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Acrocomia aculeata oil-loaded nanoemulsion: development, stability, antinflammatory properties and cytotoxicity evaluation

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Abstract

The oil from the pulp of the bocaiúva fruit may have several medical applications. However, little is known about its pharmacological activity. Therefore, this study aimed to develop and evaluate the anti-inflammatory activity of a nanoemulsion loaded with the oil extracted from the pulp of the fruit of *Acrocomia aculeata*. Griffin's method determined the hydrophilic-lipophilic equilibrium ratio of the nanoemulsion. It was shown to have an adequate particle size (173.60 nm) with excellent homogeneity (polydispersity index 0.200). The anti-inflammatory activity of the nanoemulsion was evaluated by the carrageenan-induced paw edema method. Finally, the hemolytic and cytotoxic activity of the nano formulation were determined to assess its safety. This nanoemulsion loaded with *Acrocomia aculeata* fruit pulp oil was shown to have parameters suitable to its characterization, impressive anti-inflammatory activity, and a safe profile.

Keywords

Acrocomia aculeata; inflammation; nanoemulsion; cytotoxicity; hemolysis

Introduction

Acrocomia aculeata Jacq is a palm of the Arecaceae family, commonly known as bocaiúva or macaúba. It is widespread in South America and is particularly abundant in Mato Grosso do Sul, located in the Center-West region of Brazil [1]. The rounded fruits of this palm have a pleasant aroma and flavor and are traditionally consumed by the native population [2,3].

45 Bocaiúva oil contains several antioxidant compounds such as phenols, terpenes, β-
46 carotenes, free fatty acids, monoglycerides, triglycerides, and sterols [4-6]. These
47 compounds have the potential to enhance immune response, reduce the risk of
48 degenerative diseases and contribute to anti-inflammatory activity [7,8], reducing the
49 indiscriminate use of non-steroidal anti-inflammatory drugs (NSAIDs) and
50 corticosteroids in the population [9,10] as chronic use of these drugs has been
51 observed to produce severe adverse reactions in patients [11,12]. In addition, the
52 economic impact of these degenerative and inflammatory diseases is significant,
53 underscoring the need to find effective alternative treatments for the prevention and
54 treatment of these pathologies [13-15].
55 Nanoparticle systems loaded with plant extracts have demonstrated pharmacological
56 activities that were not observed when these extracts were used naturally [16]. For
57 example, plant oil-loaded nanoemulsions exhibit high water solubility, improved
58 permeability, and enhanced bioavailability [17]. This contrasts with the limited solubility
59 and poor bioavailability of natural oil through different routes of administration [18-21].
60 To add more value to the oil obtained from bocaiúva pulp, which contains phenols and
61 carotenoids that confer excellent stability, the possibility of developing
62 nanotechnological products with potent pharmacological activity was considered.
63 Therefore, the objective of this study was to develop, characterize, and evaluate the
64 anti-inflammatory activity of the nanoemulsion loaded with *Acrocomia aculeata* oil.

65 # Results and Discussion

66 Vegetable oils are known for their high content of fatty acids, which possess a diverse
67 range of biological activities, including hypoglycemic [32], cholesterol-lowering, anti-

68 inflammatory, and antioxidant effects [8,33-35]. Bocaiúva oil is widely used to treat
69 cardiovascular, inflammatory, and renal diseases [36,37].
70 In addition, one of the main characteristics of this oil is its orange color due to the
71 presence of phenols and carotenoids, which were characterized in this study. These
72 secondary metabolites are considered to have high antioxidant activity and provide
73 high stability to the oil [38]. These metabolites have been shown to possess anti-
74 inflammatory and immunostimulant properties [1,39].

75 Physicochemical characterization and lipid profile of *Acrocomia* 76 *aculeata* fruit pulp oil

77 The physicochemical parameters of bocaiúva oil, such as acidity index, iodine index,
78 and refractive index, were analyzed. The acidity index indicates the state of
79 conservation of oils and fats and is related to the oxidation process. Our results showed
80 an acid index of 0.92 ± 0.10. The Iodine Index determines the amount of unsaturation
81 in fatty acids [33]. Our results showed an Iodine Index of 74.50 ± 1.50 g I2/100 g,
82 values that are within the range allowed (58-75) by OMS/FAO for oils with high oleic
83 acid content [40].
84 Also, quality indicators such as refractive index, solubility in different organic solvents,
85 and relative density showed that the bocaiúva oil used in that study had good purity.
86 [41]. Coimbra & Jorge analyzed *Acrocomia aculeata* oil and found similar values to the
87 refractive index in this study (1.456 ± 0.01) [33]. These results were found within the
88 reference values established for oils rich in oleic acids, such as extra virgin olive oil,
89 palm oil, and almond oil [40]. The presence of polyphenols and carotenoids was also
90 identified in this oil (see Table 1).
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92

93 **Table 1:** Physicochemical properties of *Acrocomia aculeata* fruit pulp oil.

