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Teratogen Potential Evaluation of the Aqueous and Hydroalcoholic Leaf Extracts of *Crataegus oxyacantha* in Pregnancy Rats

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Abstract: *Crataegus Oxyacantha* is used in the treatment of cardiovascular diseases. In related to your biosafety, only *in vitro* and *in vivo* genotoxicity of the fruit and the leaf is described, however, the teratogenic potential is unknown. The aim this study was evaluating the transplacental genotoxicity effect of aqueous and hydroalcoholic extract of leaves *C. oxyacantha* in a rat model and the quantification of malondialdehyde (MDA) in liver. Three different doses of the aqueous and hydroalcoholic extracts of the *C. oxyacantha* leaf were administered orally (500, 1000 and 2000 mg/kg) to Wistar rats during 5 days through the pregnancy term (16-21 days), sampling in rats were every 24 h during the last 6 days of gestation and only one sample was taken in neonates at birth. A sample of the mother's and neonate's liver was taken for the determination of MDA. The results show that, at the hepatic level, the evaluated doses of extracts *C. oxyacantha* in pregnant rats and their pups did not show cytotoxicity. However, the aqueous and hydroalcoholic extract generated cytotoxic and genotoxic damage in the short term. On the other hand, only the aqueous extract showed a teratogenic effect. Based on these results, the aqueous and hydroalcoholic extracts of the *C. oxyacantha* leaf should not be administered during pregnancy.

Keywords: *Crataegus oxyacantha*; teratogen potential; micronuclei; Malondialdehyde

1. Introduction

The World Health Organization (WHO) has reported that around 80% of the world´s population depends on the use of medicinal plants [1]. The study of plants for medicinal purposes consists of different steps in their preclinical stage, such as the selection of plants to be investigated, correct botanical identification, phytochemical characteristics, pharmacological and toxicological studies [2].

Tests for the detection of agents that damage DNA are of great importance since genotoxic compounds can alter the genetic material in organisms [3], which can manifest itself in teratogenic effects, germ cell mutations, influence aging processes [3,4], and induce somatic cell mutations that can lead to cancer development [4–7].

When the damage is generated in pregnancy, the compound is called a teratogen [8], since it can alter the genetic material, causing mutations in somatic and germ cells [9]. Various chemical agents can cause damage at birth, whether physiological or biochemical, at any stage of development of the fetus, causing either uterine death, abortion, premature birth, and neonatal poisoning [10].

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The teratogenic potential is associated with the formation of micronucleus [11,12]. Any compound that can cross the placental barrier and induce micronucleated erythrocytes in the fetus is considered a potential teratogen [10].

The micronucleus technique allows us to determine the ability of a compound to generate chromosomal damage (clastogenic or aneugenic) in the prenatal period, when the mother has been exposed to it, which would lead to a mutagenic risk [13,14].

Among the plants with medicinal purposes is *C. oxyacantha*, which is a shrub, a member of the Rosaceae family [15].

Used since an ancient time mainly in cardiovascular diseases [16–23], likewise, its activity has been described as a lipid-lowering [24–26], immunomodulator [27,28], hepatoprotective [29–31], anti-inflammatory [32,33], antioxidant [32–37], antimicrobial [33,37,38], anxiolytic and antidepressant [39,40]. These have been associated with the different types of flavonoids that are present in the leaf, bark, fruit, and flowers of *C. oxyacantha.* However, these also have a close relationship with the toxicological potential of the plant. According to the reported studies, the toxicological profile of *C. oxyacantha* has not yet been fully established, since only the genotoxicity and cytotoxicity of the fruit have been described both in vivo and in vitro, as well as the average lethal dose of the leaf. Therefore, the present study aims to determine the teratogenic potential of the aqueous and hydroalcoholic extract of the leaves of *C. oxyacantha* in Wistar rats and their babies.

2. Results

2.1. Phytochemical analysis leaf of C. oxyacantha.

The presence of flavonoids, tannins and quinines were identified by phytochemical analysis (Table 1).

The hydroalcoholic and aqueous leaf *C. oxyacantha* extract showed the presence of derivatives of gallic acid and catechols, compounds with the γ-benzopyrone nucleus (flavones, flavonols, flavanones, flavanonols, isoflavonoids and xanthones) and anthraquinones. The hydroalcoholic extract showed the presence of anthrone derivatives.

Table 1. Results of the phytochemical analysis of leaf of *C. oxyacantha.*

Test		Leaf	
		Hydroalcoholic Extract	**Aqueous Extract**
Flavonoids	Shinoda	-	-
	$HCl_{(c)}$		
	NaOH	+	+
	Gelatin	-	-
Tannins	$FeCl_3$	Green +	Green +
		Black +	Black +
	Potassium ferrocyanide	-	-
	NH_4OH	+	+
Quinines	H_2SO_4	-	-
	Bornträger reaction	Yellow +	-

+ (present), − (Absent).

