Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating: 3.53
www.plantsjournal.com
JMPS 2021; 9(3): 06-13

Received: 05-01-2021
Accepted: 09-03-2021

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Determination of total phenols and evaluation of the antioxidant activity of pulps and fruit derivatives of *Vangueria infausta* and *Strychnos spinosa*

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Abstract
Mozambique possesses a widespread variety of edible wild plants with high nutritional and medicinal value. The impact of these fruits in the diet depends on the availability, the species and varies according to the region and its cultural traditions. Initiatives were developed and technologies adapted to process indigenous fruits of spontaneous occurrence into saleable products such as spirits (liquor), sweets and jams. The products were approved for human consumption by the Ministry of Health, but it is necessary to evaluate their physical-chemical, phytochemical and nutritional properties. The method of Folin-Ciocalteu was used to the determination of total phenolic and free radical 2,2-diphenyl-1-picrylhydrazyl sequestration was used for antioxidant activity evaluation. The pulps of *Vangueria infausta* and *Strychnos spinosa* exhibited CI_{50} 1,348 e 1,137 mg/l, respectively, the derivatives, exhibited CI_{50} 13,786 e 4,485 mg/l for liquor and jams of *Vangueria infausta*, respectively and CI_{50} 5,016 mg/l for liquor of *Strychnos spinosa*. The pulps of *Vangueria infausta* and *Strychnos spinosa* showed higher total phenolics and antioxidant capacity than the corresponding derivatives.

Keywords: Native fruits, total phenolics, antioxidant activity, nutritional properties

Introduction
Recent research has demonstrated the existence of a large variety of indigenous fruits with high nutritional and medicinal potential that meet the needs of rural families [1-4]. In Mozambique, varieties of these wild food plants are widely distributed throughout the country and their fruits and almonds are sold in informal markets during the harvest season and consumed in different ways. The importance of these fruits in the diet depends on their availability and the choice of species varies according to the region and cultural traditions [5, 6].
In recent years, several initiatives have been taken in Mozambique to develop and adapt technologies for the processing of native fruits of spontaneous occurrence such as Mapfílwa (*Vangueria infausta*), Canhu (Sclerocarya birrea), Massala (*Strychnos spinosa*), Passionflower (Passiflora spp), Tamarind (*Tamarindus indica*), Malambe (*Adansonia digitata*) and Jambalão (*Syzygium cordatum*) in their derivatives (liquors, sweets and jams). Most of these derivatives circulate in a commercial format in the national market only with certification for human consumption attested by laboratories of the Ministry of Health, without knowing its physico-chemical, phytochemical and nutritional properties. It is within this scope that a physical-chemical, phytochemical and antioxidant activity evaluation of the pulps and derivatives (liquors and jams) of the fruits of Mapfilwa (*Vangueria infausta*) and Massala (*Strychnos spinosa*) were carried in the present work.
The *Vangueria infausta* belongs to the Rubiaceae family and is a source of food and medicine for rural communities [3]. Its fruits are consumed by people and wild animals while the different parts of this plant are traditionally used for the treatment of malaria, sores, menstrual cycle problems, uterine problems, genital swellings among other diseases [7, 3, 8-12].
Aqueous root extracts are used for the treatment of diarrhea [13, 14] and as anthelmintic [15]. The content of minerals found in the fruit *Vangueria infausta* is constituted by Fe, K, Ca, Mn, P, N, Mg, Na, S, Se and Zn and analyzed in the dry matter, moisture, protein, pH, (%), sugar content (° Brix), fat and ash [3, 1]. In the phytochemical study, flavonoids were identified in leaf extracts [16, 17, 10] and extracts of roots, tannins and saponins [16].