Property	Value
Relative density	0.9000 ± 0.0001
Iodine value (g I2 /100 g)	74.50 ± 1.50
Refractive index (30°C)	1.456 ± 0.001
Peroxide value (mEq/Kg oil)	4.50 ± 0.40
Saponification index (mg KOH/g)	133.00 ± 4.50
Acidity	0.92 ± 0.10
Total carotenoids (µg/g)	266,00 ± 12,00
Polyphenols (mg/g)	12.60 ± 0.30

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103 Table 2 shows the profile of fatty acids present in *Acrocomia aculeata* fruit pulp oil.
104 Oleic acid was the major component (71.25 %) among monounsaturated fatty acids
105 (73.79%). Therefore, bocaiúva oil can be considered an oil with a cardioprotective
106 effect due to its high oleic acid content [42,43]. In addition, its levels of
107 monounsaturated fatty acids are higher than those found in extra virgin olive, soybean,
108 corn, sunflower, and flaxseed oils [43,44].
109 The bocaiúva oil used in this work is of excellent quality according to the quality
110 parameters used to qualify vegetable oils reported in the literature [8,23]. These data
111 are similar to those found by Hiane et al, and Lieb et al, where the fruit pulp was also
112 rich in monounsaturated fatty acids [5,45].
113 A study by Amaral et al determined the presence of 69.07% oleic acid in the oil of
114 *Acrocomia aculeata* pulp [46]. These minor differences can be explained by the
115 different climatic conditions, temperature, and drying time during which the pulps were
116 exposed before extracting the oil. Even the extraction method can accelerate free fat
117 formation [33]. However, these data are similar and indicate the critical chemical
118 composition of the oil studied.

119

120 **Table 2**. Lipid profile of *Acrocomia aculeata* fruit pulp oil.

Fatty acids	Content (%)	RE Index	LR* Index
	Saturated		
Hexanoic acid	0.22 ± 0.02	974	975
Octanoic acid	0.25 ± 0.02	1169	1170
Decanoic acid	0.13 ± 0.01	1365	1365
Dodecanoic acid	0.85 ± 0.01	1548	1547
Tetradecanoic acid	0.70 ± 0.01	1747	1749
Hexadecanoic acid	16.52 ± 0.15	1970	1969
Octadecanoic acid	4.11 ± 0.15	2164	2165
Docosanoic acid	0.06 ± 0.03	2562	2564
Subtotal	**22.84 ± 0.05**		
	Monounsaturated		
9-hexadecenoic acid	2.54 ± 0.01	1939	1938
9-octadecenoic acid	71,25 ± 2,21	2241	2142
Subtotal	73.79 ± 1.11		
	Polyunsaturates		
9,12,15-octadecatrienoic acid	0.80 ± 0.04	2154	2155
9,12-octadecadienoic acid	2.20 ± 0.33	2176	2175
Eicosanoic acid	0.20 ± 0.03	2369	2370
Subtotal	3.20 ± 0.13		
Total fatty acids	> 99.00%		

121 *Literate retention rate (from NIST chemistry webbook, SRD 69).

122 Preparation of nanoemulsions required hydrophilic-lipophilic 123 balance, particle size and zeta potential, and shelf stability.

124 To develop a nanoemulsion, it is necessary to determine the required hydrophilic-
125 lipophilic balance (HLBr), particle size and polydispersity index [27,47,48]. In our study,
126 the nanoemulsion of *Acrocomia aculeata* presented a homogeneous particle size
127 distribution and a stable polydispersity index when formulated with the surfactant
128 presenting a hydrophilic-lipophilic balance of 12, parameters that define the stability of
129 this nanoformulation.

