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Antihyperglycemic effect and genotoxicity of *Psittacanthus calyculatus* extract in streptozotocin-induced diabetic rats

[Efecto antihiperglicemico y genotoxico de extractos de Psittacanthus calyculatus en ratas diabeticas inducidas con streptozotocina]

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Abstract
Psittacanthus calyculatus (DC.) G. Don (Lorantaceae) is known as "Ingerto". The aerial parts are used in the treatment of diabetes and hypertension. Methanolic extract was tested with streptozotocin-induced diabetic rats. Dose of 200 mg/Kg body weight for acute experiments, as well as 200 and 400 mg/Kg for semi-chronic bioassay were used. In both experiments extract produced significant hypoglycemic activity in streptozotocin-induced rats when compared with diabetic control ($p < 0.05$). To study possible clastogenic effects of methanolic extract a mouse micronucleus test was performed (as part of the genetic toxicology trial). CD-1 white mice were administered with 200 and 400 mg /Kg of methanolic extract of *P. calyculatus* dissolved in water by intraperitoneal injection. The cytotoxic activity polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) and the induction of micronuclei in peripheral blood erythrocytes (MNPCE) was recorded with sampling times of 24, 48 and 72, h after an exposure without killing of mice. The frequency of MNPCE in the circulating blood obtained from the tail of the mouse was statistically not significant compared with its negative control animals (time zero) and the PCE/NCE ratio showed evidences of light cytotoxic activity compared with its negative control animals (time zero). Thus, in this test, the methanolic extract of *Psittacanthus calyculatus* dissolved in water did not induce chromosomal damage resulting in micronucleus formation in peripheral blood erythrocytes and showed light cytotoxic activity.

Keywords: *Psittacanthus calyculatus*; hypoglycemic effect; methanolic extract, micronucleus assay; streptozotocin.

Resumen
En la zona del bajío mexicano la planta *Psittacanthus calyculatus* (DC.) G. Don (Lorantaceae) es conocida popularmente como "ingerto". Las partes aéreas de este vegetal se utilizan para tratar enfermedades como la diabetes y la hipertensión. Se realizaron experimentos agudos y semi-crónicos en ratas diabéticas inducidas con estreptozotocina. El efecto hipoglucemiante del extracto metanólico se evaluó a dosis de 200 y 400 mg/Kg de peso. En ambos experimentos, el extracto redujo significativamente ($p < 0.05$) la glucemia en las ratas diabéticas. Para determinar los posibles efectos clastogénicos del extracto metanólico se administraron por vía intraperitoneal a ratones cepa CD-1 las dosis que mostraron actividad hipoglucemiante disueltas en agua y se llevó a cabo el bioensayo de micronúcleos en sangre periférica de ratón. La actividad citotóxica se determinó mediante el cálculo de la relación entre los eritrocitos policromáticos y los eritrocitos normocromáticos (PCE/NCE). La inducción de micronúcleos en eritrocitos de sangre periférica (MNPCE) fue el indicador de gentotoxicidad los cuales se midieron a las 24, 48 y 72 horas después de la administración del extracto. La frecuencia de micronúcleos en eritrocitos policromáticos no fue estadísticamente significativa con relación al control negativo (al tiempo 0) por lo tanto, el extracto no induce daño cromosómico. Asimismo la relación PCE/NCE mostró que el extracto metanólico fue ligeramente citotóxico a la dosis de 400 mg/Kg y a las 48 h posteriores a la administración.

Palabras Clave: *Psittacanthus calyculatus*, efecto hipoglucémico, ensayo de micronúcleos, extracto metanólico, estreptozotocina.

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INTRODUCTION

Diabetes mellitus is a global public health problem. This is the most common endocrine disorder characterized by hyperglycemia and carbohydrate, fat and protein metabolism disturbances. The mains symptoms are post-prandial sickness due to hyperglycemia, increased food ingestion due to loss appetite control, muscular proteolysis and adipose tissue lipolysis leading to severe body weight loss, glycosuria and osmotic polyuria and long-term damage to blood vessels, eyes, nerves, kidneys and the heart and lead to a reduced quality of life and life expectancy. Different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus (Thévenod, 2008; O´Keefe *et al*., 2009). The undesirable side effects of drugs currently used for the treatment of diabetes (Cheng and Fantus, 2005) and the limited access to public health services and due to traditional culture motivate patients to use alternative therapies. In Mexico, almost all population has made use of medicinal plants at least once (Argueta and Cano, 1994). Andrade-Cetto and Heinrich, 2005 reported that diabetic patients practically always use plants with or without biomedical medication.