The *Strychnos spinosa* belongs to the family Loganiaceae and the parts most used in traditional medicine are the roots, leaves and the fruits. The fruits are edible and the leaves are used in many African countries as analgesics [18], [19], in the treatment of venereal diseases, liver diseases, stomach diseases and in the treatment of wounded results of snake bites [19]. In Benin, leaves and roots are used for the treatment of stomach pain, abdominal pain, cramps, sterility, abscess, sleeping sickness and malaria [20]. In Mali, stem barks and roots are used for the treatment of diarrhea [21]. The inner part of the fruit is used for the treatment of warts and when mixed with leaves of *Strychnos madagascariensis* is applied for wound treatment [22]. Aqueous root extract is used for the treatment of venereal diseases, hernia, and snake bites [23]. The fruit presented a composition in minerals such as Fe, K, Ca, Mg, Zn, Na and P, and the percentage of ash, lignins, proteins, fibers, pH, acidity and vitamin C were analyzed [24], [25].

In the qualitative analysis of the phytochemicals of the different parts of the plant *Strychnos spinosa*, the seeds presented chemical compounds as strychnine and other alkaloids [18], the aqueous, ethanolic and methanolic extracts of the leaves and stem bark revealed the presence of alkaloids, steroids, terpenoids, tannins, reducing sugars and saponins [26]. The reported pharmacological properties of this plant, are associated to the existence of these various secondary metabolites [19].

The antioxidant property of extracts and leaf fractions determined using the DPPH and ABTS methods showed varied and higher IC_{50} values compared to ascorbic acid and Trolox standards [27].

Materials and Methods

Acquisition of Samples and Extraction

The fruits of *Vangueria infausta* (Mapfilwa) were purchased in June 2013 in Gaza province at two sites: (a) Chongoene locality in Xai-Xai district; and (b) Chimodzo locality in the Macia district. Washing, selection, refrigeration (4°C), separation of pulp and seeds, drying (at room temperature for 48 hours and artificial in circulating air oven at 40°C for 72 hours) and fruit spraying was carried in the Chemistry of Natural Products laboratory.

The fruits of *Strychnos spinosa* (Massala) were purchased in the province of Maputo at two sites: (a) in the Chiboene Quarter - Moamba district in August 2013, and (b) in the Esperança - Manhiça district in December 2013. Pre-selection, washing, separation of pulp and seeds and storage (4°C) was carried in the Chemistry of Natural Products laboratory. The derivatives of these fruits (liqueurs and jam) were produced by the Agro-services company.

The extraction methods applied were soxhlet and maceration and the solvents used were methanol (99.5%). A rotary evaporator was used for recovery of extraction solvents. The extracts were submitted to physical-chemical, phytochemical and antioxidant tests.

Determination of Total Phenol Content

The determination of the total phenol content present in the fruit pulp extracts of *Vangueria infausta* and *Strychnos spinosa* and its derivatives (liqueurs and jam) was done by means of spectroscopy in the visible region at 750 nm using the Folin-Ciocalteu (Merck) method [28], [29], [30], [31].

The total phenol content (FT) was determined by interpolating the absorbance of the samples against a calibration curve constructed with gallic acid (Merck) standards (0 to 350 mg/ml) prepared by dissolving 0.5 grams in 100 ml of a mixture of water and methanol (10 ml of methanol and 90 ml distilled water) and expressed as mg EAG (gallic acid equivalents) per g extract of fruits and their derivatives, obtained from the application of equation 1. All analyzes were carried in triplicate.

$$EAG = \frac{CxV}{m} \quad (1)$$

Where: EAG is equivalent grams of gallic acid in mg/g, ***C*** is the concentration of gallic acid in mg/ml, ***V*** is the volume of the extract used in the test in ml and ***m*** is the mass of the extract in g.

Determination of Antioxidant Activity

The antioxidant capacity of the crude extracts of the pulps and their derivatives was evaluated using the DPPH free radical sequestration method, which is based on a photometric test where the DPPH free radical (Sigma-Aldrich), which presents a deep purple coloration in alcoholic solution, reduces the presence of antioxidant molecules, forming 2,2-diphenyl-1-picrylhydrazine, which is colourless [32], [33].