2.2. Proportions of polychromatic erythrocytes (PCEs) and micronucleated polychromatic erythrocytes (MNPCEs) in pregnant rats

The results of proportions of PCEs and MNPCEs of the aqueous and hydroalcoholic leaf extracts of *C. oxyacantha* in pregnant rats of the Wistar strain are shown in Table 1.

The results showed that the negative control (sterile water) did not present significant changes in the proportion of PCEs and MNPCEs at different sampling times to its baseline value (0 h). In contrast, the positive control (CP) decreased the proportion of PCEs significantly at 120 hours and statistically significantly increased the proportion of MNPCEs at 96 and 120 hours with respect to its baseline value.

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Similarly, the aqueous and hydroalcoholic leaf extracts of *C. oxyacantha* decreased the PCEs in the three doses evaluated (Table 2). The 2000mg/kg dose of the aqueous extract showed a significant decrease to its basal value from 24 to 120 hours, likewise, the dose of 2000mg/kg dose of the hydroalcoholic extract showed a decrease from 72 to 120 hours. The 1000mg/kg dose of the aqueous and hydroalcoholic extracts decreased this proportion statistically significantly from 48 to 120 hours, the 500mg/kg dose of the hydroalcoholic extract showed a statistical decrease at 48, 96 and 120 hours, however, the 500mg/kg dose of the aqueous extract only showed a significant decrease in this proportion at 120 hours.

Regarding the proportion of MNPCEs, between the aqueous and hydroalcoholic extract of the *C. oxyacantha* leaf, only at doses of 2000mg/kg showed a significant increase to its basal value, the aqueous extract at 72 and 96 hours (p-value = 0.002 and 0.025, respectively), and the hydroalcoholic extract only at 72 hours (p-value = 0.011).

Table 2. Number of PCEs and MNPCEs at different sampling times in the study groups in pregnant rats.

PCEs/1000 TEs								
			0 h	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**
Controls		**SW**	30.40 ±4.33	29.80±5.16	28.00±3. 53	30.40±3.57	30.20±2.58	28.60±2.70
		p-value	***-----***	1.00	1.00	1.00	1.00	1.00
		CP	53.00 ±5.35	52.00±5.35	55.00±9.27	52.00±12.30	46.75±14.50	12.00±4.54
		p-value	***------***	1.00	0.610	1.00	0.346	**0.0001**
C. oxyacantha	**Aqueous Ext of leaf**	**2000 mg/kg**	39.40±2.19	31.20±2.38	28.00±2.91	25.00±2.34	22.60±3.28	22.40±3.50
		p-value	***------***	**0.002**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
		1000 mg/kg	40.20±4.38	34.40±3.64	31.00±1.58	29.00±2.54	32.40±4.03	29.40±4.92
		p-value	***------***	0.065	**0.0001**	**0.0001**	**0.033**	**0.002**
		500 mg/kg	38.00±4.41	40.20±4.91	33.60±2.96	34.40±2.79	35.00±2.34	27.80±3.03
		p-value	***------***	1.00	0.172	1.000	1.000	**0.004**
	Hydroalcoholic Ext	**2000 mg/kg**	39.80±3.83	38.00±2.91	36.00±2.12	32.60±3.04	28.80±1.92	24.80±1.64
		p-value	***------***	1.00	0.405	**0.025**	**0.001**	**0.0001**
		1000 mg/kg	41.40±2.60	37.20±2.16	34.20±1.09	31.00±1.58	27.20±3.49	24.60±2.50
		p-value	***-------***	0.498	**0.002**	**0.0001**	**0.0001**	**0.0001**
		500 mg/kg	49.60±2.07	44.00±2.91	44.20±5.63	44.40±2.96	40.20±1.09	39.20±2.48
		p-value	***------***	0.085	**0.037**	0.278	**0.005**	**0.003**
MNPCEs /1000 PCEs								
Controls		**SW**	3.20 ± 1.48	4.00 ±1.00	3.40 ±1.51	3.00 ±1.22	2.80 ± 1.09	4.20 ± 0.44
		p-value	***-----***	1.00	1.00	1.00	1.00	1.00
		CP	5.00±0.81	5.50 ±1.73	6.75 ± 1.70	6.000 ±2.58	10.75± 2.21	18.50 ±4.35
		p-value	----	1.00	0.446	1.00	**0.0001**	**0.0001**
Leaf of C. oxyacantha	**Aqueous Ext.**	**2000 mg/kg**	2.60±0.89	3.40±0.89	4.40±1.67	5.20±1.48	5.00±2.12	3.60±1.34
		p-value	***-----***	1.00	0.202	**0.002**	**0.025**	1.00
		1000 mg/kg	3.00±1.22	4.20±1.64	4.80±1.92	3.60±0.54	5.00±2.34	4.20±2.16
		p-value	***-----***	0.567	0.202	1.00	0.111	1.00
		500 mg/kg	2.80±1.09	3.00±0.70	3.80±1.30	4.40±0.89	2.40±1.14	1.40±0.54
		p-value	***-----***	1.00	1.00	0.155	1.00	1.00

