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Antiangiogenic potential of *Jatropha curcas* latex in the chick chorioallantoic membrane model

Potencial antiangiogênico do látex de Jatropha curcas em modelo de membrana corioalantoica de embrião de galinha

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ABSTRACT

AIMS: To perform a physicochemical and phytochemical characterization of *Jatropha curcas* latex and to investigate its antiangiogenic potential.

METHODS: We performed an initial physicochemical characterization of *J. curcas* latex using thermal gravimetric analyses and Fourier Transform Infrared spectroscopy. After that, phenols, tannins and flavonoids were quantified. Finally, the potential of *J. curcas* latex to inhibit angiogenesis was evaluated using the chick chorioallantoic membrane model. Five groups of 20 fertilized chicken eggs each had the chorioallantoic membrane exposed to the following solutions: (1) water, negative control; (2) dexamethasone, angiogenesis inhibitor; (3) Regederm®, positive control; (4) 25% *J. curcas* latex diluted in water; (5) 50% *J. curcas* latex diluted in water; and (6) *J. curcas* crude latex. Analysis of the newly-formed vascular net was made through captured images and quantification of the number of pixels. Histological analyses were performed to evaluate the inflammation, neovascularization, and hyperemia parameters. The results were statically analyzed with a significance level set at $p<0.05$.

RESULTS: Physicochemical characterization showed that *J. curcas* latex presented a low amount of cis-1.4-polyisoprene, which reduced its elasticity and thermal stability. Phytochemical analyses of *J. curcas* latex identified a substantial amount of phenols, tannins, and flavonoids (51.9%, 11.8%, and 0.07% respectively). Using a chick chorioallantoic membrane assay, we demonstrated the antiangiogenic potential of *J. curcas* latex. The latex induced a decrease in the vascularization of the membranes when compared with neutral and positive controls (water and Regederm®). However, when compared with the negative control (dexamethasone), higher *J. curcas* latex concentrations showed no significant differences.

CONCLUSIONS: *J. curcas* latex showed low thermal stability, and consisted of phenols, tannins, and flavonoids, but little or no rubber. Moreover, this latex demonstrated a significant antiangiogenic activity on a chick chorioallantoic membrane model. The combination of antimutagenic, cytotoxic, antioxidant and antiangiogenic properties makes *J. curcas* latex a potential target for the development of new drugs.

KEYWORDS: physic nut; purging nut; anticancer agents; chorioallantoic membrane.

RESUMO

OBJETIVOS: Realizar uma caracterização físico-química e fitoquímica do látex de *Jatropha curcas* e investigar o seu potencial antiangiogênico.

MÉTODOS: foi realizada uma caracterização físico-química inicial do látex de *J. curcas* utilizando as análises termogravimétricas e a espectroscopia com a Transformada de Fourier. Depois disso, fenóis, taninos e flavonoides foram quantificados. Finalmente, o potencial do látex de *J. curcas* em inibir a angiogênese foi avaliado através do uso de modelo de membrana corioalantoica de embrião de galinha. Cinco grupos, cada um com 20 ovos de galinha fertilizados, tiveram a membrana corioalantoica exposta às seguintes soluções: (1) água, controle negativo; (2) dexametasona, inibidor da angiogênese; (3) Regederm®, controle positivo; (4) 25% de látex de *J. curcas* diluído em água; (5) 50% de látex de *J. curcas* diluído em água; e (6) látex bruto de *J. curcas*. A análise da rede vascular recém-formada foi feita por meio de imagens capturadas e quantificação do número de pixels. Análises histológicas foram realizadas para avaliar os parâmetros de inflamação, neovascularização e hiperemia. Os resultados foram analisados estaticamente com nível de significância estabelecido em $p<0,05$.

