UHPLC-ESI-Orbitrap-HR-MS Analysis of Cyclopeptide Alkaloids From Ziziphus joazeiro

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Abstract

Ziziphus joazeiro Mart., popularly known as “juazeiro”, is a tree widely found in the northeast of Brazil. It is commonly used as an anti-inflammatory, antibacterial, antifungal, and analgesic agent. The stem extract exhibited, beside cytotoxic properties, substantial activity against the Gram-negative bacterium Allivibrio fischeri. UHPLC-ESI-Orbitrap-HR-MS analysis of the alkaloidal fraction of the crude methanolic stem extract of this species enabled the detection and putative identification of sixteen cyclopeptide alkaloids (CPAs), including four possibly new structures. According to the MS² fragmentation analysis, from the sixteen identified CPAs, three possess a type-Ia1, one a type-Ia2, and twelve a type-Ib cyclopeptide alkaloid core. The structures of paliurine-C and -D were supported by NMR data.

Keywords

Ziziphus joazeiro mart., Rhamnaceae, cyclopeptide alkaloids, ansa-type cyclopeptide, UHPLC-ESI-orbitrap-HR-MS

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Introduction

Cyclopeptide alkaloids (CPAs) are macrocyclic compounds (13-15 members), defined as heterocyclopeptides due to the presence of a styrylaminine moiety.¹ They are commonly found in the Rhamnaceae family, especially in Ziziphus species, but are also present in other families like Asteraceae, Celastraceae, Euphorbiaceae, and Rubiaceae. According to their ring characteristics, CPAs are divided into subtypes. These compounds have been reported as being active on the central nervous system, e.g. as an analgesic, in addition to anti-inflammatory, antibacterial, antimycobacterial, antifungal, antiplasmodial, and immunostimulant properties. This class of secondary metabolites is found in almost all plant parts, like roots, stems, seeds, fruits, and flowers, but most commonly in the bark derived from roots or stems.¹³

Ziziphus joazeiro Mart., popularly known as “juazeiro”, is a tree that often occurs in the northeast of Brazil (Caatinga biome). Its leaves and stem-bark are used in folk medicine against fever, bacterial and fungal infections, chronic bronchitis, and gingivitis, while its stem is used to treat fungal mouth infection, flu and tooth decay.¹⁴,¹⁵ Phytochemical investigations led to the isolation and identification of several compounds, especially triterpenes (lupane-, ceanothane- and dammarane-type), flavonoids and phenolic derivatives.¹⁶,¹⁷ Despite the high number of phytochemical studies with Z. joazeiro, there are only few studies describing the isolation and structural elucidation of cyclopeptide alkaloids from this species (nummularine-K, zoazzerine and nummularine-M).¹⁰,¹¹ Triggered by the widespread use of Z. joazeiro and following up on our earlier analytical and synthetic work on such ansa-type cyclopeptide alkaloids from Rhamnaceae and Ziziphus spp. in general, we take a deeper look on such compounds for this species.⁹,¹²,¹³-¹⁵

Results and Discussion

To characterize cyclopeptide alkaloids, the alkaloidal fraction of the crude methanolic extract of stems from Z. joazeiro was analyzed by UHPLC-ESI-Orbitrap-HR-MS. It is noteworthy that in most cases, a high amount of botanical material is necessary
to obtain feasible amounts of isolated compounds to evaluate its biological activities.\textsuperscript{16,17}

The positive ion UHPLC-ESI-HR-MS base peak chromatogram (Figure 1) shows approximately twenty peaks. Among them, sixteen cyclopeptide alkaloids (CPA) could be detected and putatively identified\textsuperscript{18} based on their elemental composition and fragmentation behavior. The detailed results are summarized in Table 1.

The fragmentation patterns of compounds 1 to 16 (Figure 2, see also Figures S1–S42) were proposed according to the rules of mass fragmentation under ESI conditions (Figure 3), and some models from the literature.\textsuperscript{31-33} From the sixteen detected CPAs, three possess a type-Ia1, one a type-Ia2 and twelve a type-Ib cyclopeptide alkaloid core. As usual for cyclic peptides, the fragmentation process goes through the protonation of a nitrogen atom of an amide moiety, which weakens the nitrogen-carbonyl bond of the amino acid.\textsuperscript{34} Fragmentation of type-Ia (Figure 3A), in general, is characterized by the loss of the hydroxystyrylaminine moiety (which generates fragment a), followed by the loss of carbon monoxide (CO, C-4) which leads to a relatively unstable intermediate (usually showing low intensity under the applied conditions). The successive loss of the HNC-R\textsuperscript{1} side chain forms fragment b.