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	Hydroalcoholic Ext.	**2000 mg/kg**	2.40±0.54	2.20±1.30	3.00±1.00	4.60±0.89	3.60±0.54	3.00±1.22
		P value	-----	1.00	1.00	**0.011**	1.00	1.00
		1000 mg/kg	4.00±0.70	3.60±1.34	2..80±0.83	3.20±1.30	2.40±0.89	2.80±1.30
		p-value	----	1.00	1.00	1.00	0.433	1.00
		500 mg/kg	2.60±1.14	2.20±0.44	2.60±0.89	3.20±1.30	4.00±1.22	3.20±1.30
		p-value	-----	1.00	1.00	1.00	0.806	1.00

The results are expressed as mean ± standard deviation. Comparisons were made between each group and their respective baseline value (0h), using the analysis of variance (ANOVA) for repeated means and the Bonferroni adjustment test was used for multiple post hoc comparisons, it was considered statistically significant when p <0.05. SW: Steriel wáter; CP: Cyclophosphamide; Ext: extract; PCEs: polychromatic erythrocytes: TEs: total erythrocytes; MNPCEs: micronucleated polychromatic erythrocytes; h: hour.

2.3. Proportion of PCEs, MNPCEs and micronucei (MNs) in neonates of rats

The teratogenic potential was evaluated in the peripheral blood of the neonates of rats exposed and not exposed to the aqueous and hydroalcoholic leaf extract of *C. oxyacantha* by the MN test. The results obtained are presented in Table 3.

Concerning to the proportion of PCEs, only the neonates of the rats exposed to CP and the dose of 2000mg/kg of the aqueous leaf extract of *C. oxyacantha* showed a statistically significant decrease to the negative control with a *p-value*=0.0001 (Table 2).

The proportion of MNPCEs in the neonates of rats exposed to the leaf extracts of the *C. oxyacantha* showed a dose-dependent increase, which is more noticeable in the aqueous extract. The neonates of the rats exposed to CP and the 2000mg/kg dose of the aqueous leaf extracts of *C. oxyacantha* showed a statistically significant increase to the negative control proportion with a *p-value* =0.0001. In contrast, the neonates of rats exposed to the 500mg/kg dose of hydroalcoholic leaf extract of *C. oxyacantha* showed a significant decrease in this proportion.

The proportion of MNEs obtained from neonates exposed to CP compared to neonates not exposed rats (negative control) showed a statistical increase of this proportion (p-value= 0.0001). The neonates of rats exposed to the aqueous leaf extract of *C. oxyacantha* showed results very similar to the three doses evaluated in the neonates of the not exposed rats, for which no significant differences were found between them. In contrast, the neonates of rats exposed to the evaluated doses of hydroalcoholic leaf extracts showed a statistically significant decrease in this proportion to the neonates of the not exposed rats (Table 2)

Table 3. Proportion of PCEs, MNPCEs, and MNEs in peripheral blood of rat neonates exposed and not exposed to the aqueous and hydroalcoholic leaf extracts of *C. oxyacantha.*

		Number of newborns	PCEs/1000 TEs	MNPCEs /1000 PCEs	MNEs /10000 TEs
Controls	Negative control (SW)	30	678.56 ±72.57	4.87±1.35	7.00±2.01
	Positive control (CP)	30	500±93.23	27.07±10.63	13.33±5.33
	p-value		**0.000**	**0.000**	**0.000**
Aqueous Ext of	2000 mg/kg	30	532.66±84.48	8.30±1.98	8.17±1.70
	p-value		**0.000**	**0.000**	0.384
	1000 mg/kg	30	618.70±78.05	6.03±1.92	6.73±1.63
	p-value		0.083	0.208	1.000
	500 mg/kg	30	658.30±95.59	5.50±1.30	7.43±1.87

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	Valor de p		1.00	0.835	1.000
Hydroalcoholic Ext. of	2000 mg/kg	30	636.70±98.84	4.40±1.07	5.40±1.65
	p-value		0.813	0.977	**0.038**
	1000 mg/kg	30	652.73±80.84	4.50±1.35	5.27±1.66
	p-value		0.995	1.000	**0.017**
	500 mg/kg	30	661.06±107.06	3.23±0.93	4.23±1.38
	p-value		1.00	**0.000**	**0.000**

The results are expressed as mean± standard deviation. Intergroup comparisons were made with respect to the negative control values, by means of the one-way analysis of variance (ANOVA) and the Dunnett´s adjustment test was used for multiple post hoc comparisons, it was considered statistically significant when p <0.05. Ext: extract; PCEs: polychromatic erythrocytes: TEs: total erythrocytes; MNPCEs: micronucleated polychromatic erythrocytes; MNEs: micronucleated erythrocytes; SW: Sterile water; CP: Cyclophosphamide.