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RESULTADOS: A caracterização físico-química mostrou que o látex de *J. curcas* apresenta uma baixa quantidade de cis-1,4-poliisopreno, o que reduz sua elasticidade e estabilidade térmica. Análises fitoquímicas do látex de *J. curcas* identificaram uma quantidade significativa de fenóis, taninos e flavonoides (51,9%, 11,8% e 0,07% respectivamente). Usando o modelo de membrana corioalantoica de ovo de galinha embrionado, demonstrou-se o potencial antiangiogênico do látex de *J. curcas*. O látex induziu a diminuição da vascularização das membranas, em comparação aos grupos controle neutro e positivo (água e Regederm®).

CONCLUSÕES: O látex de *J. curcas* apresentou baixa estabilidade térmica, ausência ou pouca quantidade de borracha e presença de fenóis, taninos e flavonoides em sua composição. Além disso, apresentou alta atividade antiangiogênica no modelo de membrana corioalantoica de embrião de galinha. A combinação de propriedades antimutagênicas, citotóxicas, anti-inflamatórias, antioxidantes e antiangiogênicas faz com que o látex de *J. curcas* seja um alvo potencial para o desenvolvimento de novos medicamentos.

DESCRITORES: pinhão-manso; pinhão de purga; agentes antineoplásicos; membrana corioalantoide.

Abbreviations: CAM, chick chorioallantoic membrane(s); DE, dexamethasone solution; FTIR, Fourier Transform Infrared; JC25, 25% *J. curcas* latex diluted in water; JC50, 50% *J. curcas* latex diluted in water; JC100, *J. curcas* crude latex; RE, solution with Regederm®; TGA, thermogravimetric analyses; WA, pure water (negative control solution).

INTRODUCTION

Currently, the search for compounds that inhibit angiogenesis (development of blood vessels from pre-existing vascular tissue) has garnered increasing attention from the scientific community. It has been suggested that antiangiogenic drugs can potentiate the activity of co-administered chemoradiotherapies [1]. Treatment with antiangiogenic drugs has become part of the standard care for various tumors and has helped several patients worldwide with advanced cancers [2].

Plants produce a variety of compounds that can be important for the development of new drugs [3, 4]. Among the plant exudates, latex has been shown to have high antiangiogenic potential [5]. A scientometric analysis found that the latex of 16 plants had antiangiogenic activity [5], of which the two most prominent ones are *Calotropis procera* and *Ficus carica*. However, there are many others lactiferous species without any information about pharmacological or allergenic potential. Importantly, the natural rubber latex extracted from *Hevea brasiliensis* caused an allergic reaction in approximately 0.3% to 1% of the global population [6].

Jatropha curcas L. (Euphorbiaceae) is a perennial lactiferous species, also known as physic nut [7, 8]. Ethnobotanical surveys have shown that traditional use of *J. curcas* latex is to treat burns, hemorrhoids, ringworm, and ulcers [9]. It is known that latex of this species contains secondary metabolites which exhibit a wide range of medicinal properties, such as antioxidant, anti-inflammatory and anticancer activities [10]. The anticancer potential of *J. curcas* latex was demonstrated against cancerous human cell lines, such as colon adenocarcinoma, cervix carcinoma, and ovarium carcinoma [10, 11]. However, the manner in which *J. curcas* latex biocompounds act as antiproliferative substances remains unknown.

The first objective of this study was to perform a physicochemical characterization of *J. curcas* latex using two different techniques, and quantifying some classes of secondary metabolites. The second objective was to evaluate the potential of *J. curcas* latex as an *in vivo* angiogenesis inhibitor, using the chick chorioallantoic membrane (CAM) model. CAM assays are among the most common *in vivo* models used to study different aspects of angiogenesis [12, 13]. Several advantages make this model attractive, such as: fast results, low cost, simplicity, high reproducibility, easy dynamic observation, and no major ethical concerns involved [14, 15].

