Analgesic effect of methanolic extract obtained from *Schinus terebinthifolius* leaves

_Efeito analgésico do extrato metanólico obtido das folhas de* *Schinus terebinthifolius*

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Abstract

Previous studies from our group showed the analgesic effects of essential oil and extracts of *Schinus terebinthifolius*. The aims of this research were to evaluate the anti-nociceptive, anti-arthritic properties of methanolic extract obtained from *S. terebinthifolius* (MEST) leaves in animal models of experimental pain and arthritis. The MEST (30 and 100 mg/kg) reduced both phases of formalin induced licking behavior in Swiss mice. The MEST (100 mg/kg) reduced mechanical hyperalgesia induced by carrageenan in swiss mice. The MEST (100 mg/kg) oral administration reduced significantly the mechanical hyperalgesia (day 2, 5, 10, and 20), knee oedema (day 2), cold (day 15) and heat sensitivity (day 20), but not depressive-like behavior (Forced Swim Test), after Complete Freund’s Adjuvant (CFA) in C57Bl6 mice. The MEST prevented the anti-nociceptive effects in formalin nociception and did not interfere with locomotor activity in Swiss mice (open field test). The MEST was effective for the inhibition of pain and arthritic parameters without altering locomotor activity or depressive-like behavior. The present results may open new possibilities for the development of new analgesic agents from *S. terebinthifolius*.

Keywords: *Schinus terebinthifolius*, Anacardiaceae, rodents, nociception, arthritis.

Resumo

Estudos anteriores do nosso grupo mostraram os efeitos analgésicos do óleo essencial e extratos de *Schinus terebinthifolius*. Os objetivos desta pesquisa foram avaliar propriedades anti-nociceptivas e antiartríticas do extrato metanólico obtido das folhas de *S. terebinthifolius* (MEST) em modelos animais experimentais de dor e artrite. O EMST (30 e 100 mg/kg) reduziu comportamento de lamber a pata como reflexo à dor nas fases neurogênica e inflamatória induzidas pela formalina em camundongos *Swiss*. O EMST (100 mg/kg) reduziu a hiperalgésia mecânica induzida por carragenina em camundongos *Swiss*. A administração oral do EMST (100 mg/kg) reduziu significativamente a hiperalgésia mecânica (dia 2, 5, 10 e 20), edema do joelho (dia 2), sensibilidade ao frio (dia 15) e sensibilidade ao calor (dia 20), mas não o comportamento depressivo (Teste de nado forçado), após o adjuvante completo de Freund (CFA) em camundongos C57Bl6. O EMST previniu efeitos antinociceptivos na nocicepção de formalina e não interferiu na atividade locomotora de camundongos Swiss (teste de campo aberto). O EMST foi eficaz na inibição da dor e dos parâmetros artríticos, sem alterar atividade locomotora ou comportamento depressivo. Os presentes resultados podem abrir novas possibilidades para o desenvolvimento de novos agentes analgésicos a partir de *S. terebinthifolius*.

Palavras-chave: *Schinus terebinthifolius*, Anacardiaceae; roedores; nocicepção, artrite.
Introduction

Pharmacological and non-pharmacological pain treatment is complex and performed based on pain classification (1). This pain process is a clinical sign observed in inflammatory diseases, being one of the first manifestations in acute inflammation (2). Inflammatory diseases and neuropathies determine nociceptor sensitization, implying the occurrence of different subtypes of pain such as allodynia and hyperalgesia (3).

In this way, the currently available therapeutic treatments for pain are generally non-specific and have limited efficacy and safety, particularly with chronic pain and arthritis treatments, such as NSAIDs and cytokine blockers (4). However, many side effects are observed with the use of these drugs, including immunosuppression, opportunistic infections, and gastrointestinal, renal, and hepatic lesions that may increase morbidity and mortality (4). In addition, some painful conditions such as neuropathic pain are refractory to currently available drugs, including opioids (5).