2.4. Hepatic peroxidation

In Figure 1 shows the results obtained on the concentration of MDA at the liver level in rats at the term of gestation. The group treated with CP showed the highest concentration of MDA in the liver compared to the other groups evaluated.

The aqueous extract leaf of *C. oxyacantha* tends to increase the concentration of MDA as the dose increases, in contrast, the groups treated with the hydroalcoholic extract of *C. oxyacantha* show a tendency to decrease MDA as the dose increases, not being significant statistically for these differences.

When comparing the three evaluated doses of the aqueous and hydroalcoholic extracts to the CP group, the medium and low dose of the aqueous and the high dose of the hydroalcoholic leaf extract of *the C. oxyacantha* showed a statistically lower concentration of MDA compared to the CP group (p-value=0.047, 0.047 and 0.009, respectively).

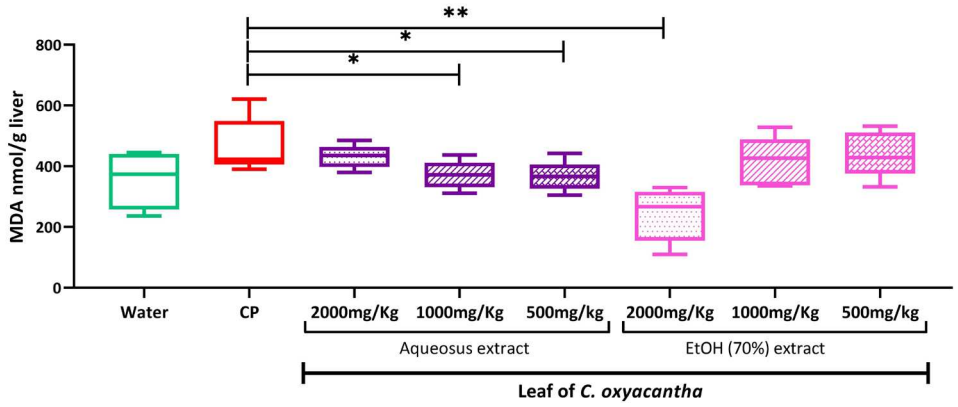

Figure 1. MDA concentration in the liver of Wistar rats exposed to aqueous and hydroalcoholic extracts of the *C. oxyacantha* leaf at the end of pregnancy. The results are expressed as median with minimum and maximum. Intergroup comparisons were made using the Kruskall Wallis analysis with Dunn's post hoc and were estimated to be statistically significant when * $p < 0.05$ and ** $p < 0.001$. MDA: malondialdehyde; nmol; nanomole; g: grams; kg: kilograms.

In the Figure 2 shows the MDA concentration in the liver of neonates of rats exposed to the different doses of the aqueous and hydroalcoholic extract leaf of *C. oxyacantha.*

The neonates of mothers exposed to CP presented a higher concentration than that presented by the neonates of mothers exposed only to water, this difference being statistically significant (p-value= 0.032). The MDA concentrations of the neonates of the rats exposed to the 2000 and 1000mg/kg doses of the aqueous extract were statistically lower than those of the neonates of mothers exposed to CP

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(p-value=0.028 and 0.027, respectively). Similarly, the neonates of rats exposed to the 2000mg/kg dose of the hydroalcoholic extract also presented MDA concentrations lower than those of the neonates of rats exposed to CP (p-value=0.0014).

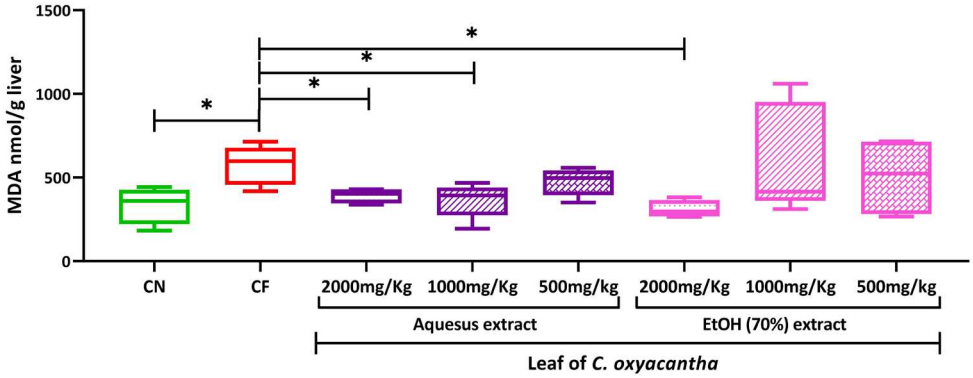

Figure 2. MDA concentration at a hepatic level in neonates of Wistar rats exposed to different doses of aqueous and hydroalcoholic leaf extracts of *C. oxyacantha*. The results are expressed as median with minimum and maximum. Intergroup comparisons were made using the Kruskall Wallis analysis with Dunn's post-hoc and were estimated to be statistically significant when *p<0.05. MDA: malondialdehyde; nmol; nanomole; g: grams; kg: kilograms.