METHODS

Latex extraction

J. curcas latex was obtained from trees of the collection belonging to Universidade Estadual de Goiás, in the city of Ipameri, state of Goiás, Brazil (17°43’19’’S, 48°9’35’’W, 773 m). After botanical identification, a voucher specimen was deposited at the Universidade Estadual de Goiás Herbarium (Anápolis, Goiás, Brazil) under voucher number 10.042. Latex was extracted in a sterile tube by drilling in the tree trunk. To collect latex, a knife was used to make a cut of approximately 5 cm length and 0.5 cm depth in the bark. The latex was centrifuged for 5 min at 3,000 rpm to remove the debris derived from the collection process, and the supernatant was transferred to a new collection tube.

Latex biofilm production

For the physicochemical characterization it was necessary to obtain a solid compound from the

J. curcas latex sample. Thus, *J. curcas* latex solution was carefully deposited on a sterile petri plate to polymerize at room temperature. After a period of three days, a vitreous and brittle material was obtained, and pulverized into small pieces in the order of few millimeters, (hereafter "biofilm"). A *H. brasiliensis* (rubber tree) latex biofilm was obtained in a similar manner to the *J. curcas* latex biofilm, except for being polymerized at 55 ºC, and this biofilm was used as a control. Some properties of the *H. brasiliensis* latex biofilm have already been characterized, such as its elasticity, chemical composition, and thermal stability [15].

Physicochemical characterization

The physicochemical properties of the biofilms were evaluated using two different techniques: (1) thermogravimetric analyses (TGA), to evaluate the thermal stability of compounds; and (2) Fourier Transform Infrared (FTIR) spectroscopy that allowed the evaluation of functional chemical groups of compounds. TGA analyses were performed using a thermoanalyzer (Shimadzu, model DTG 60/60 H). Dynamic scans were conducted for a temperature range from 27 to 950 °C, at a constant heating rate of 10 °C/min, and under a nitrogen atmosphere. For the FTIR analyses, we used a Vertex 70 spectrophotometer (Bruker).

Total phenolic content

The total phenolic content of *J. curcas* latex was determined using $FeCl_3$ following the modification adapted by Mole and Waterman [17] of the Hagerman and Butler method [18]. For the spectrophotometric analysis, the latex solution (10 mg/ml) was added to 2 ml of sodium dodecyl sulfate/triethanolamine and 1 ml of $FeCl_3$. The colorful complex obtained was analyzed in a spectrophotometer at 510 nm. The solutions were analyzed in triplicate. Tannic acid and its dilutions (1.5, 2.0, 2.5, 3.0, and 3.5 mg/ml) were used to obtain the standard curve.

Total tannins content

A protein precipitation method was used for tannins content quantification, according to the Hagerman and Butler [18] method, adapted by Waterman and Mole [19]. For this, a Bovine Serum Albumine (BSA) solution (1 mg/ml) in 0.2 M acetate buffer (pH 4.9) was prepared. Next, the latex solution (5 mg/ml) was added to 2 ml of BSA and maintained for 15 min at room temperature. Then, this solution was centrifuged at 3,000 rpm for 15 min and the precipitate was dissolved in 4.0 ml of sodium dodecyl sulfate/triethanolamine solution and 1.0 ml of $FeCl_3$. The colored complex was analyzed by a spectrophotometer at 510 nm. All solutions were analyzed in triplicate. Tannic acid and its dilutions (1.5, 2.0, 2.5, 3.0, and 3.5 mg/ml) were used to obtain the standard curve.

Total flavonoids content

For the quantification of total flavonoids, we used an ultraviolet spectrophotometric method, adapted from the assay described by Rolim et al. [20]. The latex solution (5 mg/ml) was added to 0.02 M metanol:acetic acid (99:1). This mixture was analyzed using the spectrophotometer at 361 nm. The concentration of total flavonoid content was determined in comparison to rutin. Standard rutin was dissolved in 0.02 M methanol:acetic acid (99:1) to obtain concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml. All samples analyzed were performed in triplicate.