The calibration curve was constructed from the absorbance values at 515 nm of all solutions (0 to 40 µg/ml), measured in glass cuvettes with an optical path of 1 cm and with methanol as the "blank". Absorbance measurements were taken at intervals of 1 min between each reading.

The solutions of the methanolic extracts (100 mg/l) and the methanol positive controls were diluted at the concentrations of 75, 50, 25 mg/l. The absorbance measurements of the reaction mixtures (0.3 ml of the sample solution or the positive control and 2.7 ml of the stock solution of 40 µg/ml DPPH) were made at 515 nm after 30 minutes. The mixture of methanol (2.7 ml) and methanolic solution of the extract (0.3 ml) was used as white. From the equation of the calibration curve and the 30 minutes absorbance values for each concentration tested, the percentages of remaining DPPH (% $DPPH_{REM}$) were determined, according to equation 2:

$$\%DPPH_{REM} = [DPPH]_{T=t} / [DPPH]_{T=0} \times 100 \quad (2)$$

Where: $[DPPH]_{T=t}$ corresponds to the concentration of DPPH in the medium (30 minutes), after the reaction with the extract and $[DPPH]_{T=0}$ is the initial concentration of DPPH, that is, 40 µg/ml.

The ability of a particular compound to inhibit the oxidation of oxidizable substrate can be simulated by the chemical reduction of the DPPH radical obtained by the donation of hydrogen protons from the compound to the radical. This simulation has been called "percent inhibition" or "percentage of DPPH sequestration" being calculated by equation 3:

$$\%\ Inibição = ((Abs_{DPPH} - Abs_{Extract}) / Abs_{DPPH}) \times 100 \quad (3)$$

Where: Abs_{DPPH} is the absorbance of the DPPH solution at 40 µg/ml and $Abs_{Extract}$ is the absorbance of the sample in solution. $Abs_{Extract}$ was calculated based on the difference in absorbance of the test sample solution with its blank.

The inhibition coefficient (IC_{50}), the concentration required to inhibit 50% of the oxidizable substrate, was obtained by the equation of the line generated from percent inhibition associated with four concentration levels (100, 75, 50 and 25 mg/l) for each sample.

The lower the IC_{50}, the better the antioxidant activity [30].

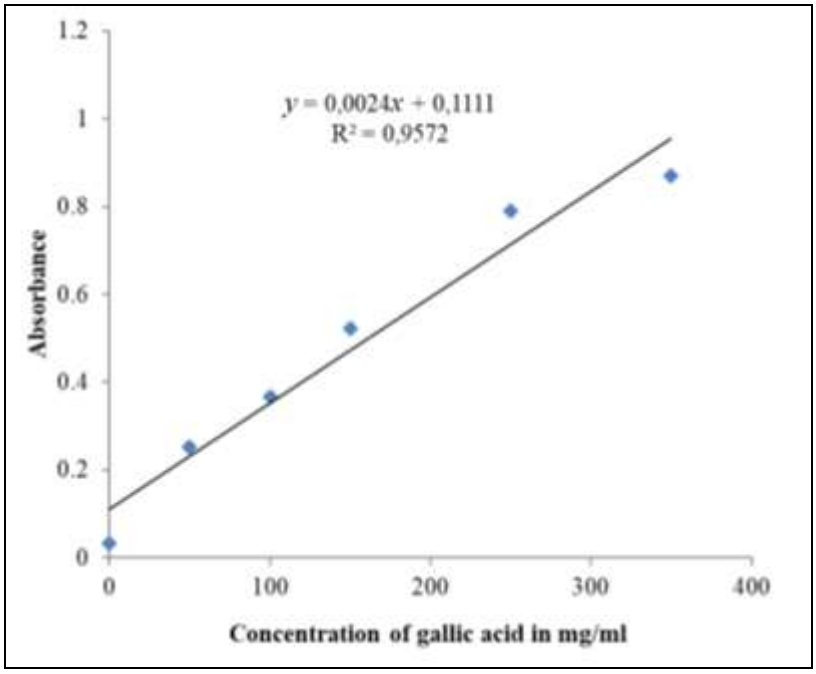
The concentration of the sample, equivalent to 50% of the DPPH absorbance, was calculated graphically [34].
Absorbance values at all concentrations tested, at 30 minutes, can also be converted to percentage of antioxidant activity (% AA), using equation 4:

$$\%AA = \{[Abs_{control} - (Abs_{sample} - Abs_{blank})]x100\}/Abs_{control} \quad (4)$$

Where: $Abs_{control}$ is the initial absorbance of the methanolic solution of DPPH and Abs_{sample} is the absorbance of the reaction mixture (DPPH + sample).

Results and discussion

The quantification of phenolic compounds was performed through a calibration curve of gallic acid, constructed from the absorbance values at 750 nm and represented by graph 1.



Graph 1: Gallic acid calibration curve in mg/ml

The equation for the line $y = 0.0024x + 0.1111$ was used to determine the concentration in mg of gallic acid, where ***x*** corresponds to the concentration of gallic acid and ***y*** corresponds to the absorbance of the sample ($R^2 = 0.9572$).

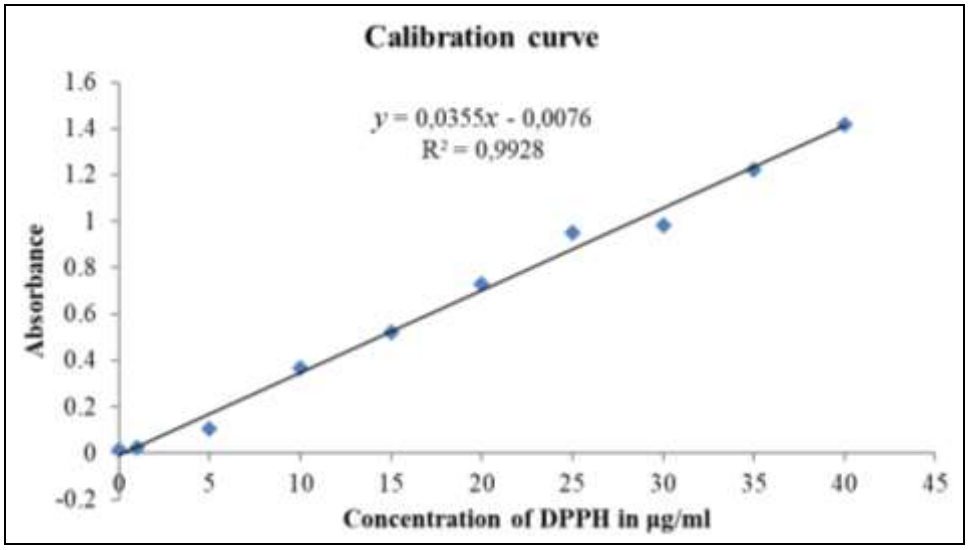
The concentrations of total phenolic compounds in mg of gallic acid/g of extract (EAG), obtained for the pulps and derivatives of fruits *Vangueria infausta* and *Strychnos spinosa* are presented in table 1.

Table 1: Total phenolic compounds in pulps and derivatives of *V. infausta* and *S. spinosa*

	Vangueria infausta			***Strychnos spinosa***	
	Pulp	**Liquor**	**Jam**	**Pulp**	**Liquor**
Abs (sample)	0,128±0,001	0,055±0,008	0,084±0,012	0,176±0,006	0,078±0,012
Con A.G (mg/ml)	7,04±0,42	<DL	<DL	27,04±2,53	<DL
EAG (mg/g)	0,528±0,030	<DL	<DL	2,028±0,190	<DL