Herbal products (fresh medicinal plants, herbal extracts, plant drugs and phytotherapics) are included in the search for new drugs with fewer adverse effects in the treatment of different pathophysologies, including pain. The Brazilian small tree “aroeira-vermelha”, “aroeira-pimenteira” or “poivre-rose” and “aroeira” (6) is known scientifically as Schinus terebinthifolius (Anacardiaceae). The empirical therapy with this plant as an anti-inflammatory, antipyretic, analgesic, purifying astringent, diuretic and diarrhea medication was made by the population (7-9).

However, the literature also reports pharmacological evidence that this plant, administered in the form of different preparations, has anti-hyperalgesic (10), antimicrobial (11-12) and anti-histaminergic actions (13). Previous studies in our research group have shown methanolic extract from leaves of S. terebinthifolius (collected in Dourados, MS, Brazil) exhibited marked antioxidant, antiproliferative and in vivo anti-edematogenic activity, and chemical analysis revealed the predominance of phenolic compounds (14).

The aim of this study was to evaluate the potential anti-nociceptive (in a formalin model), anti-hyperalgesic (in a carrageenan model), antidepressant and anti-arthritic (in the Complete Freund’s Adjuvant persistent (CFA) model) effects of the methanolic extract (MEST) of S. terebinthifolius. The present research extends the work of Araujo et al. (14).

Introdução

O tratamento farmacológico e não farmacológico da dor é complexo e realizado com base na classificação da dor (1). Esse processo doloroso é um sinal clínico observado em doenças inflamatórias, sendo uma das primeiras manifestações na inflamação aguda (2). As doenças inflamatórias e neuropatias determinam a sensibilização dos nociceptores, implicando a ocorrência de diferentes subtipos de dor, como alodinia e hiperalgésia (3).

Dessa forma, os tratamentos terapêuticos atualmente disponíveis para a dor geralmente são inespecíficos e têm eficácia e segurança limitadas, principalmente em tratamentos crônicos de dor e artrite, como AINEs e bloqueadores de citocinas (4). No entanto, muitos efeitos colaterais são observados com o uso desses medicamentos, incluindo imunossupressão, infecções oportunistas e lesões gastrointestinais, renais e hepáticas que podem aumentar a morbimortalidade (4). Além disso, algumas condições dolorosas, como a dor neuropática, são refratárias às drogas atualmente disponíveis, incluindo os opioides (5).

Os produtos a base de plantas (planta medicinal in natura, extratos a base de plantas, droga vegetal e fitoterápico) são incluídos na busca de novos medicamentos com menos efeitos adversos no tratamento de diferentes fisiopatologias, incluindo a dor. A pequena árvore brasileira “aroeira-vermelha”, “aroeira-pimenteira” ou “poivre-rose” (6) é conhecida cientificamente como Schinus terebinthifolius (Anacardiaceae). A terapia empírica com essa planta como remédio anti-inflamatório, antipirético, analgésico, adstringente, diurético e diarreia é realizada pela população (7-9).

No entanto, a literatura também relata evidências farmacológicas de que esta planta, administrada na forma de diferentes preparações, possui ações anti-hiperalgésica (10), antimicrobiana (11-12) e anti-histaminérgica (13).

Estudos anteriores em nosso grupo de pesquisa demonstrou que o extrato metanólico de folhas de S. terebinthifolius (coletadas em Dourados-MS) apresentou atividade antioxidante, antiproliferativa e anti-edematogênica in vivo, e as análises químicas revelaram a predominância de compostos fenólicos (14).

O objetivo deste estudo foi avaliar os potenciais anti-nociceptivo (no modelo de formalina), anti-hiperalgésico (no modelo de carrageenina), antidepressivo e anti-
Materials and Methods

Chemicals and drugs

λ-carrageenan (Cg), Complete Freund adjuvant (CFA), Tween 80 and dexamethasone (DEXA) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Plant material

Leaves of the plant *S. terebinthifolius* were collected at the Federal University of Grande Dourados (UFGD) and identified by Maria do Carmo Vieira, Ph.D. (researcher of the College of Agronomical Sciences of the UFGD). A voucher specimen (DDMS 4600) was deposited in the herbarium of the UFGD in Mato Grosso do Sul state of Brazil. This specie was registered on National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under the code A51F665.