The indiscriminate drugs consumption of vegetal origin by the population is correlated with the occurrence of diseases like renal damage and severe complications including increased hospitalizations, ketoacidosis and acute hyperglycemia (Andrade-Cetto and Heinrich, 2005). These drugs are made of vegetal extracts that constitute complex mixtures that potentially could contain a great number of substances with mutagenic and/or carcinogenic properties (Alvarez *et al*., 2004). At the moment in the scientific literature few studies exist on the mutagenic action that own the medicinal plants consumed by the population. Therefore, it is essential to assess the genotoxic implications of traditionally used medicinal plants.

Psittacanthus calyculatus is a hemiparasite shrub, which lives upon trees of the genera *Acacia, Prosopis*, *Quercus* and *Prunus* (Rzedowski and Rzedowski, 1987). *P. calyculatus* is known as "injerto" or "muerdago verdadero" in central region of México and is used for the treatment of hypertension, cicatrizing wound, purgative and hypoglycemic agent (Martínez, 1959; Rodríguez-Cruz *et al.*, 2003; Andrade-Cetto and Heinrich, 2005). Traditional usage often recommends boiling the leaves (10 g dry weight approx) in a liter of water for tea preparation (Argueta and Cano, 1994). The people diabetic sick drink this tea as well as usage water, the beneficial effects on glucose homeostasis of this herbal preparation is recognized. A hypoglycemic effect of the water extract only was demonstrated in alloxan diabetic mice (Andrade-Cetto and Heinrich, 2005). There are however a few scientific studies about the chemical composition of polar extract of *P. calyculatus*. The aim of this study was to investigate the hypoglycemic effect of methanol extracts from *P. calyculatus* in streptozotocin-induced diabetic rats in acute and sub-chronic model, to identify the main chemical constituents in the tested extract, and tries to determine if the active extract of *P. calyculatus* has or not genotoxic properties through a test of micronuclei in peripheral blood of CD1 mice.

MATERIAL AND METHODS

Plant

Plant materials were obtained from the woods of the state of Guanajuato, México, during flowering season in November 2008. *P. calyculatus* collected for this study was growing over *Prosopis juliflora*, and was authenticated by Izta herbarium of the Universidad Nacional Autónoma de México where a voucher specimen was deposited with number 42601.

Preparation of the extract

The extract was prepared by percolation successively of dried powered leaves (100 g) with hexane, ethyl acetate and methanol. The extracts were evaporated under reduced pressure and dry in an oven at 36º C. The hexanic and ethyl acetate extracts were not actives. The yield of methanolic extract gave a residue of 12 g.

Preliminar phytochemical screening

The methanolic extract was subjected to preliminary phytochemical analysis to determine the presence of secondary metabolites groups such as alkaloids, saponnins, steroids, tannins, flavonoids and anthraquinones following standard published protocols (Evans, 2002; Sampietro *et al*., 2009). Thin layer chromatography was performed using TLC Silica gel plates (Merck,Germany), 0.25 mm; elution system: n-butanol–acetic acid–water (BAW 4:1:5, upper phase); detection: 1% MeOH solution of diphenyl-boric acid-ethanolamine complex and additionally with 5% EtOH solution of PEG 400. After drying the plates were visualized under UV366. The methanolic extract (4.6 g) was subjected to open CC on Si-gel G (for thin

layer chromatography), employing a gradient elution with AcOEt (solvent A) and MeOH (solvent B), from 100% to 0% A. A total of 125 fractions were collected. Fractions AcOEt-MeOH (85:15 and 75:25), gave (+)-catechin (40 mg), and gallic acid (28 mg) respectively. (+)- Catechin and gallic acid were identified by comparison with reported physical and spectroscopic data (Nawwar *et al*., 1982; Salem *et al*., 2011). In the most polar fractions were detected a mixture of condensed tannins (1.8 g) spectrophotometrically by oxidative cleavage reaction (Sampietro *et al*., 2009).

Animals

Male Wistar rats weighing 210-230 g were used. They were housed in standard environmental conditions and fed with standard rodent diet with water *ad libitum*.