Values obtained by mean ± standard deviation, <DL = less than detection limit

The method of Folin-Ciocalteau allows to quantify phenolic compounds and anthocyanins present in the samples, which have the capacity to bind to free radicals, inhibiting oxidative processes.
The methanolic extracts of the pulps of *Vangueria infausta* and *Strychnos spinosa* presented values of phenolic compounds of 0.528 ± 0.030 and 2.028 ± 0.190 mg EAG per gram of extract, respectively (Table 1). Taking into account figure 1, the contents of the phenolic compounds of the derivatives (liquor and jam of *Vangueria infausta* and liquor of *Strychnos spinosa*) were not determined because they had absorbance values lower than that registered for the blank which was approximately 0.1.
In this study, the antioxidant activity was analyzed according to the stable radical DPPH sequestration method, where the antioxidant compound transfers electrons to the DPPH and it loses the characteristic intense purple coloration.
The assay was chosen because it is a simple and sensitive method. The calibration curve was constructed from the absorbance values at 515 nm of DPPH solutions (0 to 40 µg / ml) (graph 2).



Graph 2: DPPH calibration curve in µg/ml

Table 2 shows the absorbance values, in triplicate, at 515 nm, at different concentrations of the pulp samples and fruit derivatives of the plants *Vangueria infausta* and *Strychnos spinosa*.

Table 2: Mean values of the absorbances of the different dilutions of the extracts

Samples		Abs **DPPH**	Abs **White**	**Concentrations (mg/l)**				
				6,25	**25**	**50**	**75**	**100**
V. infausta	Pulp	1,419	0,119		0,682±0,074	0,297±0,108	0,174±0,075	0,115±0,019
	Liquor	1,419	0,088		1,227±0,019	1,212±0,004	1,121±0,039	1,109±0,082
	Jam	1,419	-0,001		1,133±0,034	0,936±0,012	0,805±0,009	0,621±0,023
S. spinosa	Pulp	1,419	0,019		0,658±0,039	0,274±0,010	0,131±0,009	0,123±0,018
	Liquor	1,419	0,007		1,134±0,035	0,980±0,011	0,843±0,004	0,719±0,025
	Vit. C	1,419	0,035	0,068±0,004				

Absorbance expressed on average three measurements ± standard deviation

Table 3 presents values of the percentage of DPPH remaining after 30 minutes of reaction, obtained from the equation of the calibration curve (graph 2) and of equation 2.

Table 3: Percentage of DPPH remaining after 30 minutes of reaction

% DPPH Remnant (Remaining) by concentration						
Samples		**Concentration (mg/l)**				
		6,25	**25**	**50**	**75**	**100**
Vangueria infausta	Pulp		49,21	21,71	12,93	8,71
	Liquor		88,14	87,07	80,57	79,71
	Jam		81,43	67,36	58	44,86
Strychnos spinosa	Pulp		47,5	20,07	9,86	9,29
	Liquor		81,5	70,5	60,71	51,86
	Vit. C	5,36				

From these results it was observed that the extracts of the pulps at concentrations of 75 and 100 mg/l were more efficient, with the percentage of the DPPH concentration after 30 minutes of reaction much lower and their efficiency is close to that of standard vitamin C (Merck) used in this study, in relation to the same radical. As a diagram, these results can be observed in figure 1.