Type-Ib CPA fragmentation (Figure 3B) is initiated at the side chain attached to the proline moiety by a [1,3] sigmatropic rearrangement of hydrogen to N-19, which generates the protonated ion species c attributed to the 13-membered cycle plus the side chain R\textsuperscript{1} attached at C-5. R\textsuperscript{1} can be isoleucine/leucine or phenylalanine (hydroxyphenylalanine in 4) residues as indicated by c-type fragments at m/z 374 or 408 (m/z 424 in alkaloid 4), respectively. Another proton rearrangement promotes the macrocycle opening and the loss of a CO moiety (C-7). Consequently, the removal of C\textsubscript{3}H\textsubscript{7}NO plus the amino acid residue R\textsuperscript{1}, followed by the loss of ammonia (NH\textsubscript{3}) generates low intensity peaks of m/z 233 (e, Figure 3B) and 216 (e - NH\textsubscript{3}, Figure 3B), respectively, which are characteristic for type-Ib CPA.\textsuperscript{32} In some cases, the radical fragmentation of the nitrogen-R\textsuperscript{2} bond gives the protonated side chain fragment d (Figure 3B), which is followed by the loss of CO in the next step (d - CO, Figure 3B). The m/z values of the resulting peaks are highly informative for the side chain (R\textsuperscript{2}) identification.

Alkaloids 1, 3 and 6 were found to have a type-Ia CPA core due to the observed loss of the styrylaminine moiety (C\textsubscript{9}H\textsubscript{9}NO, m/z 135), which is common for this type of CPA, as described in previous studies.\textsuperscript{33} Alkaloid 1 (tr 8.10 min, m/z 567.3167, [M+H]\textsuperscript{+}, calcd. for [C\textsubscript{31}H\textsubscript{43}N\textsubscript{4}O\textsubscript{6}]\textsuperscript{+} m/z 567.3178, Table 1), 3 (tr 8.25 min, m/z 551.3218 [M+H]\textsuperscript{+}, calcd. for [C\textsubscript{31}H\textsubscript{43}N\textsubscript{4}O\textsubscript{5}]\textsuperscript{+} m/z 551.3228, Table 1) and 6 (tr 9.00 min, m/z 535.3278 [M+H]\textsuperscript{+}, calcd. for [C\textsubscript{31}H\textsubscript{43}N\textsubscript{4}O\textsubscript{4}]\textsuperscript{+} m/z 535.3279, Table 1) showed remarkably similar fragmentation patterns (Figure 4), indicating the presence of a C\textsubscript{3}H\textsubscript{7} moiety at R\textsuperscript{3}, which corresponds to a valine or isovaline residue. However, since isovaline is not commonly found in Ziziphus CPAs, this residue is attributed to valine. The MS\textsuperscript{2} spectra of 1 and 3 exhibit the same fragment ion at m/z 416.2538 (a, calcd. for [C\textsubscript{23}H\textsubscript{34}N\textsubscript{4}O\textsubscript{7}]\textsuperscript{+} m/z 416.2544, R\textsuperscript{1}: C\textsubscript{4}H\textsubscript{8}O, R\textsuperscript{2}: C\textsubscript{4}H\textsubscript{14}NO, R\textsuperscript{3}: C\textsubscript{3}H\textsubscript{8}, Table 1). This fragment was particularly useful to assign the OH group bound to the styrylaminine moiety of alkaloid 1. Previously, several CPAs of this type were isolated bearing an OH moiety at C-1.\textsuperscript{33} The fragmentation pattern of alkaloid 3 led to the putative identification of the known CPA sanjoinine-F.\textsuperscript{21} Due to the mass difference of 16 amu (oxygen) and the fragmentation pattern (Figure S2) of

![Figure 1. UHPLC-ESI-HR-MS base peak chromatogram of crude alkaloidal fraction of Ziziphus joazeiro (positive ion mode, full MS m/z 100-2000, retention time window 7.7-10.7 min). Compounds were numbered in order of increasing retention time.](image-url)
Table 1. Ia and Ib-Type Cyclopeptide Alkaloids Characterized in the Crude Alkaloid Fraction From the Stem of *Ziziphus joazeiro*.

