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Determination of in Vitro Antioxidant Activity, Acute Toxicity in Guinea Pig and TLC-Fingerprint of *Panda oleosa* (Pierre) Bark Extract Used to Treat Diabetes in Folk Medicine

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ABSTRACT

Objective: Panda oleosa (Pierre) is a rainforest plant used in traditional medicine to treat diabetes among other diseases. The study aimed at determining TLC fingerprint profile and measuring antioxidant activity and toxicity of trunk bark extracts. *Methods*: The fingerprint was processed for saponins, flavonoids and tannins fractions. The antioxidant activity was tested with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and anti-hydroxyl radical capacity methods using quercetin, ascorbic acid and tannic acid as references. The toxicity was carried out in guinea pigs to estimate the range of LD_{50}. *Results*: Various colored spots appeared at different Rf on TLC chromatogram comprised of at least 8 spots in saponins fractions, 4 in flavonoids, and 3 catechin derivatives in tannins fractions. The EC_{50} values for DPPH radical scavenging capacity were 0.072 mg/ml, 0.156 mg/ml, 0.188 mg/ml and 0.432 mg/ml for total polyphenols, flavonoids, quercetin and tannins. For hydroxyl radical inhibition capacity, the EC_{50} values varied from 0.435 – 6.6 mg/ml for the extracts compared to 0.252 mg/ml for ascorbic acid and 0.281 mg/ml for tannic acid. The LD_{50} value was around 7892 mg/kg body BW, classifying the plant trunk bark extracts as practically non-toxic. *Conclusion*: The extract tested has a complex chemical profile, possesses a high antioxidant activity in *vitro* and a very low toxicity in guinea-pig.

1. INTRODUCTION

In modern medicine, diabetes is classified as a metabolic disease caused by an imbalance of insulin levels in the body. The therapeutic goal is to restore the normal insulin level or to improve any mechanism leading to the reduction of blood glucose. Diabetes remains a chronic disease classified as incurable but controllable [1]. Despite the development of high-efficiency synthetic drugs with controlled safety for the management of diabetes, alternative strategies based primarily on the use of traditional medicinal plants are worthy of consideration in poor populations where access to modern therapies is limit. Every country in the developing world is encouraged by the World Health Organization (WHO) to take the necessary steps to explore the therapeutic potential of plants for the purpose of integrating them into modern conventional herbalism [2]. Traditional healers are used to thinking that herbal medicines can definitely cure diabetes. So far, hundreds of plant species have been found as potential anti-diabetic candidates worldwide. In sub-Saharan African countries, a number of edible trees, shrubs and herbs have been used in traditional medicine to treat diabetes [3-5]. According to some ethnopharmacological studies conducted in the Democratic Republic of Congo (DRC), many plants have been recorded and experimentally tested [5-9]. Among these plants, *Panda oleosa* Stone of the family *Pandaceae*, syn. *Porphyranthus zenkeri* Engl. or *Sorindeia rubiflora Eng* l has been found very promising [8, 9].

P.oleosa is a special case because it is a large plant only located in the African rainforest [10, 11]. Many other plants listed are ornamental or edible vegetables from the Americas, Europe or Asia. The species is a slow-growing plant native to West and Central Africa (DRC, Congo-Brazzaville, Gabon, Central African Republic, Liberia, Ghana, Guinea, Ivory Coast, Nigeria and Cameroon). It has a dense crown, usually 10 to 20 meters high but with some specimens at 35 meters; brownish yellow to moderately hard pink-red. Many trees produce fruit each year that can persist for several months [10-13]. The infusion of leaves is used as enema to treat dysmenorrheal; the root decoction is against bronchial affections; the decoction of stem bark is used as anti-inflammatory, analgesic and aphrodisiac and to treat abdominal disorders, abortions, intestinal parasites and gonorrhea; The extracts are also externally applied to treat rheumatism, wounds, yaws, whitlow, swelling and hemorrhoids. In the literature, it is also indicated that wood is traditionally a source of timber making carpentry and canoes [10, 13]. In Gabon, crushed seeds are added to sauces, soups and stews

in the same way as fruit kernels of *Irvingia gabonensis*. It is listed among the plants used by the pygmies to develop the hunting poison and dislodge the caimans from their holes [13].