130 A hydrophilic emulsifier is known to be assigned a high HLB and a lipophilic emulsifier
131 a low HLB number. Therefore, the midpoint is approximately ten, and the assigned
132 values range from one to forty [47] see Figure 1.
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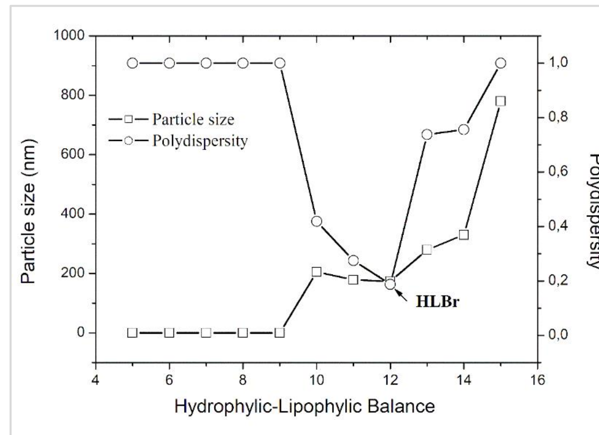

143 **Figure 1:** Particle size and polydispersity index of nanoemulsions versus the
144 hydrophilic-lipophilic balance of the surfactant system used for the preparation.
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146 Using a surfactant system with an HLB value of 12, an adequate polydispersity index
147 (0.200), necessary to emulsify bocaiuva oil, was obtained. Also, the stability of this
148 nanoemulsion was demonstrated by its ability to maintain stable Z-potential and
149 particle size parameters at 25°C ± 2°C for 180 days on shelf. The particle size
150 distribution about intensity was 173.6 ±0.70 nm (Figure. 2a). The Z potential of the
151 nanoemulsion prepared with 0.28 parts of Span 80 and 0.72 parts of Tween 80 gave
152 the value of -14.10 ±1.06 mV (Figure. 2b).
153
154 It should be noted that antioxidant compounds such as phenolics and carotenoids
155 contained in this oil are considered potent antioxidants, contributing to the stability of
156 the nanoemulsion [49,50].
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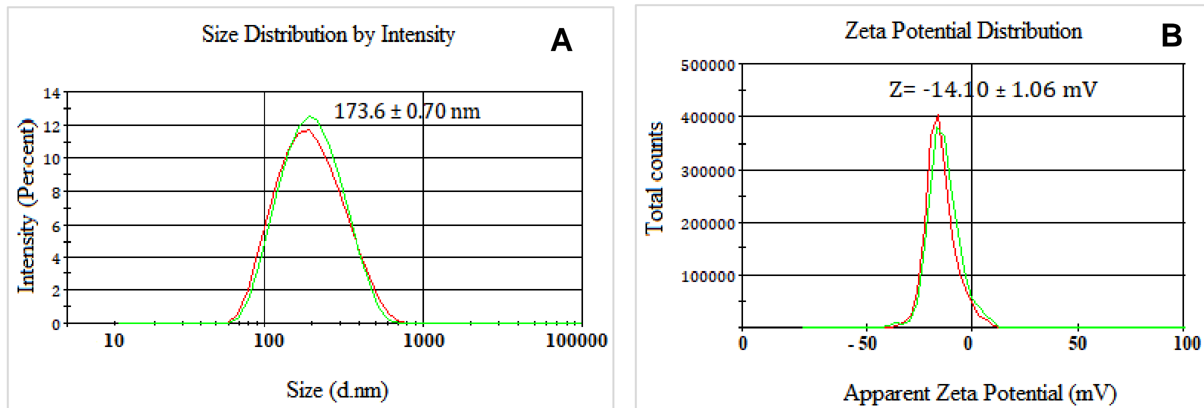

165 **Figure 2**. Particle size distribution and z-potential of the nanoemulsion prepared with

166 0.28 parts Span 80 and 0.72 parts Tween 80 (HLB = 12).

167

168 The stability of the nanoemulsion was demonstrated by its ability to maintain stable Z-

169 potential and particle size parameters at 25°C ± 2°C for 180 days on the shelf (see

170 Figure. 3).

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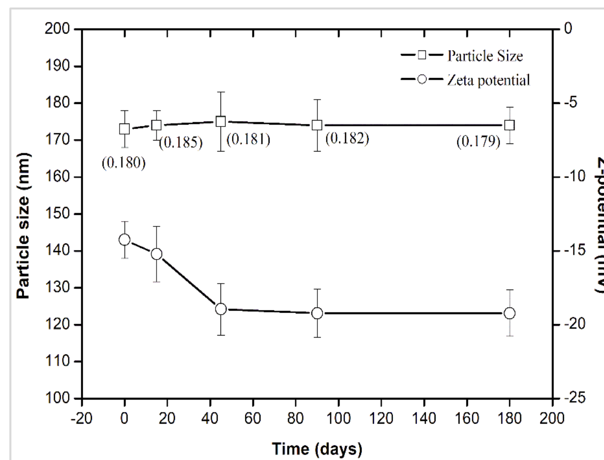