Induction of diabetes

Diabetes was induced by a single intraperitoneal injection of a buffered (0.1 M citrate, pH 4.5) solution of streptozotocin (STZ) at a dosage of 60 mg/Kg, b.w. The animals were considered diabetic if their blood glucose values were between 400 and 600 mg/dL on a 3rd day after single streptozotocin injection.

Effect of P. calyculatus methanolic extract on streptozotocin-induced hyperglycemia.

Acute treatment

After the induction of diabetes, all rats to be used for the experiments were kept in the laboratory on a normal diet for 7 days. For acute experiments, diabetic rats were divided into four groups (each, n = 6): Group 1, a diabetic control rats received orally tween 10x (10 per cent) dissolved in distilled water; Group 2, an experimental diabetic group received a subcutaneous injection of 5 units/Kg of insulin (NovoRapid®); Group 3, experimental diabetic rats received orally glibenclamide (Euglucon®) 0.1 mg/Kg. Diabetic rats of Group 4 were treated orally with 200 mg/Kg of the methanolic extract of *P. calyculatus* dissolved in tween 10x (10 per cent in distilled water). Blood samples of the all animals of each group were obtained in heparinized capillary tubes by tip of the tail puncture before and at 1, 2, 4, 6 and 8 h after treatment. The blood glucose was measured by glucose–oxidase method in a glucometer apparatus (Roche®).

Sub-chronic treatment

For sub-chronic experiments; five days after streptozotocin injection (diabetic rats), the animals were divided into four groups (each, n = 6): Group 5 diabetic control rats received orally tween 10x (10 per cent) dissolved in distilled water; Group 6 diabetic rats treated with 200 mg/Kg/day of extract suspended in tween 10x; Group 7 diabetic control rats received a treatment equal at group 5; Group 8 diabetic rats treated with 400 mg/Kg/day of extract suspended in tween 10x. The controls and experimental diabetic rats received tween 10x or extracts orally using mesogastric cannula, once day, during 5 days. Blood samples for glucose measurements were collected from tip of the tail before and at 2, 4, 6 and 8 h after treatment each day. The blood glucose was measured by glucose–oxidase method in a glucometer apparatus (Roche®).

Statistical analysis

The results are expressed as means ± S.D. The significance of the results was calculated using Student's *t*-test and were considered statistically significant when $P < 0.05$.

Mouse micronucleus test with peripheral blood

Extract sample

The methanolic extract of *P. calyculatus* was dissolved in distilled water under sterile conditions before the treatment of the animals. All treatments were done by single intraperitoneal injection. The injection volume was 0.2 mL/10 g body weight.

Animals

Healthy (4-7 weeks) males of *Mus musculus* (CD1), weighing 25-30 g, obtained from bioterio of Facultad de Estudios Superiores Iztacala-UNAM, were used in the studies. All animals were brought to the laboratory 7 days before the experiments. The mice were housed in plastic cages (35x 24x 14 cm) at 21 ± 2 °C, with a natural light-dark cycle of 12 h. Food and water were provided *ad libitum.*

Experimental procedure (micronucleus test)

For the micronucleus assay, 40 male mice were selected and distributed randomly into four groups of ten animals each. The animals were treated as follows: group 1 (positive control) received 40 mg/Kg intraperitoneally of ifosfamide (Ifoxan, Baxter Oncology) (Krishna *et al*., 2000); group 2 received distilled water; two groups received the following treatment with 200 and 400 mg/Kg of methanolic extract of *P. calyculatus*, respectively. The doses used

for this study were those that showed antihyperglycemic effect.

The assay was carried out following standard protocols (Hayashi *et al*., 2000; Rosas *et al*., 2006). Blood samples were drawn from the caudal vein of each mouse and slides prepared in triplicate prior to the administration of the test compounds (time zero), and at 24, 48 and 72 h after administration. Drops of blood were spread on pre-cleaned standard glass microscope slides, air dried, and fixed in absolute methanol for 5 min. All slides were coded prior to scoring by a person not involved in reading the slides. The smears were stained with Giemsa 5% for detecting micronucleated polychromatic (MNPCE). For each animal, three slides were prepared and 2000 polychromatic erythrocytes (PCE) were counted to determine the frequency of MNPCE. To determine the cytotoxic activity of the plant extract, 2000 normochromatic erythrocytes (NCE) were counted, as well as the frequency of PCE within the same microscope fields. The PCE/NCE ratio was then calculated. The slides were analyzed using a Nikon Labophot-2 microscope (1000 X). Slides were decoded only after all slides from an experiment had been read.