This study is part of a series of experiments designed to study the chemical components and their bioactivities in the species growing in the DRC. It aimed to evaluate the ability of extracts to trap free radicals (antioxidant activity) to explain the mechanism of antidiabetic activity and their toxicity in animals to ensure safety limits for their use. A complement to its fingerprint was also considered to account for what is known today of its chemical composition.

2. MATERIALS AND METHODS

2.1 Plant Materials

Fresh trunk bark pieces were harvested in Masako reserve forest, 14 km from the city of Kisangani. Specimens were authenticated in the herbarium of the Faculty of Sciences in the University of Kisangani under the voucher number 21243 [9**]**. The fresh barks were shade-dried, then ground and sieved before analysis (Fig.1).

Figure 1 Image of *Panda oleosa Pierre* samples

2.2 Preparation of Plant Extracts

2.2.1. Extracts for TLC fingerprint

For the saponins, 10 g of plant powder was heated under reflux in methanol/water (70 / 30v / v) for 15 minutes at 90°C; the mixture was filtered and the filtrate was evaporated to remove methanol; the remaining aqueous phase was mixed with n-butanol and then separated;

addition of diethyl ether to the butanol phase precipitated the saponins; these were collected, introduced into a silica gel column and eluted with diethyl ether/ethyl acetate (1: 1) giving 6 fractions S1, S2, S3, S4, S5, S6.

For the phenol flavonoids, an aliquot (10 g) of the plant powder was washed with petroleum ether and then extracted with methanol/water (70/30 v / v) for 15 minutes at 90°C. Under reflux in water bath; the mixture was concentrated by evaporation of methanol; the remaining water was then extracted successively with diethyl ether (F1) and ethyl acetate (F2).

For the tannins, three primary extracts were prepared: T1 (aqueous decoction at 100 ° C for 15 min reflux; T2 (maceration with methanol for 70 min at room temperature) and T3 (maceration in water for 70 min at room temperature). The T1 extract was fractionated into three subfractions: T1a (dichloromethane extraction), T1b (extraction with a diethyl ether/ethyl acetate mixture: 1/1) and T1c (extraction in methanol).

2.2.2. Preparation of Extracts for antioxidant activity

Total polyphenols (Poly): 1 g powder was defatted with petroleum ether, extracted with 10 ml methanol-water (70:30 v/v) reflux for 15 min, filtered, evaporated to dryness at 60°C.

Flavonoids methanol (Flav): 1 g powder was refluxed in 10 ml methanol, filtered, evaporated.

Flavonoids ethyl acetate and n-butanol: 1 g powder was refluxed in 10 ml of methanol, filtered and evaporated to dryness, the residue dissolved in mixture ethyl acetate + distilled water (10/10 v/v) in a separating funnel then separated (FlavE); the aqueous phase was extracted with 10 ml n-butanol (FlavB).

Tannins (Tanin): 1 g powder was refluxed 15 min with 10 ml acetone/distilled water mixture (35/15v/v), filtered, evaporated at 40°C in a water bath to remove the acetone; the remaining aqueous phase was washed with 15 ml of petroleum ether to remove pigments and lipids; the aqueous phase was extracted twice with 15 ml of ethyl acetate and evaporated at 37^{o}C.

2.2.3. Extracts for acute toxicity

40 g of the powder was put in 100 ml of distilled water and boiled for 15 min. When cool, the mixture was filtrated through whatman No.3 in a flask and completed to 100 ml with distillate water.