180 **Figure 3**. Stability of Bocaiúva oil-loaded nanoemulsion prepared with 0.28 parts of

181 Span 80 and 0.72 part of Tween 80 (HLB = 12).

182 Figure 4 illustrates the effect of temperature (from 10°C to 80°C) on AANE particle size

183 over 180 days. From 0°C to 60°C, particle size remained between 171 and 181 nm.

184 Above 60°C, the particle size decreased systematically remaining between 110 and

185 120 nm at all time points.

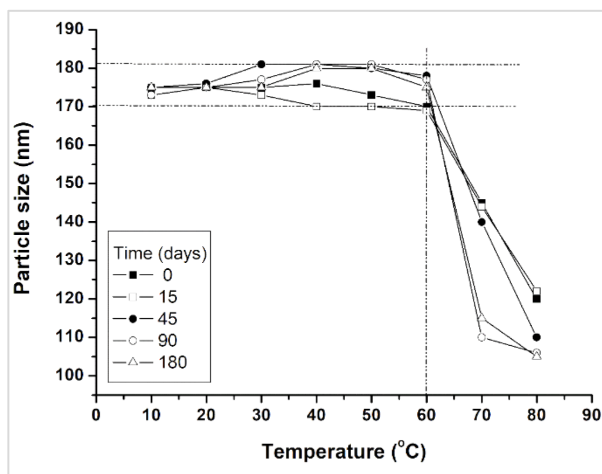

Fig 4. Effect of temperature on particle size of nanoemulsion loaded with bocaiúva oil.

Anti-inflammatory activity

Once the quality of this nanoformulation was demonstrated, its anti-inflammatory, hemolytic, and cytotoxic activity was evaluated. In inflammatory processes, pharmacological therapies focus on reducing the productive phase of the inflammatory response. This includes inhibition of leukocyte influx [51].

In our study, the inflammatory response was induced by subplantar injection of carrageenan. This is a sulfated polysaccharide that acts as an inducer of the acute inflammatory process. This is due to the action of various proinflammatory mediators associated with hyperalgesia, which induces edema [52]. Carrageenan-induced paw edema is a test that allows the evaluation of two critical parameters in the inflammatory process: leukocyte migration and protein extravasation.

Evaluation of anti-inflammatory activity revealed that *Acrocomia aculeata* nanoemulsion (50 mg/kg) possessed this pharmacological effect, which was two times higher than that of *Acrocomia aculeata* oil (100 mg/kg). This confirms that nanoformulated systems can have superior pharmacological activities compared to formulations with extracts used in their natural form [16].