The values were expressed as percentage of toxicity (% T) of the total erythrocyte counts to determine a reduction of erythroblast proliferation. The percentage of genotoxicity (% G) was calculated by counting a total of 2000 PCEs per animal, the % G was examined for the presence of micronuclei.

Statistical analysis

Results expressed as mean ± standard deviation. Data were compared using multifactorial analysis of variance (ANOVA) and the Tukey HSD test. The factors of variation were doses and time after administration of the extract. The dependent variables were the ratios PCEs/NCEs (% T) and MNPCEs/PCEs (% G). A Statistical Analysis System (Minitab version V.15) was employed for the analyses, and the level of significance was $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical screening

The qualitative phytochemical screening of the extract showed the presence of tannins, flavonoids and phenylpropanoids. The extract was negative for anthraquinones, steroids and alkaloids detection tests. In this study, two spots were detected by TLC in the methanolic extract with characteristic phenolic colour (Rf = 0.45 and 0.7).

When we fractioned the methanolic extract, the main compounds isolated were: firstly to the class of condensed tannins, and secondly, a series of metabolites identified as gallic acid and (+)-catechin. These studies correlate with those made by Bah *et al*., 2011, which also gallic acid and (+)-catechin were isolated of this specie. Furthermore, they isolated two flavonol-3-biosides and a nonprotein amino acid *N*-methyl-*trans*-4-hydroxy-L-proline.

Loranthaceae are usually regarded as autotrophic, depending on their host for water and inorganic nutrients only, although even in these mistletoes there is evidence for carbon uptake from the host (Norton and Carpenter, 1998). There are no studies demonstrating that *P. calyculatus* obtains organic compounds from the hosts, therefore it is questionable whether there is variation in the chemical composition of *P. calyculatus* regarding the chemical composition of the host.

Assessment of hypoglycemic activity on STZ diabetes rats

In this study, streptozotocin administration induced significant hyperglycemia at blood glucose level (> 400mg/dL) in all groups of rats. In acute experiment (Table 1), diabetic rats treated with 200 mg/Kg of methanolic extract of *P. calyculatus* showed a significant reduction ($P < 0.05$) of glucose level at 547 mg/dL (basal) to 370 mg/dL after 8 h of treatment. As expected, the administration of insulin induced significant hypoglycemia (< 250 mg/dL) in rats throughout of the experiment. No hypoglycemic effect showed the glibenclamide experiment. Because it was evident the reduction of glucose in rats treated with methanol extract of *P. calyculatus* in acute experiment, it was performed semi-chronic experiments. In this case the extract was administered every 24 h for 5 days, and blood glucose levels was measured similarly to the acute experiment. After of daily treatment with the methanolic extract (200 and 400 mg/Kg) there was significant fall in blood sugar level after 6-8 h of each oral administration of extract (Figures 1a and 1b), this effect was constant throughout of the experiments. It was also noted that the reduction was greater when administered in doses of 400 mg/Kg, and blood glucose levels were lower on the fourth day of experiment.

Table 1
Effect of glibenclamide, insulin and *P. calyculatus* methanolic extract (200 mg/Kg) on blood glucose levels in STZ diabetic rats. Number in the parentheses denotes percentage of reduction.

	Blood glucose levels (mg/dL)					
Diabetic rats	**Time (h)**					
	0	1	2	4	6	8
Control	494.7 ± 35. 6	469.3 ± 30.7	496.7 ± 44.7	510.7 ± 60.1	508.0 ± 52.7	516.0 ± 50.0
Glibenclamide	482.4 ± 24.6	481.8 ± 23.0 (0.1)	485.4 ± 22.9	474.4 ± 35.7 (0.2)	474.2 ± 31.0 (0.2)	481.8 ± 37.0 (0.1)
Insulin	498.0 ± 47.6	253.8 ± 61.6* (49)	229.3 ± 48.0* (54)	164.0 ± 35.8* (67)	193.5 ± 37.2* (61)	276.5 ± 13.6* (44)
P. calyculatus	546.7 ± 25.2	566.7 ± 28.8	553.3 ± 13.7	552.3 ± 7.2	409.3 ± 9.0* (25)	370.0 ± 13.2* (32)