2.3. Procedure of antioxidant activity

2.3.1. DPPH scavenging procedure [19-22]

A series of 5 concentrations (0.02, 0.04, 0.08, 0.16, 0.32 mg / ml) of each extract including quercetin as a reference were prepared. In different tubes, 0.3 ml of each sample in triplicate was mixed with 3 ml of methanol and 0.5 ml of DPPH radical (0.16 mg/ml solution). The mixture of 3.3 ml of methanol and 0.5 ml of the sample served as blank for each measurement. The mixture of 3.5 ml of methanol and 0.3 ml of DPPH (0.16 mg/ml) served as a control. After standing for 30 minutes in the dark at room temperature, the absorbance of each solution was read at 517 nm with a model SP-2100 spectrophotometer.

2.3.2. Hydroxyl radical scavenging procedure [23, 24]

A series of 5 concentrations (0.32; 0.64; 1.28; 2.56; 5.12 mg/ml) of each extract including ascorbic acid and tannic acid as references were prepared. In different tubes, 0.2 ml of each concentration was mixed with 0.2 ml of $FeSO_4$ solution (5 mM in distilled water), and 0.2 ml of H_2O_2 (1% in distilled water). A control blank solution contained 0.2 ml of water plus 0.2 ml of $FeSO_4$ solution (5 mM in distilled water) plus 0.2 ml of H_2O_2 (1% in distilled water). Each tube was set at room temperature (25°C) for 60 minutes. Then 1 ml of distilled water was added to each tube, stirred and read at 510 nm. By principle, the reaction is known as Fenton reaction ($FeSO_4$ +H_2O_2 reaction) where Fe^{+2} is oxidized by hydrogen peroxide to Fe^{+3} forming a hydroxyl radical and a hydroxide; Fe^{+3} is then reduced back to Fe^{+2} by another molecule of hydrogen peroxide, forming a hydroperoxyl radical and a proton. The net effect is a disproportionation of hydrogen peroxide to create two different oxygen-radical species, with water, H^+ + OH^- as a byproduct. Anti-radical compounds can inhibit the process.

2.4. Procedure of acute toxicity

The protocol respected recommendations for animal experimentation [25]. The acute toxicity was done with 30 male guinea pigs (200-550g weight) as described previously [26]. Five groups of 6 animals each were constituted for different doses. The guinea-pigs were placed in compartmented cages and fed for a period of one month before starting. Each animal was given per oral a volume of decoction 40% of the plant in increasing doses. The observation concerned changes in skin, hair, eyes, mucous membranes, respiratory system, circulatory system, nervous system, somatomotor activity and death.

2.5. Statistical Analysis

The radical scavenging capacity was expressed as percentage of the control solution:

$$\%RSA = 100x\frac{AbControl - AbSample)}{AbControl}$$

The EC_{50} for radical scavenging and LD_{50} for acute toxicity were determined graphically using Excel software. Values were smoothed with Solver using the following classic adapted sigmoid equation [27]:

$$\%Q=1/((Ka/L)^n+1)*(max-min)+min ;$$

Where n=slope, [L]=concentration, Ka=EC50 ; max% and min% effect. Simple correlation served to compare experimental and calculated values. Excel Windows tool was used.

3. RESULTS

3.1. Antioxidant capacity

Table 1 presents the four points used for sigmoid regression. Fig.2 presents the simulated concentration-effect curves. The correlation between experimental and calculated data was good for all extracts (R^2 >0.980). The EC_{50} values thus differed between the two procedures used. For total polyphenols extract, EC50=72.6 and 627.4 µg/mL by DPPH and $FeSO_4$. The tannins extract was smoothed with EC_{50} =221.6 by $FeSO_4$ and 970.2 µg/mL by DPPH. For flavonoids extracts there was EC_{50} =110.7 µg/mL by DPPH and 413 - 6628 µg/mL by $FeSO_4$. EC_{50} of references were quercetin (188 µg/ml) by DPPH, ascorbic acid (262.3µg/ml) and tannic acid (281.4µg/ml) by $FeSO_4$.