211 The higher anti-inflammatory activity of AANE may be attributed to the nanometer size
212 of the particle, which increases its ability to interact with receptors for uptake [53]. This
213 result suggests that bocaiuva oil-loaded nanoemulsion is a promising anti-
214 inflammatory product.
215 Carrageenan-induced paw edema model evaluated the anti-inflammatory effect of
216 AAO and AANE. The anti-inflammatory activity of AAO (100mg/kg b.w.) was much
217 lower (at 3 hours of treatment) than that of diclofenac and AANE (at doses of 5,10, 20
218 and 50mg/kg b.w.). The same behavior was observed at 6 hours of treatment with the
219 different doses of AANE (see Figure 5).
220 At 3 hours of treatment, nanoemulsion showed an anti-inflammatory response superior
221 to diclofenac at doses of 20 and 50 mg/kg. At 6 hours, AANE showed an anti-
222 inflammatory response similar to diclofenac at the 20 and 50 mg/kg doses.
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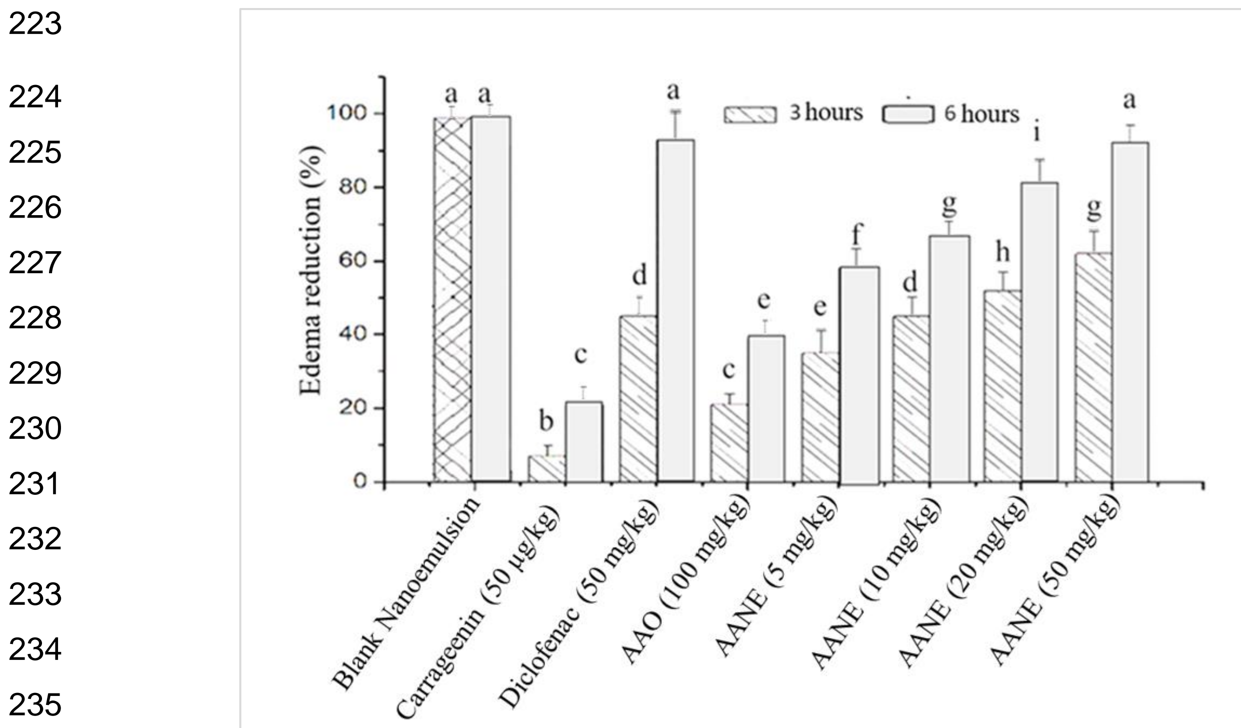
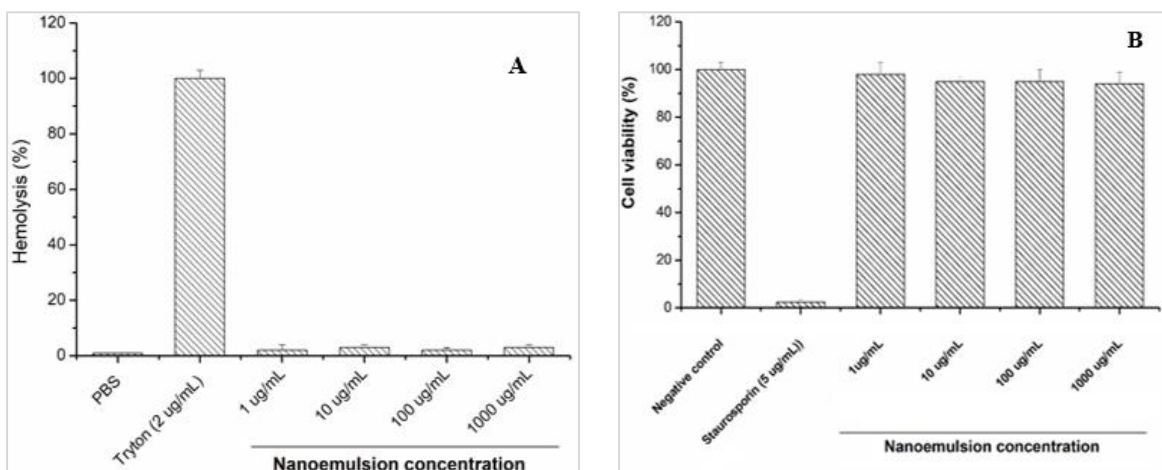


237 **Figure 5**. Anti-inflammatory effect of *Acrocomia aculeata* fruit oil and oil-loaded
238 nanoemulsion. Different letters indicate statistically significant differences at p≤0.05.