Production of MEST

After oven drying and grinding, collected leaves of *S. terebinthifolius* (620 g) were extracted by maceration with 8 L of methanol at room temperature. After filtration, concentration under reduced pressure, and lyophilization, the methanolic extract (MEST, 38 g) was obtained.

Experimental animals

The experiments were performed using male Swiss mice (25-35 g) and male C57Bl6 mice (25-35 g) provided by the Federal University of Grande Dourados (UFGD) biotherium. The animals were maintained under a 12-hour light-dark cycle, with controlled humidity (60-80%) and temperature (22 ± 1°C). The animals were acclimatized to the experimentation room for at least two hours before the experimental protocol. The experimental procedures were carried out after the approval by the ethics committee for research on laboratory animals of the UFGD (Nbr. 004/2012).
The MEST was dissolved in aqueous solution containing 1% of Tween 80. The vehicle administration was made by a solution of water plus 1% of Tween 80 (1%). All oral pretreatments (or administrations) described with the respective tests with the MEST were made via gavage.

**Formalin induced-Licking Test**

The procedure used was similar to that described by Hunskaar and Hole (18). Groups of mice (n=6) were pretreated orally with the MEST (100 mg/kg), subcutaneously with dexamethasone (1 mg/kg), or orally with vehicle solution one hour before injection of 20 µL of 2.5% formalin (0.92% formaldehyde) in the right hind paw. These animals were placed in glass cylinders, and the time that the animal spent licking and/or elevating the injected paw was recorded in blocks of five minutes for up to thirty minutes. The first five minutes were considered Phase I (neurogenic), while Phase II (inflammatory) was between fifteen and thirty minutes.

**Analysis of locomotor activity in the open-field test**

Mice were orally pretreated with the MEST (100 mg/kg), dexamethasone (1 mg/kg), or vehicle by oral route, and the open-field test was performed after fifty minutes. The open field apparatus was constructed with plywood and measured 80 x 80 cm with 40 cm walls. The walls and floor were both white. Blue lines were drawn on the floor with a marker and were visible through the clear plexiglass floor. The lines divided the floor into sixteen 20 x 20 cm squares. A central square of equal size was drawn in the middle of the open field (20 x 20 cm). The locomotor activity was measured for five minutes, analyzing the frequency with which the mice crossed one of the grid lines with all four paws.

**Carrageenan-induced hyperalgesia model**

After basal mechanical measurements, the mice were divided into groups (n = 6/group) that orally received vehicle (0.9% saline solution), MEST (100 mg/kg), or dexamethasone (1 mg/kg). After one hour, the animals received a 50 µL injection containing 300 µg carrageenan (Cg) subcutaneously in the right hind paw, and each animal was housed in the same containment boxes under the same steel mesh. Mechanical hyperalgesia was measured four hours after Cg injection using the digital von Frey analgesimeter.
CFA-induced hyperalgesia model

Mechanical measurement with a von Frey analgesimeter and respective MEST treatment (100 mg/kg, p.o.), or other treatments described below, was made once daily in C57Bl6 mice for 20 days after CFA injection. In addition, response to acetone (sensibility to cold), hot plate, forced swimming (to analyze depression), and knee oedema tests were also performed. After basal measurements, the mice were divided into groups (n = 6/group) and orally received vehicle (0.9% saline solution), MEST (100 mg/kg), or dexamethasone (1 mg/kg). One hour after this procedure, animals received an injection of 50 µL of CFA (Freund’s Complete Adjuvant) subcutaneously in the right hind paw.

Mechanical hyperalgesia analysis - One day prior to CFA treatment, and on Days 1 to 20 of CFA treatment, the mechanical measurement of all mice was performed. Mice were housed in containment boxes (W x D x H - 230 x 200 x 180 mm) under a steel mesh with 1 cm diameter spacing for a period of thirty minutes. During this time, a digital von Frey analgesimeter (Insight®, SP, Brazil) was used to determine the baseline for the mechanical stimulus in the right hind paw.

Analysis of response to acetone (sensibility to cold test) - Cold sensitivity was analyzed two days after CFA injection by the acetone drop test as described by Decosterd and Woolf (15). A blunt needle connected to a syringe was used to drop 30 μl of acetone on the paw, and the duration of the paw withdrawal (in seconds) was recorded. Minimal and maximal cut-offs were assigned at 0.5 and 20 seconds, respectively.