Table 1 Concentration-effect curves fitting parameters with Excel Solver

Parameters	Poly1	Poly2	Tan1	Tan2	Flav1	FlavB	FlavE	Flav2	Querc1	AscAc	TanAc
EC_{50} µg/mL	72.6	627.4	970.2	221.6	110.7	413.0	900	6628	189.1	262.3	281.4
Slope (n)	1.11	0.42	1.14	0.92	1.27	2.11	6.94	1.29	1.15	1.40	1.49
Max %	100	100	100	70.7	100	73.5	47.0	100	100	100	100
Min %	0	0	15.07	0	0	0	2.09	0	0	0	0
Test	$DPPH^{\cdot}$	$HO^{\cdot}$	$DPPH^{\cdot}$	$HO^{\cdot}$	$DPPH^{\cdot}$	$HO^{\cdot}$	$HO^{\cdot}$	$HO^{\cdot}$	$DPPH^{\cdot}$	$HO^{\cdot}$	$HO^{\cdot}$

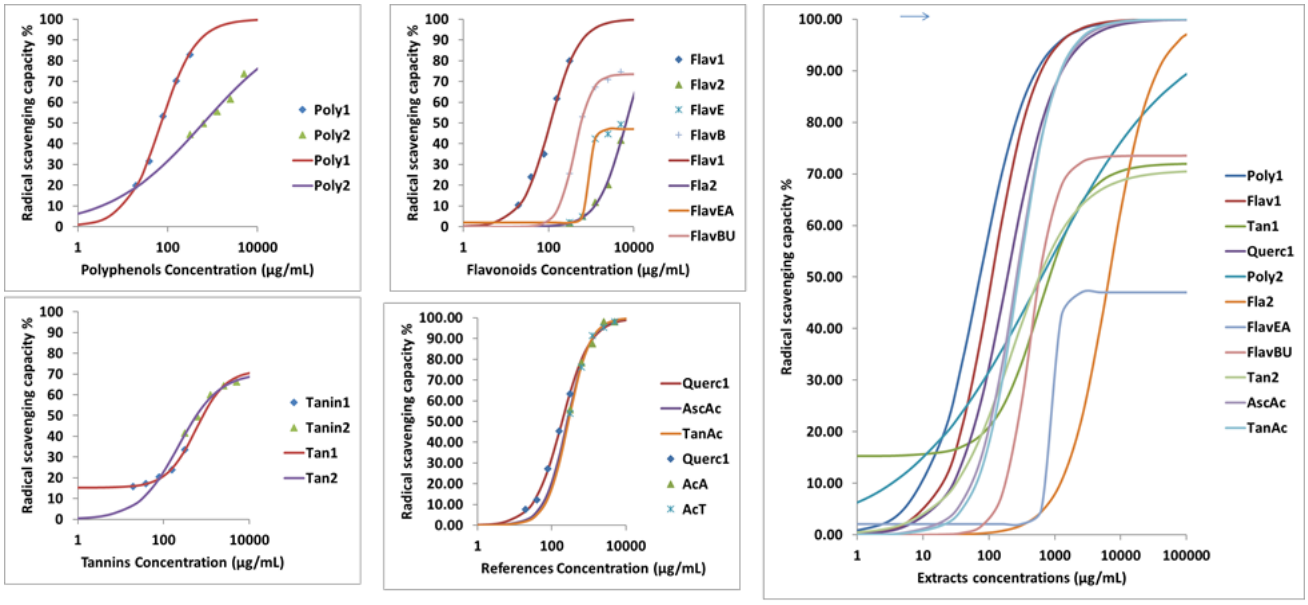

Figure 2 Concentration-Effect curves of *P. oleosa* extracts and references

3.2. Toxicity in guinea-pigs

Below 2000 mg/kg oral dose, no signs of intoxication were observed. From 6000 mg/kg, the animals presented low mobility, lack of appetite, anuria, and death. The LD_{50} was graphically estimated as 8 g/kg. The autopsy of vital organs showed the dead animals had dark organs due to hemorrhage compared to normal tissues.