239 ## Hemolytic and cytotoxic activity

240 Regarding the evaluation of the hemolytic and cytotoxic activity of the nanoemulsion
241 to check the safety for animal and human use and to assess the potential damage that
242 AANE could cause to the erythrocyte membrane, it was observed that the
243 nanoemulsion did not induce hemolysis of murine erythrocytes at concentrations of 1,
244 10, 100 and 1000 μg/ml compared to Triton X-100 which is a substance known for its
245 remarkable hemolytic effect [54]. It was observed that our nanoemulsion at
246 concentrations of 1, 10, 100, and 1000 μg/ml did not cause erythrocyte hemolysis
247 (Figure. 6A).

248 Furthermore, cytotoxicity evaluation showed that AANE did not present cytotoxic
249 effects, as all cells maintained viability (100%) at concentrations of 1, 10, 100 and 1000
250 μg/ml (see Figure. 6B). These results suggest that this nanoemulsion could be used
251 safely. This fact could be reinforced by how *Acrocomia aculeata* oil is used by the
252 population, which drinks bocaiúva oil in its natural form and spreads it over infected
253 and inflamed body areas [55]. However, further studies should elucidate the absolute
254 safety of its use.



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256 **Figure 6**. Hemolytic effect (A) and cell viability assay (B) of nanoemulsion loaded with
257 *Acrocomia Aculeata* fruit oil.

258 # Conclusion

259 A nanoemulsion loaded with *Acrocomia aculeata* oil was developed using 0.28 parts
260 of Span 80 and 0.72 part of Tween 80 (HLB = 12). The AAO-loaded nanoemulsion
261 showed a particle size of 185 nm and a stable polydispersity index of less than 0.200.
262 The particle size distribution in relation to the intensity was 173.6 ±0.70 nm, with high
263 homogeneity and excellent shelf stability, where the properties of the nanoemulsion
264 remained practically constant after storage for 6 months.
265 Our nano formulation showed a good anti-inflammatory effect since it was
266 demonstrated that after 6 h, the anti-inflammatory effect was more potent than that of
267 *Acrocomia aculeata* fruit pulp oil and similar to that of diclofenac 50 mg/kg and did not
268 show cytotoxicity or hemolytic activity. The oil from the fruit of *Acrocomia Aculeata* can
269 be transformed into a nanotechnological product, which adds value to the oil from this
270 regional fruit.

271 # Experimental

272 ## Plant material

273 The fruits of *Acrocomia aculeata* were collected in Campo Grande, Mato Grosso do
274 Sul, Brazil (-20°.50'00.1" S, -54°.36'45.7" W) after the natural fall of the first ripe fruits.
275 The fruit pulp was manually separated from the seeds and preserved until oil
276 extraction.

277 ## Bocaiúva Oil Extraction

278 One kilogram of fresh fruit pulp was placed in an Erlenmeyer flask and extracted with
279 n-hexane (1000 ml) by mechanical agitation for 24 hours. The n-hexane solution was
280 separated from the pulp and preserved. Another 500 ml of n-hexane was added to the

281 pulp for a second extraction under the same conditions. The two extractions were
282 combined in a rotary evaporator system (Ika Werke, Germany). It was subjected to a
283 slow stream of nitrogen for 24 hours to obtain the solvent-free oil.

284 Physical-chemical characterization

285 The relative density and refractive index of *Acrocomia aculeata* oil (AAO) were
286 evaluated according to the American Pharmacopoeia [22]. Iodine value, peroxide
287 value, acid value and saponification index were also evaluated following the protocols
288 of the Brazilian Pharmacopoeia [23]

289 Determination of phenolic content

290 The total phenols present in AAO were evaluated using the Folin-Ciocalteu
291 spectrophotometric method. This method mixed 3 ml of bocaiúva oil with 10 ml of a
292 75% ethanol solution. The mixture was stirred on a mechanical shaker for 2 hours and
293 allowed to stand in the dark for 24 hours. The liquid was then centrifuged at 5000 rpm
294 (LKP, Brazil). Aliquots of 1 mL of the ethanolic phase were used for analysis. The
295 calibration curve was constructed using the standard addition method and a standard
296 reference material (Sigma, USA). The results were expressed as gallic acid equivalent.