3. Discussion

According to the WHO, approximately 80% of the world population resorts to the use of medicinal plants, however, there is a great gap in the knowledge of the chemical compositions, mechanism of action, as well as the safety and efficacy of these these [41–43].

Pregnancy is a condition that should be considered a time of minimal medical intervention, even in the consumption of plant-based products. Since it has been described that a wide variety of congenital deformities usually occur in the fetus during the period of organogenesis [44]. Mainly, it is because xenobiotic-metabolizing enzymes are induced during pregnancy, which can increase the metabolism of secondary metabolites that are substrates of these enzymes, causing intoxication by them [45].

The MN test in peripheral blood allows the cytotoxicity and genotoxicity of an agent to be evaluated, based on the decrease in the number of PCEs and the increase in MNPCE in peripheral blood [46].

It has been described that the presence of MN in the peripheral blood of neonates can assess the teratogenic potential of xenobiotics administered during pregnancy, since it has been shown that many genotoxic compounds have teratogenic potential and, in turn, could involve various mechanisms of teratogenicity [47,48]. MN is easily observable in erythrocytes obtained from newborn rats, due to the immaturity and hypofunctionality of the neonatal spleen [49].

The teratogenic potential was evaluated in newborn rats, which were exposed to the different doses evaluated of the aqueous and hydroalcoholic extracts of the *C. oxyacantha* leaf at the end of the organogenesis period (from day 16 to day 21 of the gestation period).

As a positive control, CP was achieved, which is activated by cytochrome P-450 enzyme to mustard phosphoramide and acrolein. The group exposed to CP increased the proportion of PCE and increased the number of MNPCE in a statistically significant way, both in pregnant rats and in their neonates. These results confirm its cytotoxic and genotoxic effects since acrolein has been described as the metabolite with the highest cytotoxic activity of CP, generating mitochondrial dysfunction, endoplasmic reticulum stress, and activation of apoptotic transcripts [50,51].

In a previous study, it was reported that the doses of 2000 and 1000 mg/kg of the aqueous and hydroalcoholic extract of *C. oxyacantha* in 12-week-old Balb-c mice, had no effect on the proportion of EPCS [52]. However, in this study, it was observed that pregnant rats exposed to doses of 500, 1000,

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and 2000 mg/kg of the aqueous and hydroalcoholic extracts of the *C. oxyacantha* leaf statically decreased the proportion of PCE to the basal value. The fact that, in pregnant rats, at the lowest dose evaluated, which was 500mg/kg of the aqueous and hydroalcoholic extract of the *C. oxyacantha* leaf, a cytotoxic effect was observed may be because during pregnancy the xenobiotic-metabolizing some enzymes are induced, which can increase the metabolism of secondary metabolites, substrates of these enzymes [45].

The group treated with CP presented a teratogenic effect by significantly decreasing the proportion of PCEs and significantly increasing the number of MNPCEs and MNEs in the peripheral blood of rat neonates. Previously, the transplacental effect of CP has been reported, which has been visualized with induction of MN In rat neonatal peripheral blood erythrocytes, fetal liver cells, and rat and mouse amniotic fluid cells exposed to CP during gestation [53–56].

Its teratogenic effect is associated with phosphoramide mustard and acrolein, which the active forms of CP, which are obtained through the metabolism of microsomal monooxygenases of cytochrome P-450. Mainly, they have an alkylating effect on DNA, RNA, and embryonic proteins [57].

The aqueous extract of the leaf of *C. oxyacantha* in rat neonates exposed to 2000mg/kg dose showed a cytotoxic and genotoxic effect by decreasing the proportion of PCEs and increasing that of MNPCEs. In contrast, neonates of rats exposed to different doses of the hydroalcoholic extract did not show cytotoxic or genotoxic damage. A previous study showed genotoxic and cytotoxic damage to the leaf and bark of *C. oxyacantha* in 12-week-old Balb-c mice (2000mg/kg), by showing significant changes in the proportion of MNPCEs. [52].

However, so far, no reports have been found evaluating the teratogenic potential of *C. oxyacantha* to compare our results.