| Peak No. | tR (min) | m/z [M+H]+ | Elemental composition | RDB Equivalent | Error (ppm) | MS² peaks | MS² relative intensity in % | UV (nm) | Annotation (CPA type) | Ref. |
|---------|---------|------------|----------------------|----------------|------------|-----------|--------------------------|---------|----------------------|------|
| 1       | 7.95    | 567.3167   | C₂₉H₄₂N₄O₆          | 12.5           | −1.8       | 549.3068  | ([M+H-H₂O]+, 61%)         | 219;    | unknown hydroxyl derivative of sanjoinine-F (OH at C-1) (Ia1) |      |
|         |         |            |                      |                |            | 539.3226  | ([M+H-CO]+, 13%)          | 280;    |                      |      |
|         |         |            |                      |                |            | 522.2594  | ([M+H-NH(CH₃)]+ , 100%)   | 310     |                      |      |
|         |         |            |                      |                |            | 504.2479  | ([M+H-NH(CH₃)₂-H₂O]+, 12%)|        |                      |      |
|         |         |            |                      |                |            | 495.2600  | ([M+H-C₆H₁₂O]⁺, 7%)       |        |                      |      |
|         |         |            |                      |                |            | 420.2128  | ([M+H-C₄H₁₃N]⁺ , 14%)     |        |                      |      |
|         |         |            |                      |                |            | 416.2538  | (a, R₁:C₄H₈O, R²:C₆H₁₄N, R³:C₃H₇, 54%) |        |                      |      |
|         |         |            |                      |                |            | 344.1967  | (a-C₄H₈O, R²:C₁₀H₁₄N, R₃:C₃H₇, 24%) |        |                      |      |
|         |         |            |                      |                |            | 287.1753  | (b, R²:C₆H₁₄NO, R³:C₃H₇, 36%) |        |                      |      |
| 2       | 8.10    | 521.3113   | C₂₉H₄₀N₄O₄          | 12.5           | −1.8       | 490.2701  | ([M+H-CH₂NH₂]+ , 19%)     | 204;    | mauritine-L (Ia2)       |      |
|         |         |            |                      |                |            | 422.2072  | ([M+H-C₆H₁₃N]⁺ , 100%)    | 281     | or nummularine-D (Ia2)  |      |
|         |         |            |                      |                |            | 386.2440  | (a, R₁:C₄H₆O, R²:C₆H₁₄N, R³:C₆H₆, 11%) |        |                      |      |
|         |         |            |                      |                |            | 273.1600  | (b, R²:C₇H₁₄NO, R³:C₆H₅, 11%) |        |                      |      |
| 3       | 8.25    | 551.3218   | C₂₉H₄₂N₄O₅          | 12.5           | −1.8       | 533.3124  | ([M+H-H₂O]+, 52%)         | 214;    | sanjoinine-F (Ia1)      |      |
|         |         |            |                      |                |            | 523.3279  | ([M+H-CO]+, 89%)           | 280     |                      |      |
|         |         |            |                      |                |            | 506.2648  | ([M+H-NH(CH₃)]⁺ , 100%)    |        |                      |      |
|         |         |            |                      |                |            | 479.2653  | ([M+H-C₆H₁₂O]⁺ , 33%)      |        |                      |      |
|         |         |            |                      |                |            | 416.2541  | (a, R₁:C₄H₆O, R²:C₆H₁₄N, R³:C₃H₇, 83%) |        |                      |      |
|         |         |            |                      |                |            | 344.1970  | (a-C₄H₈O, R²:C₁₀H₁₄N, R₃:C₃H₇, 24%) |        |                      |      |
|         |         |            |                      |                |            | 287.1756  | (b, R²:C₆H₁₄NO, R³:C₃H₇, 36%) |        |                      |      |
| 4       | 8.62/  | 608.3068   | C₂₉H₄₁N₅O₇          | 14.5           | −1.8       | 590.2976  | ([M+H-H₂O]+, 7%)           | 206;    | unknown hydroxyl derivative of nummularine-B/nummularine-N (Ib) |      |
|         | 8.68    |            |                      |                |            | 424.1866  | (c, R₁:C₄H₆O, 73%)         | 280     |                      |      |
|         |         |            |                      |                |            | 406.1760  | (c-H₂O, R₁:C₄H₆, 100%)     |        |                      |      |
|         |         |            |                      |                |            | 378.1816  | (c-CO, R¹:C₄H₆, 2%)        |        |                      |      |
|         |         |            |                      |                |            | 318.1452  | (c-C₆H₆O, 4%)              |        |                      |      |
|         |         |            |                      |                |            | 233.1288  | (c, 1%)                    |        |                      |      |
|         |         |            |                      |                |            | 216.1022  | (c-NH₃, 6%)                |        |                      |      |
|         |         |            |                      |                |            | 204.1022  | (f, 2%)                    |        |                      |      |
| 5       | 8.86    | 558.3283   | C₂₉H₄₃N₅O₆          | 10.5           | −0.6       | 473.2674  | ([M+H-C₄H₁₃NO]+, 2%)       | 206;    | sativane-C              |      |
|         |         |            |                      |                |            | 374.2079  | (c, R¹:C₄H₆, 100%)         | 280     | or sativane-H            |      |
|         |         |            |                      |                |            | 346.2130  | (c-CO, R¹:C₄H₆, 24%)       |        | or nummularine-P (Ib)    |      |
|         |         |            |                      |                |            | 233.1289  | (c, 9%)                    |        |                      |      |
|         |         |            |                      |                |            | 216.1024  | (c-NH₃, 5%)                |        |                      |      |
|         |         |            |                      |                |            | 204.1024  | (f, 7%)                    |        |                      |      |
| 6       | 9.00    | 535.3278   | C₂₉H₄₁N₄O₄          | 12.5           | −0.2       | 507.3331  | ([M+H-CO]+, 39%)           |        | sanjoinine-A (Ia)        |      |