297 Determination of carotenoid content

298 Carotenoid content was evaluated spectrophotometrically (Shimatsu, Japan) following
299 the procedure described by Rodríguez-Amaya. The molar extinction coefficient of β-
300 carotene (β-C) in n-hexane at 453 nm (2592 mol-1cm-1) was used. Carotenoid content
301 (CT), expressed as β-carotene, was calculated by the formula:

302 $$TC\ (\mu g/100) + (A * V * 10000)/(\varepsilon * m)$$

303 Where: A is the absorbance of the sample, V is the volume of the sample, ε is the molar
304 absorbance of β-carotene in n-hexane at 453 nm, and m is the mass of the sample
305 [24].

306 **Lipid profile**

307 A derivatization process was carried out to improve the stability of bocaiúva samples
308 using a Mega 2 series gas chromatograph coupled to a SHIMADZU GCMS-QP500
309 mass spectrometer (GC/MS) (Japan). One gram of bocaiúva oil was dissolved in n-
310 hexane and vortexed for five minutes. The hexane phase was separated by
311 centrifugation, transferred to a derivatization tube, and dried under a stream of nitrogen
312 for 24 hours. Then, 3 ml of a 2% methanolic NaOH solution was added to the tube.
313 The tube was hermetically sealed and heated at 85°C for 3 minutes. After cooling to
314 room temperature, 2 ml of a BF3/methanol solution was added. The tube was resealed
315 and heated for 25 minutes.
316 Once cooled, the solution was extracted with 5 ml of n-hexane and centrifuged. Twenty
317 microliters of supernatant (hexane phase) were injected directly into the GC-MS
318 system [25].
319 GC-MS analysis was performed using a Mega 2 series gas chromatograph coupled to
320 a SHIMADZU GCMS-QP500 mass spectrometer A 30 m x 0.32 mm capillary column
321 with a 0.25 mm thick layer (66DB-5MS, Agilent Technologies, USA) was used as the
322 stationary phase. Helium gas was used as carrier gas at a flow rate of 1.0 ml/min with
323 a split ratio of 1:10. The injector temperature was adjusted and set to 250 °C. The
324 oven temperature was set at 130 °C for 10 min and then increased to 250 °C at a rate
325 of 5 °C/min, maintaining the final temperature for 10 min.
326 Mass spectra were acquired using a mass range of m/z 40-500, an interface
327 temperature of 250 °C, and an ion source temperature of 220 °C. The solvent cutoff

328 time was 3 min and the event time was 0.20 min. The sweep speed was set at 2,500
329 mL/minute. The composition (in percent) was calculated using the peak normalization
330 method.

331 Preparation of nanoemulsions

332 *Acrocomia aculeata* oil nanoemulsions (AANE) were prepared using the phase
333 inversion method [26] The formulations comprised 5% w/w bocaiúva oil, 5%
334 surfactants (Span 80: Tween 80), and 90% deionized water. The organic phase,
335 composed of bocaiúva oil and surfactants, was stirred at 400 rpm at 35°C for 20 min.
336 The aqueous phase (deionized water with conductivity < 0.4 μS and pH 6.5) was added
337 to the organic phase at 1 ml/min under continuous magnetic stirring (400 rpm). Stirring
338 was maintained for 20 min after adding the total volume of water. Finally, the initial
339 volume of the nanoemulsion (50 ml) was restored with deionized water [27].

340 Required hydrophilic-lipophilic balance (HLBr)

341 Griffin's method determined the hydrophilic-lipophilic balance (HLBr) necessary to
342 emulsify bocaiúva oil [28]. A set of nanoemulsions was prepared using HLB values
343 from 4.3 to 15, obtained by mixing different proportions of Span 80 (HLB 4.3) and
344 Tween 80 (HLB 15). The temperature was maintained at 25±1°C. The surfactant
345 mixture that produces a stable nanoemulsion with the smallest particle size will be
346 selected as the (HLBr) to emulsify bocaiúva oil [28].

347 Particle size and zeta potential

348 Particle size and polydispersity index (PDI) were measured by dynamic light scattering
349 (DLS) with a Zetasizer Nano-ZS instrument (Malvern, UK). Z-potential was determined
350 by electrophoretic light scattering with a Zetasizer Nano-ZS instrument (Malvern, UK).

351 NEAA was diluted with Milli-Q water (1:25, v:v). All measurements were performed in
352 triplicate, and results were presented as the mean ± standard deviation [27].