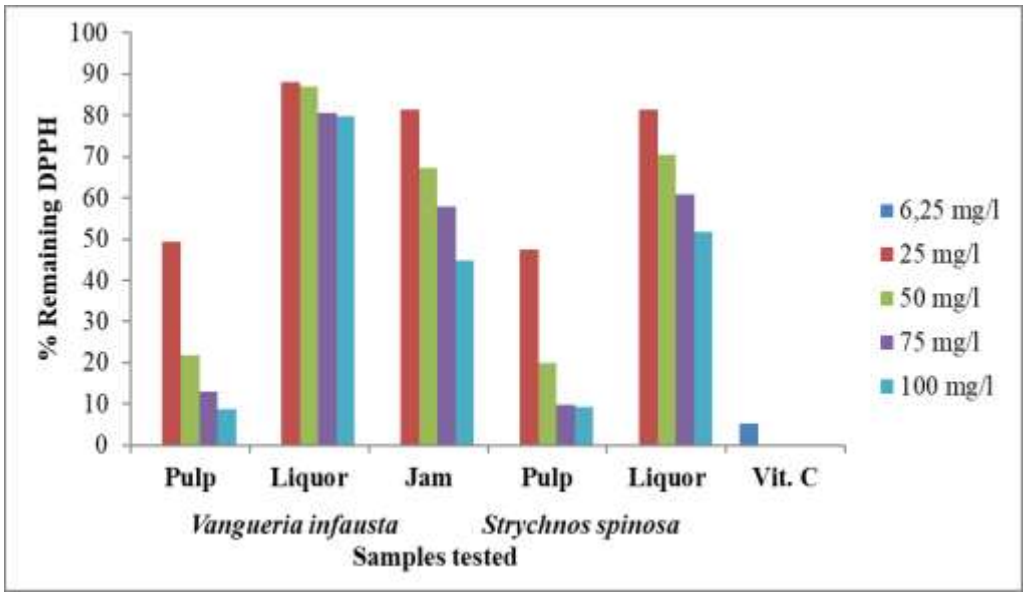


Fig 1: Percentage of DPPH remaining after 30 minutes of reaction

Table 4 presents the results obtained from equation 3, which show the antioxidant capacity of the extracts of pulps and derivatives of *Vangueria infausta* and *Strychnos spinosa*, at different concentrations and as a positive control vitamin C was used.

Table 4: Percentage of inhibition of DPPH by pulps and derivatives of *V. infausta* and *S. spinosa*

Samples		% of DPPH Inhibition				
		Concentration (mg/l)				
		6,25	**25**	**50**	**75**	**100**
Vangueria infausta	Pulp		51,94	79,07	87,74	91,89
	Liquor		13,53	14,59	21,00	21,85
	Jam		20,16	34,04	43,27	56,24
Strychnos spinosa	Pulp		53,63	80,69	90,77	91,33
	Liquor		20,08	30,94	40,59	49,33
	Vit. C	95,21				

By the assay, it is noted that all extracts have the capturing ability of the DPPH radical. However, the higher efficiency was verified in extracts of pulps at higher concentrations (75 and 100 mg/l). Figure 2 shows the results in diagram form.