Angiogenic potential – CAM assay

A CAM assay with few modifications was performed as described by Almeida et al. [21]. Five groups of 20 fertilized chicken eggs each were incubated at 37 °C in a humidified environment (around 70% of humidity). After five days of incubation, the CAM was accessed through a window cut in the egg shell, and the eggs were returned to the incubator. On day 13 of incubation, the CAM was exposed to the different treatments. For these treatments, filter paper disks were soaked in 3 µl of the following solutions: (1) water, negative control (WA); (2) dexamethasone (Laboratório Teuto Brasileiro S.A., Anápolis, Brazil) at the final concentration of the 12 µg/3 µl, angiogenesis inhibitor (DE); (3) Regederm® (Pele Nova Biotecnologia, São Paulo, Brazil) prepared with *H. brasiliensis* latex, positive control (RE); (4) 25% *J. curcas* latex diluted in water (JC25); (5) 50% *J. curcas* latex diluted in water (JC50); and (6) *J. curcas* crude latex (JC100). After exposure of CAM to different treatments, the eggs were returned to the incubator for 72 h, following which CAM were fixed in formaldehyde (3.7%) for 5 min. Then, the CAM were cut with curved blunt scissors, and maintained in Petri dishes with formaldehyde solution. Analysis and quantification of the newly-formed vascular net were made through captured images. Gimp (version 2.0.5) was used to normalize saturation, light, and contrast

of the CAM images that were obtained. ImageJ (NIH, version 1.28) was used to quantify the number of pixels in each image. The angiogenic activity of *J. curcas* latex was evaluated by comparing the treated and control groups using one way analysis of variance (ANOVA), followed by Tukey's post-hoc test to compare treatment means. A p value <0.05 was used to indicate statistical significance.

After image analysis, the CAM were embedded in paraffin for histological analysis, stained with hematoxylin and eosin, and examined by optical microscopy (40×). Different parameters were evaluated, such as inflammation, neovascularization, and hyperemia. The results were classified based on their intensity, and the data were transformed into quantitative variables by assigning the following scores: absent (0), discrete (1-25%), moderate (26-50%), and accentuated (over 51%). The results were analyzed using a Kruskal-Wallis test with a significance level of $p<0.05$ followed by Bonferroni test to compare treatment means.

RESULTS

An initial physicochemical characterization of *J. curcas* based on the TGA analysis showed that the *J. curcas* biofilm had a lower thermal stability than the *H. brasiliensis* biofilm.

Figure 1A shows that the loss of mass in *J. curcas* latex is almost continuous. The loss starts at approximately 45 °C and peaks at 90 °C (15%), due to the evaporation of organic compounds of low molecular weight. Next, a constant loss of mass was observed, with two intense peaks near 550 °C (50%) and end close above 950 °C, with a mass of 2%, which can be attributed to oxidation reactions of high molecular weight compounds and their complete combustion. Further, the TGA curve showed high thermal stability of *H. brasiliensis* biofilm up to 90 °C, following a loss of mass (10%) for temperatures close to 320 °C.

In the 320-430 °C range, a significant loss of mass was observed, which amounted to 4.5% and may have resulted from oxidation of high molecular weight compounds. The next the thermal decomposition occurred over 530 °C, resulting in a mass less than 1%.

The differential thermal behavior of *J. curcas* and *H. brasiliensis* biofilms can be explained by the FTIR data (**Figure 1B**). The results showed a strong reduction of cis-1.4-polyisoprene in the *J. curcas* biofilm when compared with *H. brasiliensis*. Cis-1.4-polyisoprene was the main component of *H. brasiliensis* biofilms and was responsible for *H. brasiliensis* biofilm elasticity after polymerization.

After physicochemical characterization, we performed phytochemical prospection and quantified the amount of phenols, tannins, and flavonoids present in the latex. The total phenols, tannins and flavonoids estimated were 51.9%, 11.8% and 0.07%, respectively.