3.3. TLC fingerprint result

Figure 3 shows the TLC footprint and the structures of the second potential metabolites. In fractions S1-S6 (Fig. 3a), different products were observed with different colors (green, sky blue, blue sea, red) at Rf 0.13, 0.25, 0.40, 0.43, 0, 55, 0.66, 0.70, 0.77, 0.80, 0.85. In fractions F1 and F2 (Figure bb), 4 to 6 spots were observed). In the tannin extracts (Fig.2b), 3 brown products were observed at Rf 0.32; 0.74; 0.80, the Rf of the last two were comparable to those of epigallocatechin and catechin used as references; the product with Rf 0.32 was identified as being similar to flavonoid rutin. The chemical structures of these compounds are shown in Figure 4.

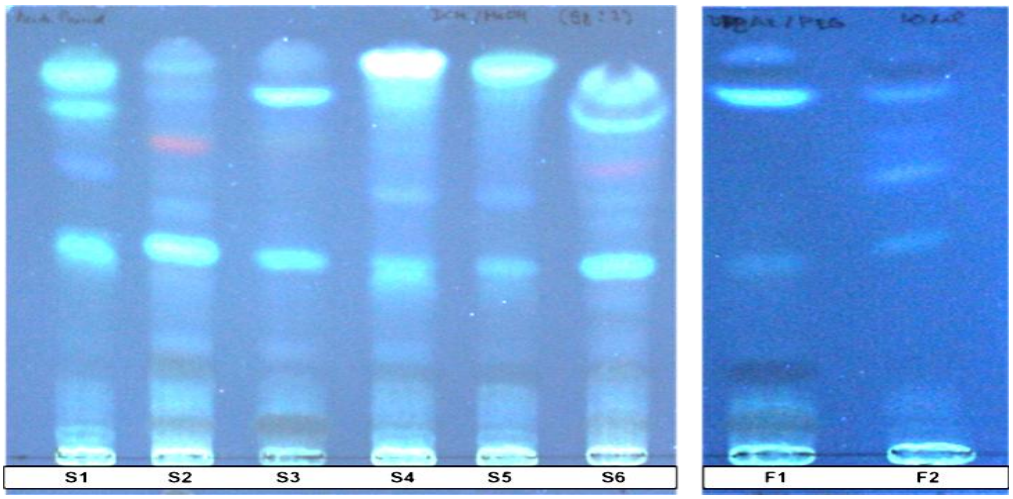

Figure 3a Saponins & Flavonoids TLC Fingerprint

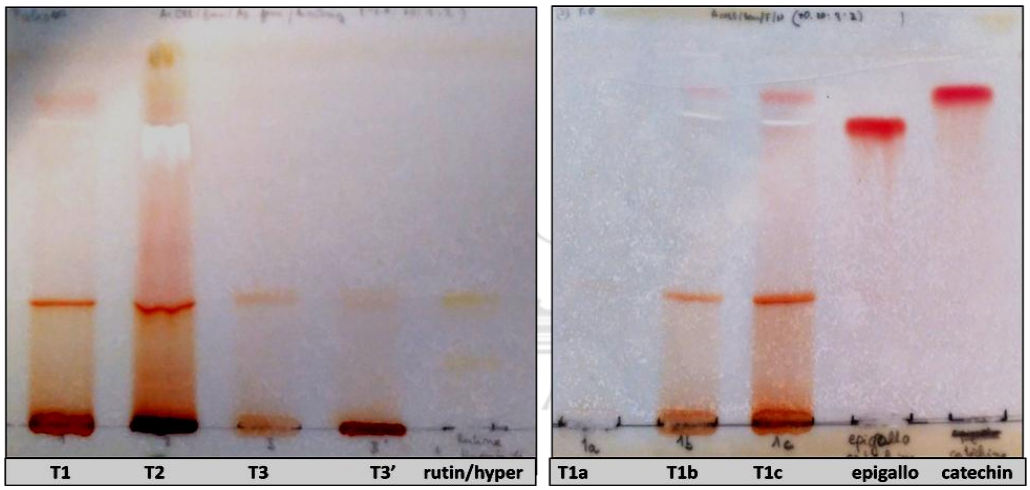

Figure 3b Tannins TLC Fingerprint

TLC conditions:

Saponins & Flavones fractions: Mobile phase: Dichloromethane/methanol (98:2 v/v). Revelation: UV 366 nm; then sprayed with sulfuric vanillin solution (1g/100 mL).