353 **Shelf stability**

354 The selected AANE was transferred to amber vial and stored at 25±2°C for 180 days.
355 Particle size, polydispersity index and Z-potential were measured at 0, 15, 45, 90 and
356 180 days. Measurements were performed in triplicate and results were presented as
357 the mean ± standard deviation.
358 The effect of temperature on particle size was also evaluated, from 10 to 70°C, at the
359 same time intervals mentioned above. Measurements were performed with the
360 Zetasizer instrument (Malvern, UK). The nanoemulsion was equilibrated at
361 temperatures of 10, 20, 30, 40, 40, 50, 60, and 70 °C for five minutes prior to
362 measurement [27].

363 Anti-inflammatory activity

364 **Animals**

365 The anti-inflammatory effect was evaluated using carrageenan-induced paw edema.
366 Six- to eight-week-old female Swiss mice weighing 22 to 28 g were used [29]. Animals
367 were acclimatized under laboratory conditions (25 ± 3°C, 65 ± 5% humidity) with a
368 12/12 h light/dark cycle. Animals had free access to food and water at all times and
369 were deprived of food six hours before the experiment.

370 **Formation of experimental groups and induction of leg edema.**

371 Eight experimental groups were randomly formed, with five animals per group (n=5).
372 Thirty minutes before edema induction, groups 3, 4, 5, 6, 7, and 8 received the test
373 substances (diclofenac sodium, AAO, or AANE) except group 1, which received blank
374 nanoemulsion (nanoemulsion obtained under the same conditions, but without AAO),

375 and group 2 which received distilled water. After this time, 50 µL of Carrageenan was
376 injected substantially into the right hind paw of animals in groups 2, 3, 4, 5, 6, 7, and 8
377 to induce edema, except for animals in group 1 that received only 50 µL of 0.9% saline
378 in the right hind paw as well.
379 Therefore, the experimental groups were organized as follows: Group 1: Blank
380 nanoemulsion (50 mg/kg blank nanoemulsion orally +50 µl injected saline), Group 2
381 (200 µl distilled water orally +50 µl carrageenan), Group 3: (diclofenac sodium 50
382 mg/kg +50 µl carrageenan), Group 4 (AAO 100 mg/kg +50 µl carrageenan); Groups 5,
383 6, 7 and 8 (AANE:5, 10, 20 and 50 mg/kg respectively +50 µl carrageenan).
384 Edema volume was measured by plethysmometry (NovaLab, Brazil) at 3 and 6 hours
385 after carrageenan injection [30]. Edema reduction was expressed (in percentage) as
386 the difference between the control value (paw volume of each animal before
387 carrageenan injection) and the volumes measured at each time point after the
388 treatments [28].

389 Hemolytic activity

390 Hemolytic activity was assessed using a murine erythrocyte suspension as described
391 by Amado et al. Briefly, 190 µl of erythrocyte suspension was added to the wells of a
392 96-well polycarbonate plate. Then, ten µl of nanoemulsion solutions at different
393 concentrations in PBS buffer (0, 5, 10, 10, 20, and 50 µg/ml) were added to each well.
394 The plates were incubated for one hour at 37 °C, followed by centrifugation [26].
395 The amount of hemoglobin was determined at 540 nm using docetaxel (DTX) 880
396 multi-mode detector (Beckman, UK). A solution of 10 µg/ml Triton X-100 was used as
397 a positive control, and 10 µl of PBS buffer was used as a negative control. The assay
398 was performed in triplicate.

399 ## Cytotoxic activity

400 The cytotoxicity of AANE was evaluated in murine macrophages. Staurosporine (5
401 μg/ml) was used as a positive control, whereas, cells from the culture without the
402 nanoemulsion served as a negative control.
403 Different concentrations of the nanoemulsion (1.65, 3.30, 6.60, 12.5, 25, 50, and 100
404 μg/ml) were added to the cultured cells and kept in contact for 24 hours. Assays were
405 performed in triplicate, and cell viability was expressed as a percentage according to
406 International Organization for Standardization ISO 10993-5 guidelines [31].

407 ## Statistical analysis

408 A one-way ANOVA followed by Tukey's HSD test was performed to determine
409 statistical differences between experimental groups. A statistically significant
410 difference was considered at $p \leq 0.05$. StatGraphics® Centurion XV.1 software
411 (StatEase, USA) was used for the analyses.

412 ## Ethical approval

413 All experiments were performed in accordance with the Ethics Committee for the
414 Experimental Use of Animals of the Federal University of Mato Grosso do Sul, Brazil
415 (Reference number: 1.250/2022).

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420 ## Conflict of Interest

421 The authors confirm that there is no conflict of interest related to the article.

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