There are reports of the antioxidant effect of flavonoids, which have been related to other types of pharmacological activity, such as anti-inflammatory, and its protective effect on the liver, brain, and cardiovascular levels. However, it has also been described that they have pro-oxidant effects, which lead to DNA damage and the formation of MN, chromosomal aberrations, and mutations. These effects are closely related to the experimental conditions under which the compounds are evaluated. Added to this, in a study by Schröder-van der Elst et al., they showed that flavonoids can cross the placenta in rats and accumulate in fetal tissues [58].

The difference in genotoxic and cytotoxic effects between the aqueous and hydroalcoholic extract of the leaf of *C. oxyacantha* may be due to the concentration of secondary metabolites, which varies according to the type of solvent used, both extracts showed flavonoids, tannins and quinones, however, we do not know in what proportion they were found and which one specifically contained.

Benabderrahmane et al., in 2018, determined some polyphenols present in the leaves of *C. oxyacantha*, such as caftaric acid, caffeic acid, chlorogenic acid, orientin, miquelianin, routine and apigenin [59].

Other authors have also reported the presence of epicatechin (dimer B2, B4, B5; trimer C1; tetramer D1; pentamer E1), isoquercitrin, hiperoside, isovitexin, and vitexin in the leaf of *C. oxyacantha* [60–62]. Apigenin, one of the compounds present in the leaves of *C. oxyacantha*, has been described as having a slow metabolism, which allows its accumulation in the body [63], there are also studies that demonstrate that it generates a teratogenic potential in rat embryos by causing a decrease in the weight, the size of the skull and tail [64]. This can be related to the antiestrogenic effect that has apigenind, which makes it difficult for the gestation process to be carried out correctly [65].

On the other hand, some epicatechin derivatives, which are also found in the leaves of *C. oxyacantha*, at low concentrations activate signaling pathways that regulate homeostasis, however, when concentrations increase, other pathways are activated, such as caspases that lead to a cytotoxic effect mediated by apoptosis [65]. Likewise, it has been described that the metabolism of flavonoids forms phenoxyl radicals which cause toxicity in the mitochondria, leading the cell to a state of apoptosis [66]. It has been shown that the methanolic extract of *C. oxyacantha* fruit has genotoxic effects in cultured human lymphocytes and generates mutations in bacteria of the Salmonella typhimurium strain [67].

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When determining the concentration of MDA in the liver, the group treated with CP showed the highest concentration of MDA in the liver. It has been described that the secondary metabolites of CF, such as phosphoramide mustard and acrolein, have prooxidant activity, which is related to its toxicity. Acrolein has a short half-life, however, it is considered the metabolite that unchains a higher production of reactive oxygen species, which causes lipid peroxidation and oxidative DNA damage [68,69]. Similarly, it has been reported that approximately 10% of CF is metabolized to reactive aldehydes, such as chloroacetaldehyde and dichloroethylcyclophosphamide, which also generates a prooxidant effect. CF exposure during gestational organogenesis has also been reported to cause a variety of fetal abnormalities in mice, rats, rabbits, and humans [70]. El-Dakdoky (2015) showed that CF administered intraperitoneally at a dose of 12mg/kg in rats on the 13th day of gestation caused damage to the products by showing an increase in the concentration of MDA in the fetal liver [71].

The present study shows that the evaluated doses of the aqueous and hydroalcoholic extracts of *C. oxyacantha* in pregnant rats and their neonates did not show hepatic cytotoxicity.

There are few studies on the evaluation of the safety of medicinal plants during pregnancy, for which no reports were found in which the quantification of MDA at the liver level in pregnant and neonatal rats exposed to these extracts has been evaluated.

It has been described that the fruit of *C. oxyacantha* at doses of 200 mg/kg administered orally for 7 days in mice generates cytotoxicity at the liver level (hepatocytes with more acidophilic cytoplasm, formation of vacuoles and space in intercellular cells, increased lumen of sinusoidal capillaries and increased hepatic tissue defense cells) [72].

However, the cytoprotective effect of the hydroalcoholic extract (EtOH) of the fruit of *C. oxyacantha* has also been described by decreasing the concentration of MDA in rats exposed to doses of 10 and 20mg/kg for ten days and a dose of 50mg/kg for twelve weeks [29].

Moreover, it was described that the n-butanol extract of *C. oxyacantha* leaves at a dose of 100mg/kg in rats decreased MDA concentrations in the liver [31]. Vanhees and collaborators investigated the effects of maternal quercetin exposure in mice. Showing that during embryonic development, increased iron levels and significantly decreased oxidative stress at the liver level [73].

Although the antioxidant effect of flavonoids is known, which are the main chemical compounds present in *C. oxyacantha*, however, some studies show that they have a dual effect, such as the case of apigenin, which is a flavone present in *C. oxyacantha* leaf, this compound has a pro-oxidant effect when administered alone in murine models [58,66].