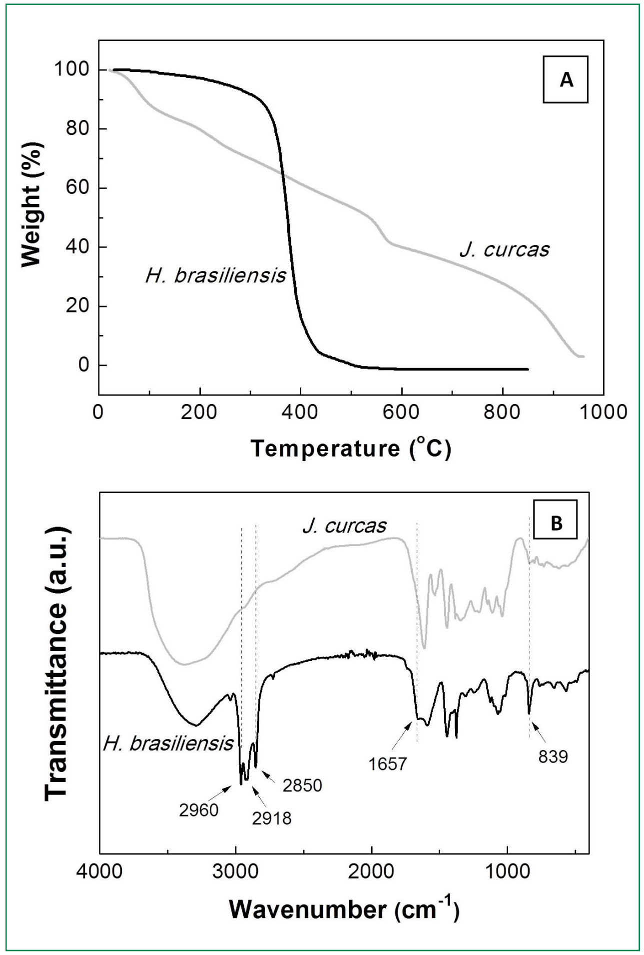

Figure 1. Physical chemistry characterization of *Jatropha curcas* and *Hevea brasiliensis* biofilms: **(A)** TGA curves and **(B)** FTIR spectra. The FTIR bands indicated with arrows are attributed to cis-1.4-polyisoprene [44].

To evaluate the angiogenic potential of *J. curcas* latex, the percentages of CAM vascular area in the latex treatments and controls were calculated. Representative images of the vascular nets of each condition are shown (**Figure 2**). The amount of blood vessels was substantially decreased in a dose dependent manner. The mean percentage of vascularization obtained for each group was: WA 12.03±2.31; DE 5.80±1.46; RE 22.68±2.03; JC25 10.4±1.97; JC50 7.03±1.01; and JC100 6.79±0.86.