Forced Swim Test (FST) - This experimental test was performed on the twentieth day using the method previously described by Porsolt (16). Animals were individually forced to swim in an open cylindrical container with a volume of 1,932 cm³ containing water at 25 ± 1°C, and the total time duration of immobility was observed for five minutes.

Knee Oedema Test - Knee measurements were performed with a micrometer (Mitutoyo®) on the twentieth day after CFA injection in the experimental group.

Análise de hiperalgésia mecânica - Um dia antes do tratamento com CFA e no dia 1 ao dia 20 após o tratamento com CFA, foi realizada a mensuração mecânica de todos os camundongos machos. Os camundongos foram alojados em caixas de contenção (L x P x A - 230 x 200 x 180 mm) sob uma grade de aço com espaçamento de 1 cm de diâmetro por um período de trinta minutos. Durante esse período, um analgesímetro digital de von Frey (Insight®, SP, Brazil) foi utilizado para determinar a linha de base do estímulo mecânico na pata traseira direita.

Análise da resposta à acetona (teste de sensibilidade ao frio) - A sensibilidade ao frio foi analisada dois dias após a injeção de CFA pelo teste de gota de acetona, conforme descrito por Decosterd & Woolf (15). Uma agulha cega conectada a uma seringa foi usada para soltar 30μl de acetona na pata, e a duração da retirada da pata (em segundos) foi registrada. Os pontos de corte mínimo e máximo foram atribuídos em 0,5 e 20 segundos, respectivamente.

Teste de nado forçado (FST) - Este experimento foi realizado no vigésimo dia, utilizando o método descrito anteriormente por Porsolt (16). Os animais foram forçados a nadar individualmente em um recipiente cilíndrico aberto com volume de 1.932 cm³ com água a 25 ± 1 °C, e o tempo total de imobilidade foi observado por cinco minutos.

Teste de edema do joelho - As medidas do joelho foram realizadas com um micrômetro (Mitutoyo®) no vigésimo dia após a injeção de CFA no grupo experimental.
Heat-Induced Hyperalgesia Test - This test was performed on Day 15 using the method of Eddy and Leimbach (17). The experimental animals were placed on an aluminum plate heated at a fixed temperature (55 ± 0.5°C), and the response to the heat latency time was assessed. They measured the time it took to the animal to withdraw its hind paw from the hot plate and lick it. Minimal and maximal cut-offs were assigned at 0.5 and 30 seconds, respectively.

Statistical Analysis
The results represent the mean ± standard error of the mean (SEM). Data were compared by one and two-way analysis of variance (ANOVA), followed by Student-Newman Keuls test. P values lower than 0.05 (p <0.05) were considered significant.

Results
Effects of MEST on formalin-induced nociception and measured motor activity in the open field test
Gavage administration of MEST reduced formalin-induced nociception in the neurogenic phase (Phase I, 0 to 5 minutes) and the inflammatory phase (Phase II, 15 to 30 minutes) (Figure 1). In the control group, the average time licking in the neurogenic phase was 103.5 seconds, and in the inflammatory phase was 219.5 seconds.

After one hour of treatment with MEST, the doses of 30 and 100 mg/kg significantly inhibited the neurogenic phase (56% and 70%, respectively). In the inflammatory phase, the observed inhibitions were 87% and 92%, respectively. Dexamethasone treatment (1 mg/kg) also significantly reduced effects in both phases (Figures 1A and 1B). The treatments did not determine changes in the locomotor activity in the open field test (data not shown).

Effects of MEST on carrageenan-induced mechanical hyperalgesia
Intraplantar Cg treatment induced mechanical hyperalgesia in mice after four hours. MEST (100 mg/kg) treatment prevented the carrageenan induced-mechanical hyperalgesia with statistical significance compared to the respective control group. Dexamethasone treatment (1 mg/kg) eliminated the mechanical hyperalgesia (Figure 2).
Figure 1 - Effect of the oral administration of MEST at 30 and 100 mg/kg on pain-related behaviors in the formalin-induced nociception model. The results are presented as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group (vehicle). Differences between groups were analyzed by analysis of variance (one-way ANOVA) followed by the Newman–Keuls test.