Mean ± SD, n = 6 in each group, *p < 0.05 statistical significance compared to time zero

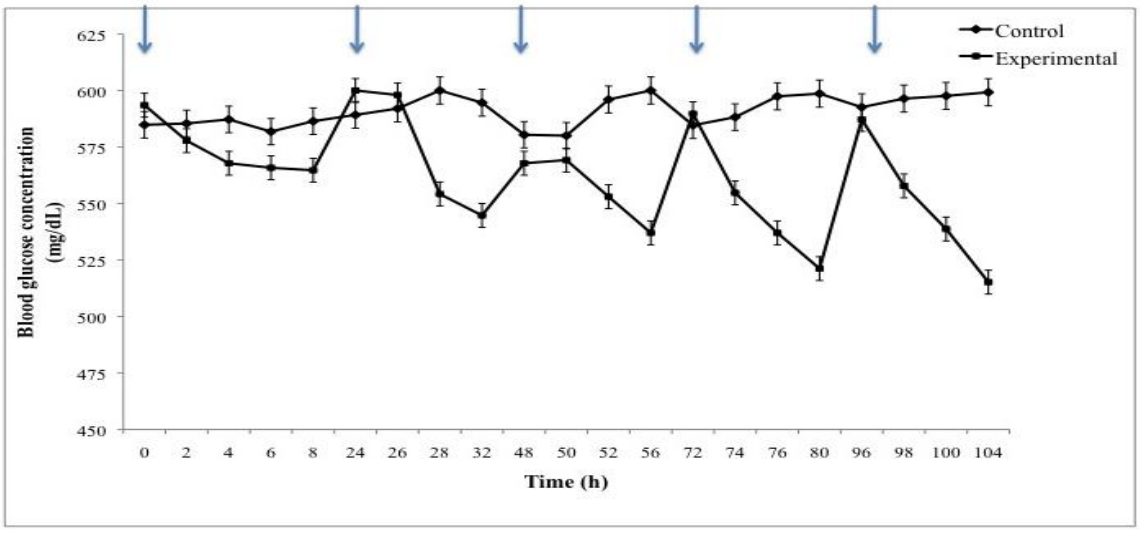
P. calyculatus methanolic extract was shown to exhibit an antihyperglycemic activity in streptozotocin-induced diabetic rats. The hypoglycemic activity *of P. calyculatus* could be due to phenolic compounds such as condensed tannins, catechin and gallic acid (Mei-Hsiang *et al*., 2011; Zhaolian *et al*., 2011; D'Andrea, 2010). Further studies are necessary to elucidate in detail the mechanism of action of the plant extract and/ or isolate compounds at the cellular and molecular levels.