Quercetin is another of the metabolites present in *C. oxyacantha*, this is one of the most abundant flavonols and is distributed in different foods. Various studies have shown that its consumption is safe during pregnancy, in addition to helping to reduce the concentration of MDA at the cardiac level and increasing the activity of antioxidant enzymes in embryos of rats treated with theophylline [74]. Another study reveals that rutin (a flavonol glycoside composed of quercetin) administered during gestation and lactation to female C57BL/6J mice modifies the concentrations of minerals, such as calcium, at the hepatic level in their offspring [75].

4. Materials and Methods

4.1. Materials and reagents

The reagents employed were of the commercial brand J. T Baker (Mexico) and Golden Bell (Mexico). Cyclophosphamide (CAS 6055 19-2) and acridine orange (CAS 10127-02-3) were from Sigma-Aldrich (St. Louis, MO, USA).

4.2. Plant material

The leaf of *C. oxyacantha* was obtained from the supplier Nutra Herbal de Mexico (Convento de Balvanera #24, Col. Jardines de Santa Monica, Mexico, Tlalnepantla C.P. 54050, Mexico).

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4.3. Preparation of the aqueous and hydroalcoholic leaf extracts of C. oxyacantha

The dried leaves of *C. oxyacantha* were pulverized. A decoction was made to obtain the aqueous extract, with a ratio of 1g per 10mL of water, and boiled for 15 minutes, then filtered and lyophilized.

For the hydroalcoholic extract of *C. oxyacantha*, 70% ethanol was used, and one was carried out by mechanical maceration for 48 hours. Refluxed for 2 hours and filtered. Activated carbon was added to remove chlorophyll and the ethanol was removed with a rotary evaporator, finally, it was lyophilized.

4.4. Phytochemical analysis leaf of C. oxyacantha

The phytochemical screening evaluation was performed through colorimetric tests to detect the presence or absence of phytochemical constituents (flavonoids, tannins and quinines).

Phytochemical screening of the extracts was performed using the following reagents and chemicals: Flavonoids with sodium hydroxide reagent test and Shinoda test and Z; Tannins with Gelatin test, ferric chloride reagent test and potassium ferrocyanide reagent test; They were identified by characteristic color changes and precipitation reactions using standard procedures [76].

4.5. Animals

Forty clinically healthy 3-month-old pregnant Wistar rats were placed in polycarbonate cages with food and water (Harlan Teklad Lab Block) ad libitum. The animals were provided by the Claude Bernard Biotherium of the Health Sciences Area, Campus UAZ, Siglo XXI, of the Universidad Autónoma de Zacatecas.

4.6. Study groups

The teratogenic potential was evaluated in the neonates of 40 female rats of the Wistar strain between 2-3 months of age, with an average weight of 205.10g ±10.75g, as well as the genotoxic and cytotoxic damage of the aqueous and hydroalcoholic extract of *C. oxyacantha* in mothers. The animals were divided into 8 experimental groups: group 1, received sterile water (negative control); Group 2, 60 mg/kg of cyclophosphamide (CP) divided into two doses (positive control); Group 3, high dose, 2000mg/kg of the aqueous extract; Group 4, medium dose, 1000mg/kg of the aqueous extract; Group 5, low dose, 500mg/kg of the aqueous extract; Group 6 also received a high dose of the hydroalcoholic extract; Group 7, medium dose of the hydroalcoholic extract; Group 8, low dose of the hydroalcoholic extract. The administration of the extracts was carried out orally through the esophageal cannula for 5 days, with a volume 0.1mL/10g of weight.

4.7. Mating

The rats were mated with the male for one week. Pregnancy was confirmed by a vaginal flush with 0.1mL of sterile water using a micropipette. The flush was placed on a slide, which was observed by 10x optical microscopy to detect the presence of sperm, which indicated the onset of gestation (day zero), in addition, the visualization of the vaginal plug confirmed pregnancy. Once the pregnancy of the female was confirmed, the gestation period was scheduled and the administration of the corresponding dose will be scheduled in the last days of pregnancy (days 16 to 21), as shown in Figure 3.