Figura 1 - Efeito da administração oral de EMST 30 e 100 mg/kg nos comportamentos relacionados à dor no modelo de nocicepção induzida por formalina. Os resultados são apresentados como a média ± SEM. * P<0,05, ** P<0,01, *** P<0,001 em comparação com o grupo controle (veículo). As diferenças entre os grupos foram analisadas pela análise de variância (ANOVA one-way) seguida pelo teste de Newman–Keuls.

Figure 2 - Effect of the MEST at 100 mg/kg on carrageenan-induced mechanical hyperalgesia in mice. The results are presented as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group (vehicle). Differences between groups were analyzed by analysis of variance (one-way ANOVA) followed by the Newman–Keuls test.

Figura 2 - Efeito do EMST a 100 mg/kg na hiperalgésia mecânica induzida por carragenina em camundongos. Os resultados são apresentados como a média ± SEM. * P<0,05, ** P<0,01, *** P<0,001 em comparação com o grupo controle (veículo). As diferenças entre os grupos foram analisadas pela análise de variância (ANOVA one-way) seguida pelo teste de Newman–Keuls.
Effects of MEST on mechanical hyperalgesia, thermic sensitivity, depressive behavior and knee oedema induced by CFA

A single intraplantar injection of CFA induced persistent mechanical hyperalgesia, cold sensibility, heat sensibility, and knee oedema as well as depressive behaviour.

Mechanical hyperalgesia was observed from the first day following application of CFA and persisted for at least 20 days (Figure 3).

Oral administration of MEST (100 mg/kg) or dexamethasone (1 mg/kg) reduced the mechanical hyperalgesia induced by CFA compared to the respective control group. The maximal inhibition was observed on the 5th, 10th and 20th days following the induction of hyperalgesia by CFA (Figure 3).

Cold sensitivity development was evaluated on the second day. After the administration of CFA and MEST, cold sensitivity decreased significantly compared to the respective control group (Figure 4A).

Figure 3 - Effects of oral administration of MEST at 100 mg/kg on mechanical hyperalgesia induced by Complete Freund’s Adjuvant (CFA). Mechanical hyperalgesia was analyzed until 20 days after CFA injection. The results are presented as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the vehicle group (CFA). Differences between groups were analyzed by analysis of variance (two-way ANOVA) followed by Bonferroni test variance (one-way ANOVA) followed by the Newman–Keuls test.

Efeitos do EMST na hiperalgesia mecânica, sensibilidade térmica, comportamento depressivo e edema do joelho induzido por CFA

Uma única injeção intraplantar de CFA induziu hiperalgesia mecânica persistente, sensibilidade ao frio, sensibilidade ao calor e edema do joelho, além de comportamento depressivo.

A hiperalgesia mecânica foi observada desde o 1º dia após a aplicação do CFA e persistiu por pelo menos 20 dias (Figuras 3).

A administração oral de EMST (100 mg/kg) ou dexametasona (1 mg/kg) reduziu a hiperalgesia mecânica induzida pela CFA em comparação com o respectivo grupo controle. A inibição máxima foi observada no 5º, 10º e 20º dia após a hiperalgesia ter sido induzida por CFA (Figura 3).

A hiper sensibilidade ao frio foi avaliado no segundo dia. Após a administração de CFA e EMST, a sensibilidade ao frio diminuiu significativamente em comparação com o respectivo grupo controle (Figura 4A). O tratamento...
Figure 4 - Effects of oral administration of the MEST at 100 mg/kg on cold sensitivity (A), hot plate test (B), and knee oedema induced by Complete Freund’s Adjuvant (CFA). The results are presented as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the vehicle group (CFA). Differences between groups were analyzed by one-way ANOVA followed by the Newman–Keuls test.