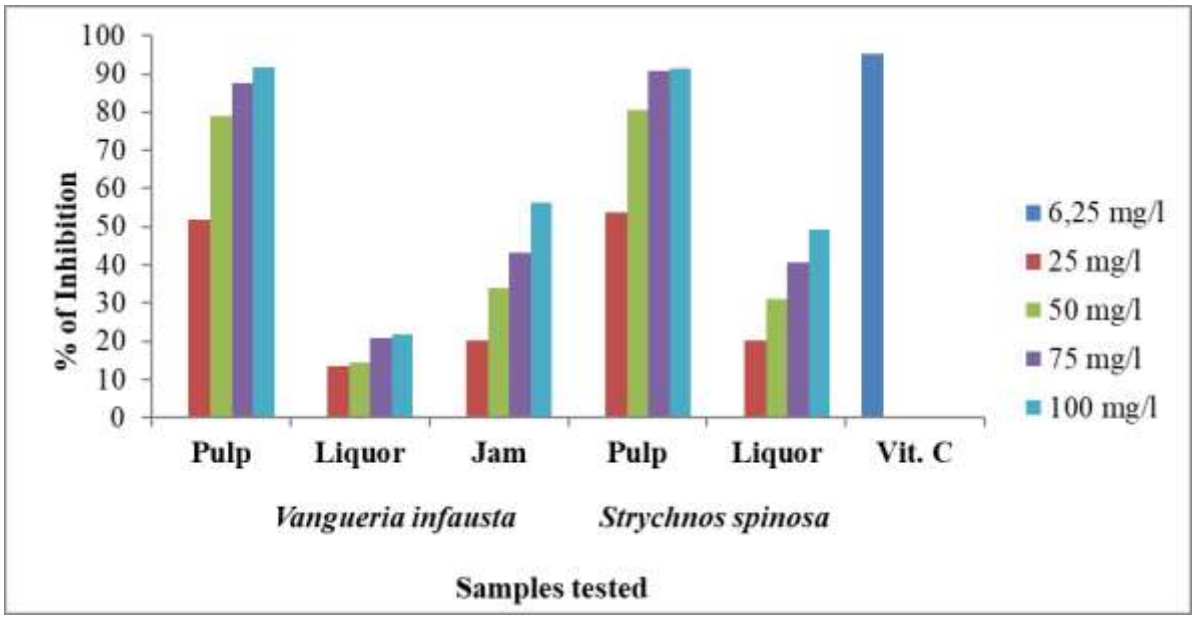


Fig 2: Percentage of inhibition of DPPH by pulps and derivatives of *V. infausta* and *S. spinosa*

The IC_{50} values of each extract are shown in table 5 and for their calculation, the equations of the lines obtained from the graphs of figure 3 were used, where the **y** value was replaced by 50 to obtain the concentration of sample with a capacity to reduce 50% of DPPH.

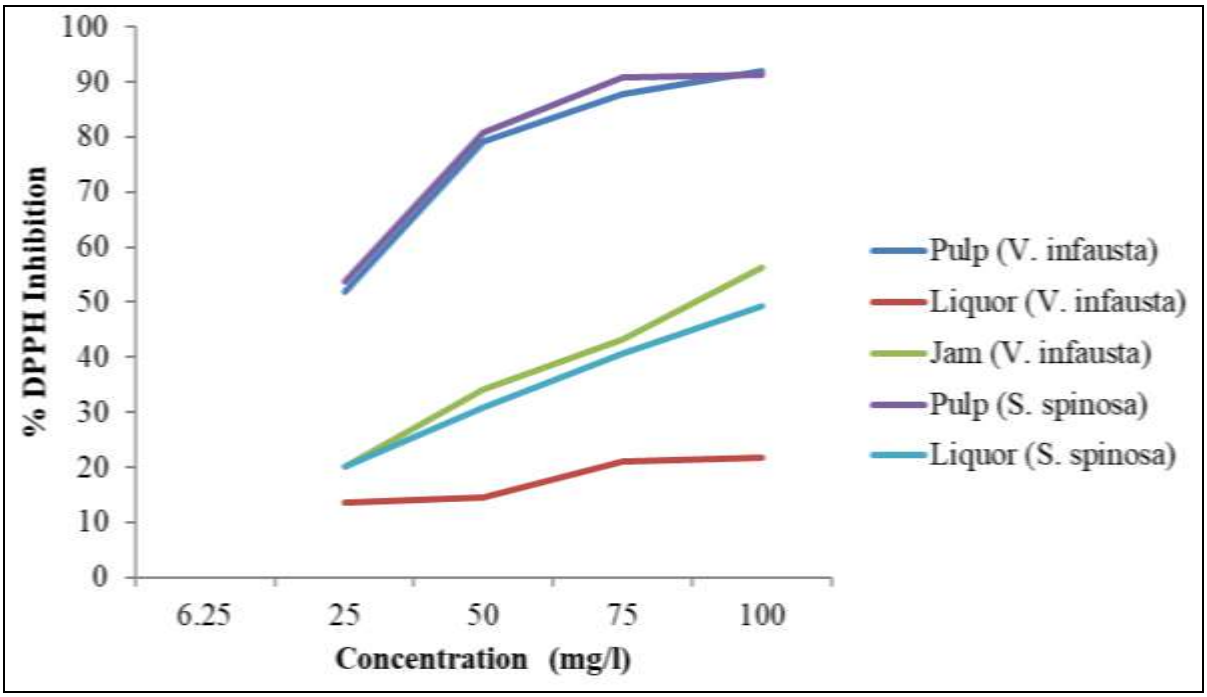


Fig 3: Effect of DPPH free radical capture by pulps and derivatives of *V. infausta* and *S. spinosa*