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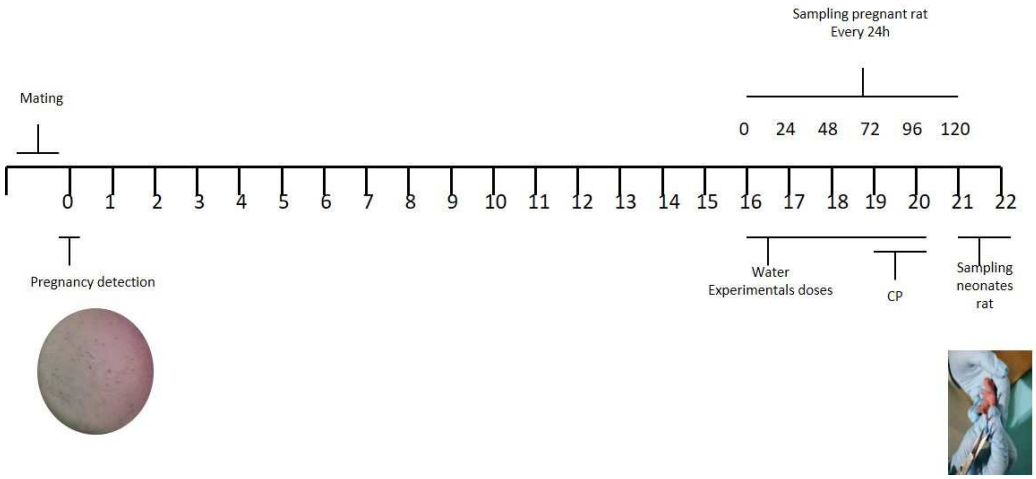

Figure 3. Scheme for the evaluation of genotoxicity of the aqueous and hydroalcoholic leaf extracts of *C. oxyacantha* in rats and their neonates (Taken and modified from Morales-Velazquez, G., et al., 2019).[56].

4.8. Sample preparation and micronucleus analysis in pregnancy rats and its neonate

The evaluation of cytotoxic and genotoxic damage in pregnant rats was determined by the micronucleus test (MN) [76]. Peripheral blood smears of the rats were made at 0, 24, 48, 72, 96, and 120 hours after the administration of the different doses, for which a drop of blood was obtained from the tip of the tail of the animals, of each group.

Once their gestation time was completed, 6 neonates were selected per rat and a blood sample was taken from the tail of each neonate and a duplicate spread was made, the smears were fixed in ethanol for 10 minutes and stained with acridine orange. An Olympus CX31 microscope equipped with epifluorescence and an oil immersion objective (100x) was used to evaluate the genotoxic and cytotoxic damage. The number of polychromatic erythrocytes (PCEs) was counted in 1000 total erythrocytes (TEs), the number of micronucleated polychromatic erythrocytes (MNPCEs) in 1000 PCEs, and the number of micronucleated erythrocytes (MNEs) in 10,000 TEs [77].

4.9. Hepatic peroxidation (Malondialdehyde quantification, MDA)

The quantification of MDA in the liver was carried out by the modified method of Mihara and Uchiyama in 1978. A 10% liver homogenate was prepared with 1.15% KCl, 0.05mL of the homogenate was taken and added to a tube, 3mL of 1% H3PO4 and 0.3mL of 0.6% of TBA was added, the mixture was put in a water bath for 45 minutes, cooled and 1-butanol was added. The MDA concentration was determined using a spectrophotometer at a wavelength of 534nm [78].

4.10. Statistical analysis

For the frequency of PCEs, MNPCEs, and MNEs, the results obtained were expressed as mean ± standard deviation per group. For rats, comparisons were made between each group and its respective baseline value (0 h), using the analysis of variance (ANOVA) for repeated measures and the Bonferroni adjustment test was used for multiple post hoc comparisons. In the case of neonates, intergroup comparisons were made concerning negative control values, using one-way analysis of variance (ANOVA), and the Dunnett adjustment test was used for multiple post hoc comparisons.

Data for MDA concentrations were expressed as a median with maximum and minimum. Intergroup comparisons were made using the Kruskall Wallis analysis with Dunn's post hoc.

Statistical significance was set at $p < 0.05$. Data analysis was performed using IBM SPSS (V25) statistics program for Windows.

4.11. Ethical Considerations

The handling of the animals was based on the Official Mexican Standard NOM-062-ZOO-1999, which shows the specifications and techniques for the production, care, and use of institutional laboratory animals. The sacrifice was based on the NOM-033-SAG/ZOO-2014 and the NOM-087-ECOL-SSA1-2002. The project has the endorsement of Bioethics of the Health Sciences Area of the Autonomous University of Zacatecas with the number ACS/UAZ/051/2019

5. Conclusions

The aqueous and hydroalcoholic leaf extracts of *C. oxyacantha* showed cytotoxic effect at the three doses evaluated and genotoxic at the doses of 2000mg/kg in pregnant rats Wistar Similarly, the dose the 2000mg/kg dose of the aqueous extract of the leaf of *C. oxyacantha* was shown to have a teratogenic potential. The pregnant rats and their neonates exposed to the aqueous and hydroalcoholic leaf extracts of *C. oxyacantha* did not show hepatic cytotoxicity. Based on the results obtained in this model, it is recommended not to administer aqueous and hydroalcoholic extracts of *C. oxyacantha* leaves to pregnant women. The importance of these findings is to contribute to the safety profile of *C. oxyacantha* leaf extracts during pregnancy for both the mother and the fetus.

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