Tannins fractions: Mobile phase: Ethyl acetate/Water/Formic acid/nitric acid (70:20:3:2; v/v/v/v). Reference: (−)-Catechin and (+)-Epigallocatechin; - Solution of Rutin + Hyperoside. Spot:20-100µL. Revelation: UV at 366 nm; then sprayed with hydrochloric vanillin solution (1g/100 mL) and observed directly in the visible. The presence of catechic derivatives is marked by pink spots after revelation with hydrochloric vanillin.

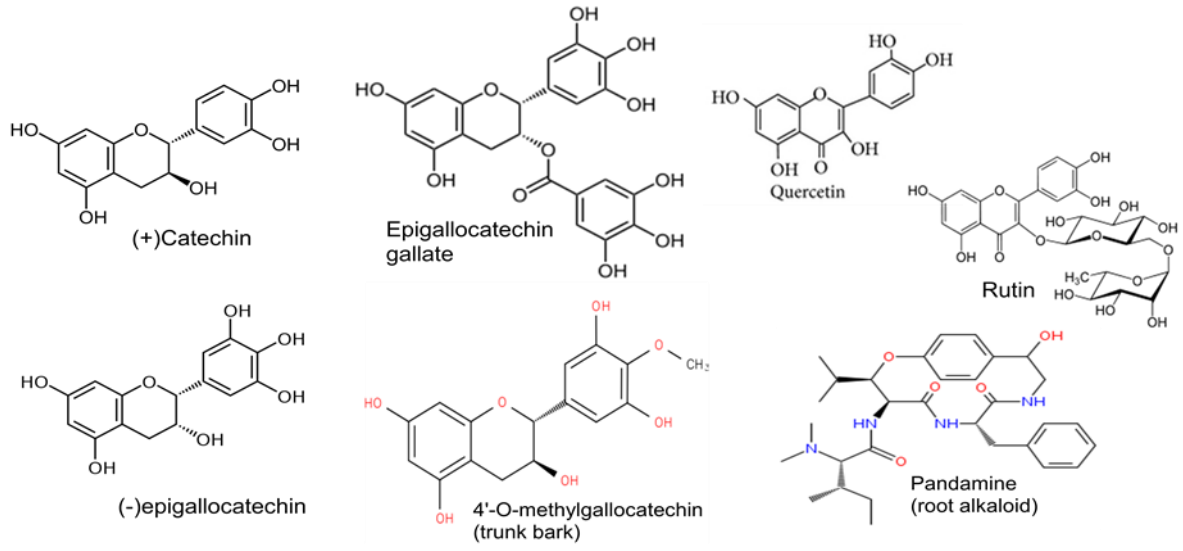

Figure 4 Possible active second metabolites from *P. oleosa*

4. DISCUSSION

As indicated in the introduction, this study aimed to evaluate the ability of *P. oleosa* stem bark extract to trap free radicals (antioxidant activity) to explain the mechanism of antidiabetic activity, to determine the toxicity of the animal to guarantee the limits of toxicity and to trace the fingerprint of the TLC chromatography for identification.