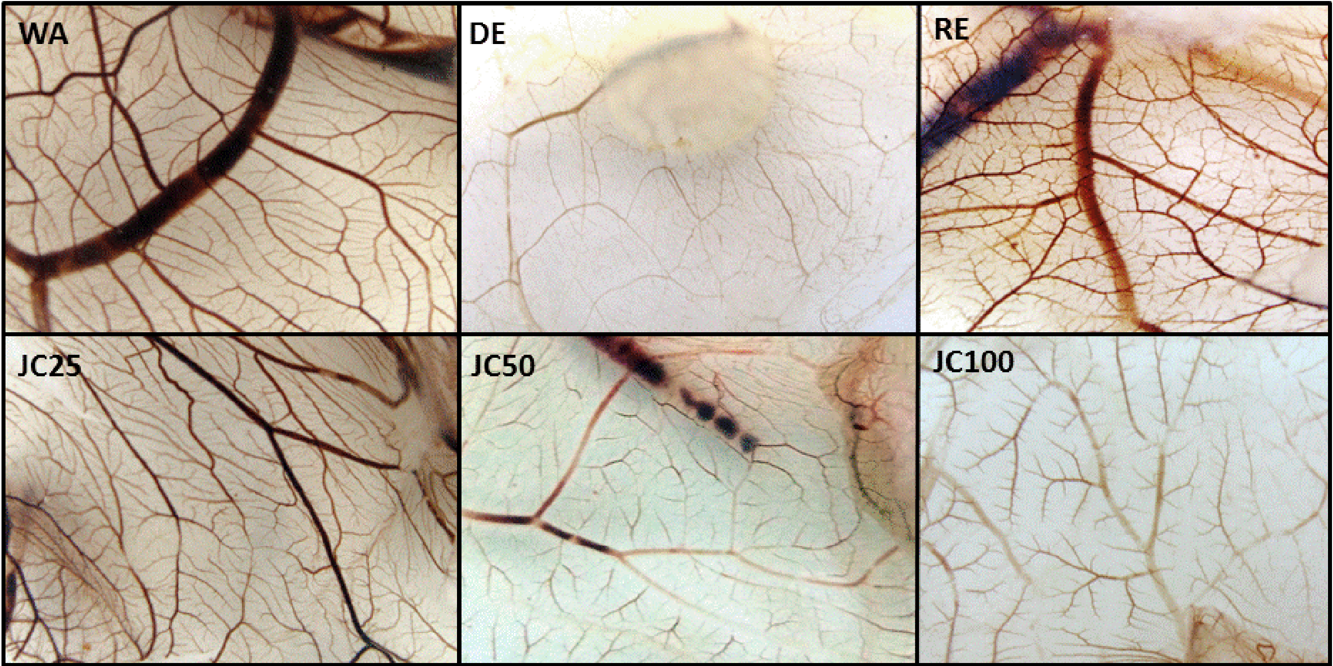

Figure 2. Antiangiogenic potential of *Jatropha curcas* latex on chorioallantoic membrane (CAM) model. Representative images of different CAM treatments: WA (water, neutral control); DE (dexamethasone, angiogenesis inhibitor); RE (Regederm®, positive control); JC25 (*J. curcas* latex 25%); JC50 (*J. curcas* latex 50%); and JC100 (*J. curcas* latex 100%).

J. curcas latex showed a significant decrease in percentage vascularization compared with both Regederm® and the negative control. However, when compared with Dexamethasone, the higher *J. curcas* latex concentrations (i.e., JC50 and JC100) showed no significant differences in the percentage of vascularization.

After CAM morphology analysis, the histological analyses were performed (**Table 1**). A decrease in the number of blood vessels for the latex (JC25, JC50 and JC100) and dexamethasone groups occurred after 72 h of treatment, when compared with the Regederm® and negative control groups (**Figure 3**). Significant differences were also observed in terms of hyperemia and neoangiogenesis. Thus, the histological results were in agreement with the findings based on CAM images, indicating that *J. curcas* latex presented antiangiogenic activity, similar to dexamethasone.

Table 1. Histological parameters of the chorioallantoic membranes treated with the control soutions and *Jatropha curcas* latex.

Solution	Inflammation		Hyperemia		Neovascularization	
	Median*	Bonferroni test	Median*	Bonferroni test	Median*	Bonferroni test
Water	0.8	B	1.6	AB	0	B
Dexamethasone	0.2	B	0.2	C	0	B
Regederm®	2.4	A	2	A	2.2	A
JC25	0.2	B	0.6	BC	0.6	B
JC50	0.0	B	0.8	BC	0.0	B
JC100	0.4	B	0.6	BC	0.0	B
P value	p≤0.08		p≤**0.03**		p≤**0.002**	

* The data were submitted to non-parametric Kruskal-Wallis analysis.
Same letters represent no significant difference by the Bonferroni t-test.