Figura 4 - Efeitos da administração oral do EMST a 100 mg/kg na hipersensibilidade ao frio (A), teste de placa quente (B) e edema do joelho induzido pelo adjuvante completo de Freund (CFA). Os resultados são apresentados como a média ± SEM. * P <0,05, ** P <0,01, *** P <0,001 em comparação com o grupo veículo (CFA). As diferenças entre os grupos foram analisadas por ANOVA unidirecional, seguida pelo teste de Newman–Keuls.
In studies carried out on Day 15 of the experiment, MEST treatment also showed a significant increase in hot plate latency compared to the respective control (Figure 4B). Significant decreases of oedema in tibiofibular articulation in groups treated with MEST and dexamethasone were observed compared to the respective control group (Figure 4C).

There were no statistically significant differences between the groups that received MEST and the control group in the forced swim test. Neither the extract nor the pure compounds exhibited an antidepressant effect (data not shown).

**Discussion**

This study showed that the extract obtained from *S. terebinthifolius* negatively modulates the carrageenan-induced persistent mechanical hyperalgesia and formalin-induced spontaneous pain. In the same way, MEST also significantly reduced CFA-induced knee oedema and cold- and hot-hypersensitivity, but not depression, without changing locomotor activity (open field test).

Nonsteroidal anti-inflammatory (indomethacin, naproxen) and immunosuppressive (hydrocortisone) drugs elicit significant inhibition of the second phase of the formalin-induced nociception (18). In addition, the MEST also inhibited the neurogenic and inflammatory phase of the formalin test, suggesting that MEST has anti-nociceptive effects against inflammatory and pain mediators. Thus, we have demonstrated the anti-nociceptive property of *S. terebinthifolius* extract without the modification of the locomotor performance.

In carrageenan model, which induced mechanical hyperalgesia, the MEST presents an anti-hyperalgesic effect. The release of several mediators could induce the peripheral nociceptor sensibilization. Dexamethasone treatment inhibited carrageenan-induced hyperalgesia, and similar results were shown by Ferreira *et al.* with a different dose (19) showing that anti-inflammatory agent reduce hyperalgesia. These results showed that MEST also resulted in a reduction of nociceptor sensibilization induced by carrageenan. The animal model of CFA-induced inflammation is widely used in chronic arthritis experiments, and the single injection of CFA used in our study induced hyperalgesia, knee oedema, myeloperoxidase activity, depression, and cold- and hot-hypersensitivity in mice, corroborating rother studies in the literature (20-22).

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Dexamethasone is typically considered a positive control drug because this drug reduces hyperalgesia, knee oedema, and myeloperoxidase activity in experimental studies (20). On the other hand, the extract obtained from *S. terebinthifolius* inhibited four parameters (mechanical hyperalgesia, knee oedema, cold- and hot-hypersensitivity) but not the depression induced by unilateral paw injection of CFA injection. The anti-arthritic and decreased sensitivity to cold and heat effects induced by MEST treatment suggest an alteration of fibers responsive to mechanical stimulus and thermal (hot and cold) thresholds. These data suggest that *S. terebinthifolius* demonstrates anti-hypergesic mechanical and thermic effects without inferring in depression-like behavior induced by CFA.

**Conclusion**

The results obtained in this study suggest that the methanolic extract of *S. terebinthifolius* leaves may be valuable for the development of new active drugs against pathophysiological processes related to pain and inflammation.

**Authors Contributions**

EB, JA, MMS and CALK performed pharmacological assays. ASNF and PCOJr prepared the extract and did the phytochemical analysis. PCOJr and CALK ensured statistical analysis, wrote, and corrected the manuscript.

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**Conflict of Interests**

The authors declare there are no financial and/or personal relationships that could present a potential conflict of interest.

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**Conclusão**

Os resultados obtidos neste estudo sugerem que o extrato metanólico de folhas de *S. terebinthifolius* pode ser valioso para o desenvolvimento de novos fármacos ativos contra processos fisiopatológicos relacionados à dor e inflamação.

**Contribuições dos autores**

EB, JÁ, MMS e CALK realizaram ensaios farmacológicos. ASNF e PCOJr prepararam o extrato e fizeram a análise fitoquímica. PCOJr e CALK, garantiram a análise estatística, escreveram e corrigiram o manuscrito.

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**Conflito de Interesses**

Os autores declararam não haver relações financeiras e/ou pessoais que possam apresentar um potencial conflito de interesses.
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