

compounds was obtained with the extract of the unripe fruits of *S. viarum* prepared in acetone:water (1:3). The extract of the unripe fruits of *S. viarum* contain varying amounts of phytochemicals, considerable antioxidant activity, and the fruits have appreciable amounts of polyphenols. It can be concluded that although the unripe fruits are highly toxic, and when they are available in pastures or roadside, they can be consumed by the cattle. This extract can therefore be used to screen for potential activities against many diseases such as cancer which are triggered by free radicals. Further studies are underway to identify the active compounds responsible for the activities exhibited by the fruits of the plant and to evaluate the anticancer efficacy against cancer cell lines and animal models. Based on the relationship between brine shrimp lethality and plant bioactivity, this work provides subsidies for new biochemical, pharmacological and phytochemical research.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

ABBREVIATIONS

LC_{50}: Lethal Concentration 50; **h:** hour; SI: Stiasny's Index; **TS:** Total Solids; **CTE:** Condensed Tannins in the Extract; **CTF:** Condensed Tannins in the Unripe Fruits.

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