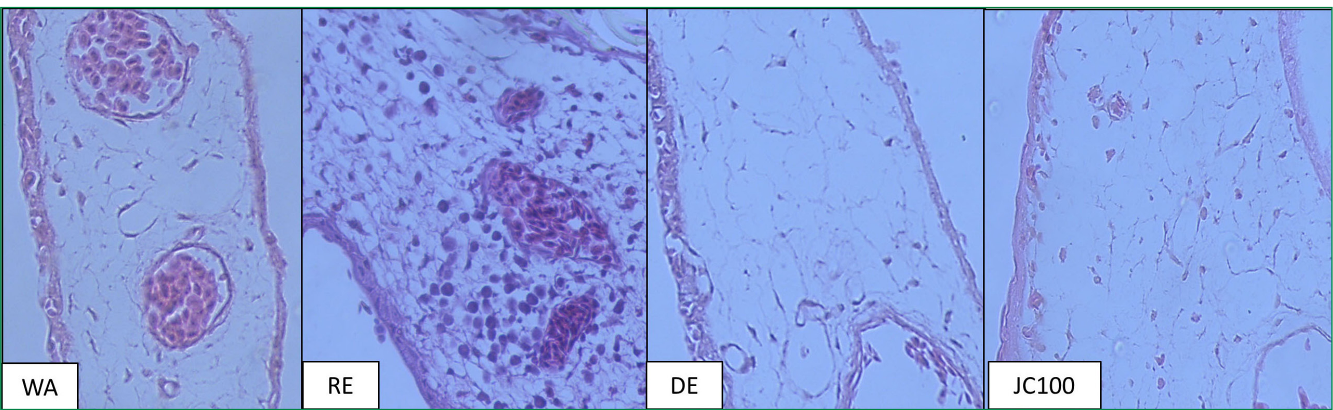

Figure 3. Histological patterns of chorioallantoic membranes (CAM) treated with *Jatropha curcas* latex. Paraffin sections stained with hematoxylin-eosin after different CAM treatments: WA (water); RE (Regederm®); DE (dexamethasone); and JC100 (*J. curcas* latex 100%). The slides were observed on optical microscopy (40×).

DISCUSSION

J. curcas exhibits biotechnological potential, mainly for biodiesel production [7], however little is known about its latex potentialities. To address this, we did a physicochemical characterization of *J. curcas* latex. TGA analysis showed that *J. curcas* latex had a lower thermal stability than natural rubber latex [21]. This difference can potentially be explained by the small amount of cis 1.4-polyisoprene detected by FTIR. Also, this result explains the loss of elasticity from *J. curcas* latex after polymerization and its vitreous appearance. To our knowledge, this is the first report of the complete absence of or the presence of only a very small quantity of cis 1.4-polyisoprene in *J. curcas* latex.

The following secondary metabolites have previously been described for *J. curcas* latex: phenols [22], tannins [22-23], saponins, and flavonoids [9]. In this study, we identified the presence of some these metabolites and we quantified their concentrations. The high secondary metabolite content are in agreement with those reported by Oskoueian et al. [10]. The high content of phenols and tannins may be the reason behind the use of *J. curcas* latex in traditional medicine [24], as it is known that phenol compounds can prevent or attenuate some human disorders [25-28].

Given the high phenolic content of *J. curcas* latex, we decided to evaluate the other biological properties of this exudate. Phenolic compounds have antimutagenic, antiangiogenic, antioxidant, anti-inflammatory, and antimicrobial properties [4]. In relation to the genotoxic activity of *J. curcas* latex, it was observed that the crude latex has genotoxic effects on the *Allium cepa* root model [29]. Further, the mutagenic and antimutagenic potential of *J. curcas* latex was demonstrated in mouse bone marrow cells by our research group. However, when *J. curcas* latex was co-administrated with a mutagenic drug (Doxorubicin®), it showed an antimutagenic effect [unpublished data]. These results suggest that *J. curcas* latex can play a dual role: mutagenic and antimutagenic.