Assessment of the mouse micronucleus test with peripheral blood

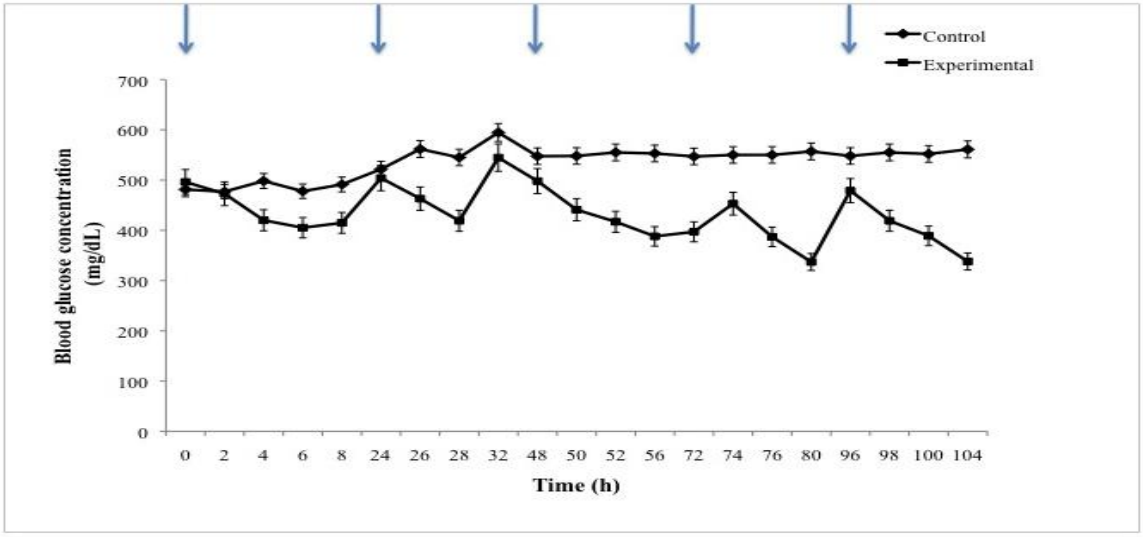
In order, an assessment of their cytotoxic and mutagenic potential of *P. calyculatus* methanolic extract was necessary to ensure a relatively safe to use of medicinal plant. The applied doses of 200 and 400 mg/Kg body weight were compared with the negative control (time zero- distilled water). The present results indicate that methanolic extract showed evidences of light cytotoxic activity with respect to negative control ($p < 0.05$) but did not induce a mutagenic or genotoxic effects (Figure 2), since not registered significant differences. This showed that the frequency of micronuclei was very low or null in the polychromatic cells of CD1 mice. This indicates that the extract had no a clastogenic effect and therefore did not turn it into a mutagenic agent. The lack of clastogenic activity of methanolic extract of *P. calyculatus* after intraperitoneal injection may be due to detoxification, lack of metabolic activation, and/or lack of distribution of the chemical components of methanolic extract to mice bone marrow. It is possible that common mechanisms of action of the reticuloendotelial system particularly the spleen and mouse bone marrow were able to neutralize the administered chemical agent (MacGregor, 1990). The spontaneous incidence of MNPCEs in CD1 mice (collected from all mice at 0 h) were 0.04%. The maximum frequencies of MNPCEs were observed at 48 h (positive control) after treatment of a single intraperitoneal injection of ifosfamide in CD1 mice, thus, demonstrating the reproducibility of positive control response in the MN assay in our laboratory. That helps verify staining and scoring approaches (Wild, 1978).

Figure 1
Effect of *P. calyculatus* methanolic extract on blood glucose level of STZ diabetic rats after 5 days treatment.