Comparing the pulp, jam and liquor of *Vangueria infausta*, we can observe that the pulp extract stands out in the inhibition of DPPH, presenting a low IC_{50} value (IC_{50} = 1.348 mg/l), in relation to the liquor extracts (IC_{50} = 13.786 mg/l) and jam (IC_{50} = 4.485 mg/l). As for pulp and liquor from *Strychnos spinosa*, the pulp extract also has an IC_{50} = 1.137 mg/l and low than the IC_{50} of the liquor extract (IC_{50} = 5.016 mg/l).

Table 5: Line equations used to calculate IC_{50} of the antioxidant activity of *V. infausta* and *S. spinosa* pulps and derivatives

Samples		Equation of the line	R^2	IC_{50} (mg/l)
Vangueria infausta	Pulp	$Y = 12{,}854x + 32, 671$	0,8536	1,348
	Liquor	$Y = 3{,}136x + 6{,}7653$	0,8916	13,786
	Jam	$Y = 11{,}748x - 2{,}692$	0,9946	4,485
Strychnos spinosa	Pulp	$Y = 12{,}319x + 35{,}99$	0,8097	1,137
	Liquor	$Y = 9{,}7393x + 1{,}1487$	0,9976	5,016

About antioxidant activity expressed as inhibition coefficient (IC_{50}), extracts from *Vangueria infausta* and *Strychnos spinosa* pulps had IC_{50} of 1.348 and 1.137 mg/l, respectively, *Vangueria infausta* derivatives showed an IC_{50} of 13.786 mg/l (liquor) and 4,485 mg/l (jam) and the *Strychnos spinosa* liquor an IC_{50} of 5.016 mg/l (table 5). These results show a high antioxidant power of the pulps that their derivatives. Same flavonoids isolated in the leaves of *Vangueria infausta* showed greater activity in the elimination of DPPH radicals and was used ascorbic acid as standard [10]. The vitamin C, present in large amounts in the fruits of *Vangueria infausta*, *Strychnos spinosa*, *Adansonia digitata*, *Sclerocarya birrea*, is responsible for its antioxidant activity [25]. In extracts of leaves of *Vangueria infausta* were isolated some flavonoids with antioxidant activity and the oxidants used for evaluation were DPPH and H_2O_2 and as standard the routine was used [17]. Higher the DPPH intake per sample, the lower it will be inhibitory concentration and the higher it wil be antioxidant activity [35], [31], this action is identified as responsible for the neutralization of reactive oxygen species, thus protecting the appearance of many chronic diseases in the human body [36].

Conclusion

Several authors have found a strong relationship between phenolic compounds and antioxidant activity, but others have not evidenced this correlation. For the latter, the antioxidant activity of an extract cannot be explained solely on the basis of its total phenolic compounds content, and it is necessary to characterize the structures of the active compounds present in the sample.

However, based on the mechanism of action of the DPPH radical and with prior knowledge of the phenolic compounds present in fruits (pulp and derivatives) of *Vangueria infausta* and *Strychnos spinosa*, it can be deduced that the antioxidant activity they present may be related to the presence of phenolic compounds.

From the results obtained in the tests it can be concluded that: The content of phenolic compounds present in the extracts of the pulp is greater than in the extracts of the derivatives in the two fruits. The inhibition coefficient (IC_{50}) has shown that the pulp extracts of the two fruits have a higher antioxidant capacity than the extracts of their derivatives.

Acknowledgment

To FNI (Fundo Nacional de Investigação) for the financing of the "Frutas Nativas" Project, which allowed the acquisition of samples, reagents and materials necessary to carry out this investigation.

To Asdi for the master's scholarship granted.

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