The pathogenesis of many diseases such as cancer, diabetes and mental illnesses, to name a few, has been associated with the production of highly reactive free radicals related to oxidative stress. Thus, the use of substances capable of capturing these radicals or blocking their production has emerged as an excellent strategy for treating or preventing these diseases [28]. The efficacy of plant extracts in the treatment of various diseases has been attributed to their second polyphenolic metabolites which have such a potential for scavenging, including flavonoids and tannins [29-33]. In the present study, the three classes of second metabolites have a radical elimination capacity. For the trapping of the DPPH radical, the total polyphenol extract is more active (EC_{50} = 72 µg / ml) than flavonoids (EC_{50} = 112), tannins (EC_{50} = 420) and quercetin taken as reference (EC_{50} = 188). The IC_{50} values by anti-hydroxyl test, however, were higher than the values of the DPPH test.

The anti-free radical IC_{50} values of the DPPH of *P. oleosa* polyphenols are comparable to the Catharanthus roseus values reported in the literature [30]: 83 µg / ml in the root, 90 µg / ml in the stem and 120 µg / ml in the leaves. Catharanthus roseus contains significant amounts of

phenolic and volatile compounds, including flavonol glycosides and caffeoylquinic acids, known for their high antioxidant activity. *P.oleosa* also contains interesting antioxidant substances.

According to many studies, the chemical structure of the substance depends on the chemical structure of the given flavonoid [34, 35]. The number, positions and types of substitutions on the flavan nucleus influence the activity of trapping and radical chelation. Multiple hydroxyl groups give the molecule important antioxidant, chelating and pro-oxidant activity [34, 35]. Methoxy groups introduce adverse steric effects and increase lipophilicity and partitioning of the membrane. A double bond and a carbonyl function in the heterocycle or polymerization of the nuclear structure increase the activity by providing a more stable flavonoid radical by electron conjugation and delocalization. Thus, the diversity and multiple mechanisms of flavonoid action, as well as the numerous methods of analysis, plausibly explain the existing divergences in antioxidant capacity levels of different plant extracts [34,35].

The WHO classification [36] of acute oral toxicity based on the determination of LD50 in rats defines the following classes: Ia with an LD50 <5 mg / kg body weight; Class Ib with a dose of 5 to 50 mg / kg; Class II with dose between 50 and 2000 mg / kg; Class III with a dose greater than 2000 mg / kg. The extract of *P.oleosa* with an LD50 of about 7892 ± 1262 mg/kg is class III or "practically non-toxic". Examination of the autopsy revealed dark organs due to haemorrhage. Saponins are known for their ability to induce hemolysis of red blood cells, which can contribute to toxicity at very high doses.

The TLC fingerprint revealed the complex composition of these secondary metabolites. Fractions S1-S6 showed that at least 8 products can be present. Fractions F1 and F2 showed the presence of at least 4 products. Two spots corresponding to 4'-O-methylepigallocatechin and catechin were detected in the tannin fraction, which corresponds to what was found by others. Previous studies of *P.oleosa* by other researchers have identified 4'-O-methylepigallocatechin from bark of the trunk [37] and pandamine of the roots [38]. The first is the fused tannin nucleus with anti-HIV activity in vitro and the second is a new class of peptide alkaloids with a mild laxative effect. Our study shows that *P.oleosa* contains small amounts of rutin. In addition, the chemical study found in the literature indicates that the seeds contain about 50% oil on a dry matter composed of myristic acid 1%, palmitic acid 26%, stearic acid 6%, arachidic acid 0.5%, oleic acid 33.5% and linoleic acid 32.5% [13].

Therefore, the plant contains a mixture of components that need to be isolated and characterized.

5. CONCLUSION

The extracts tested have a high antioxidant effect in vitro and a low toxicity in the guinea pig. The antidiabetic potential may be much more related to flavonoids than to tannins. The acute toxicity of the aqueous extract was nil at doses <2000 mg/kg. The thin layer chromatography system developed can help identify the plant extract by the presence of at least eight possible components recognizable by their different Rf and their colors. Additional studies are needed to describe the chemical structures of all secondary metabolites and their relative bioactivities.

CONSENT

It is not applicable.

ETHICAL ISSUES

The study protocol was approved by the ethical committee of the University FMP 130/2016 and fulfilled the requirements of EEC Directive applicable to animal experiment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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