a) ↓Administration of 200 mg/Kg the extract



b) ↓Administration of 400 mg/Kg the extract

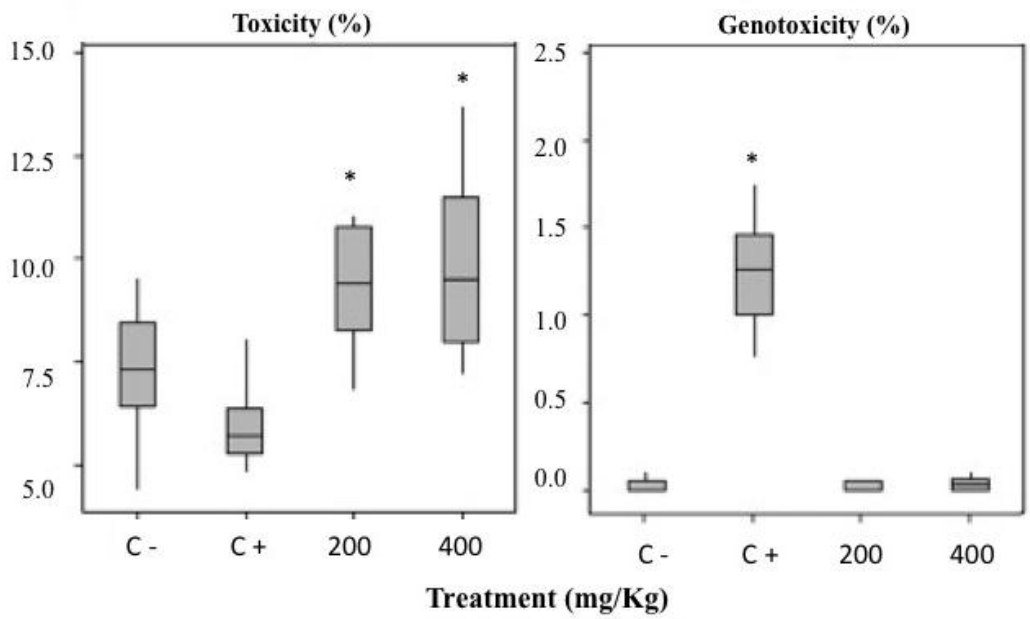


The degree of toxicity of the concentrations was another variable to compare. The results shown the dose of 400 mg/Kg of the methanolic extract of *P. calyculatus* is slightly toxic with respect to the dose of 200 mg/Kg (Table 2). It was more evident to 48 hours of exhibition where there is a significant increase of the means values, compared with the exposure times 24 and 72 hours ($p < 0.05$). On the other hand, the exposure times also were a variable important to know the degree toxicity of the extract. The toxic effect of the highest dose (400 mg/Kg) of methanolic extract was time-dependent because induced maximum percentage of toxicity 48 h after treatment (Figure 3). The present results demonstrate a light toxicity only in the exposure times of 48 hours on the sanguineous cells of CD1 mouse.

The results shown *P. calyculatus* methanolic extract is not clastogenic to polychromatic erythrocytes of the peripheral blood of CD1 mice. Therefore the methanolic extract cannot be considered

like a mutagenic agent. Further studies should be carried out to evaluate the safety of the compounds from *P. calyculatus* in other animal models.

Figure 2
Frequencies of PCE/NCE ratio (Toxicicty %) and MNPCE (Genotoxicity %) in peripheral blood erythrocytes of CD1 mice after treatment whit 200mg/Kg and 400 mg/Kg of *P. calyculatus* methanolic extract.



Mean ± SE, n = 10 in each group, *p < 0.05 statistical significance compared to negative contro

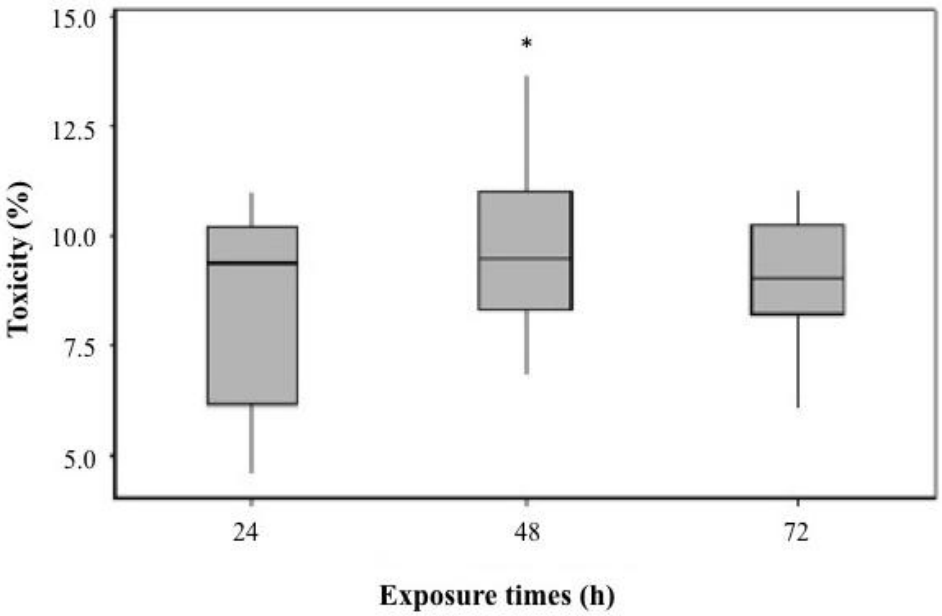
Table 2
Toxicity rate (% T) in CD1 mice peripheral blood erythrocytes after the exposure whit 200mg/Kg and 400 mg/Kg of *P. calyculatus* methanolic extract.

Variable	Treatment	Mean	SE Mean	STDev	Coef Var
(%)T	200 mg	8.66	0.36	1.97	22.8
	400 mg	9.34	0.31	1.715	18.35

CONCLUSION

The methanolic extract showed antihyperglycemic effect in streptozotocin-induced diabetic rats in both acute and semi-chronic experiments. In addition, this extract did not cause genotoxic effects in peripheral blood erythrocytes of mice. The main components of the active extract were condensed tannins, (+)-catechin and gallic acid.

Figure 3
Frequencies of PCE/NCE ratio in peripheral blood erythrocytes of CD1 mice after treatment (24, 48 and 72 h) whit 400 mg/Kg of *P. calyculatus* methanolic extract.



Mean ± SE, n = 10 in each group, *p < 0.05 statistical significance

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