Besides the mutagenic and antimutagenic potential of *J. curcas* latex, it also presented cytotoxic effects on plant cells [29]. These cytotoxic effects make *J. curcas* an interesting target of study for its anticancer and antimitotic properties [30]. Other important characteristics of *J. curcas* latex are its antioxidant and anti-inflammatory activities [10]. Previous studies have demonstrated the critical role of free radicals in the initiation and development of cancer, and how antioxidants may slow cancer development and progress [31]. Also, there are reports showing how inflammation is involved in cancer initiation and metastasis, and epidemiological research suggests that as many as 25% of all cancers may be due to chronic inflammation [32-34].

Previously, only a few compounds had been isolated from *J. curcas* latex; protein curcain [35], curcacycline A [36], curcacycline B [37], and jatrophidin I [38]. Among these, curcacycline A and B showed cytotoxicity and anti-proliferative action in tumor cells [11,39]. Thinking in new drugs against tumors, it would be interesting to create a product that has antimutagenic, cytotoxic, antioxidant, anti-inflammatory, and antiangiogenic properties.

We evaluated the antiangiogenic potential of *J. curcas* latex using a CAM assay. Angiogenesis

is a fundamental physiological process with strong implications in tissue homeostasis [40]. Extensive efforts have been made to develop therapeutic strategies to promote or inhibit angiogenesis [41]. For instance, a compound that inhibits angiogenesis might be a promising agent to target the abnormal vasculature found in several types of solid tumors [3]. One widely adopted strategy to maximize the efficacy of tumor treatment is to combine anti-angiogenesis agents with chemotherapy or radiotherapy [2].

To determine the potential of *J. curcas* latex in inhibiting angiogenesis, we submitted CAM to different doses of this latex and found that the vascularization percentages decreased after latex treatment. The histological and morphological results were in agreement, suggesting that *J. curcas* latex showed antiangiogenic activity. Materials that inhibit angiogenesis are important for antiangiogenic cancer therapy. In this therapy, the blood supply to the cancer cells is cut off, thereby depriving the tumor of nutrients and impeding its development [42].

In contrast to our results, Salim et al. [43] and Balqs et al. [44] observed an angiogenic and anti-inflammatory activities for a topical cream composed of 10-15% of *J. curcas* latex. The skin of mice subjected to the application of this *J. curcas* cream showed improved wound healing and a decrease in inflammatory cell infiltration. Our results, in contradiction to this previous report, showed that the high concentrations of the latex or the crude nature of the latex had an antiangiogenic activity in the CAM assay model. The contrasting results can be explained by the latex concentrations used. Dose-dependent effect has been previously described. For example, a dose-dependent dual effect of baicalin on angiogenesis was demonstrated by Zhu et al [45], who observed that high doses of baicalin showed anti-angiogenesis effects through the induction of apoptosis; however, low dosages of baicalin promoted angiogenesis through stimulation of cell proliferation.

We conclude that *J. curcas* latex showed low thermal stability and an absence of or the presence of a very small amount of rubber in its composition. These characteristics are unexpected when comparing *J. curcas* with other *Euphorbiaceae* family species, such as *H. brasiliensis.* The chemical characterization of this latex identified and quantified phenolic compounds, tannins, and flavonoids, which have previously been shown to exhibit antimutagenic, cytotoxic, and antioxidant activities. Moreover, this latex demonstrated a significant antiangiogenic activity on a CAM model. The combination of antimutagenic, cytotoxic, antioxidant, and antiangiogenic properties of *J. curcas* latex makes this exudate a potential target for the development of new drugs.

NOTES

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Conflicts of interest disclosure

The authors declare no competing interests relevant to the content of this study.

Authors' contributions

All the authors declare to have made substantial contributions to the conception, or design, or acquisition, or analysis, or interpretation of data; and drafting the work or revising it critically for important intellectual content; and to approve the version to be published.

Availability of data and responsibility for the results

All the authors declare to have had full access to the available data and they assume full responsibility for the integrity of these results.

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