(Continued)
| Peak No. | $t_R$ (min) | $m/z$ [M + H]$^+$ | Elemental composition$^2$ | RDB Equiv. | Error (ppm) | MS$^2$ peaks $m/z$ (relative intensity in %) | UV (nm) | Annotation (CPA type) Ref. |
|---------|-------------|-----------------|--------------------------|------------|------------|---------------------------------|---------|--------------------------|
| 7       | 9.13        | 592.3114        | C$_{32}$H$_{41}$N$_5$O$_6$ | 14.5       | −2.6       | 408.1917 (c, R$_1$: C$_7$H$_7$, 100%) | 206;    | or lotusamine-A (Ia)    |
|         |             |                 |                          |            |            | 380.1972 (c-CO, R$_1$: C$_7$H$_7$, 13%) | 278     |                          |
|         |             |                 |                          |            |            | 233.1289 (c, 4%)                 |         |                          |
|         |             |                 |                          |            |            | 216.1023 (c-NH$_3$, 4%)          |         |                          |
|         |             |                 |                          |            |            | 204.1022 (f, 4%)                |         |                          |
|         |             |                 |                          |            |            | 185.1289 (d, R$_3$: C$_3$H$_7$, C$_3$H$_8$N, <1%) |         |                          |
|         |             |                 |                          |            |            | 175.0754 (d-CO, R$_3$: C$_3$H$_7$, C$_3$H$_8$N, <1%) |         |                          |
| 8       | 9.20        | 487.2791        | C$_{27}$H$_{30}$N$_2$O$_6$ | 9.5        | −2.4       | 148.1122 (100) | 205;    |                          |
|         |             |                 |                          |            |            | 207; nummularine-B or nummularine N (Ib) | 279     |                          |
|         |             |                 |                          |            |            | 233.1289 (e, 8%)                |         |                          |
|         |             |                 |                          |            |            | 216.1023 (e-NH$_3$, 6%)         |         |                          |
|         |             |                 |                          |            |            | 213.1602 (d, R$_2$: C$_3$H$_7$, R$_3$: C$_3$H$_8$N, 4%) |         |                          |
|         |             |                 |                          |            |            | 185.1652 (d-CO, R$_2$: C$_3$H$_7$, R$_3$: C$_3$H$_8$N, <1%) |         |                          |
| 9       | 9.28        | 586.3587        | C$_{31}$H$_7$N$_3$O$_6$   | 10.5       | −2.1       | 501.2715 ([M + H-C$_6$H$_7$N]$^+$, <1%) | 203;    | unknown (Ib)            |
|         |             |                 |                          |            |            | 473.2762 ([M + H-C$_6$H$_7$NO]$^+$, 3%) | 281     |                          |
|         |             |                 |                          |            |            | 374.2078 (c, R$_1$: C$_7$H$_7$, 100%) |         |                          |
|         |             |                 |                          |            |            | 346.2129 (c-CO, R$_1$: C$_7$H$_7$, 30%) |         |                          |
|         |             |                 |                          |            |            | 233.1289 (c, 8%)                |         |                          |
|         |             |                 |                          |            |            | 216.1023 (c-NH$_3$, 6%)         |         |                          |
|         |             |                 |                          |            |            | 204.1023 (f, 8%)                |         |                          |
|         |             |                 |                          |            |            | 213.1602 (d, R$_2$: C$_3$H$_7$, R$_3$: C$_3$H$_8$N, 4%) |         |                          |
|         |             |                 |                          |            |            | 185.1652 (d-CO, R$_2$: C$_3$H$_7$, R$_3$: C$_3$H$_8$N, <1%) |         |                          |
| 10      | 9.51        | 620.3431        | C$_{34}$H$_{43}$N$_5$O$_6$| 14.5       | −1.9       | 507.2607 ([M + H-C$_6$H$_7$NO]$^+$, 4%) | 208;    | unknown (Ib)            |
|         |             |                 |                          |            |            | 408.1918 (c, R$_1$: C$_7$H$_7$, 100%) | 280     |                          |
|         |             |                 |                          |            |            | 380.1972 (c-CO, R$_1$: C$_7$H$_7$, 18%) |         |                          |
|         |             |                 |                          |            |            | 233.1289 (c, 4%)                |         |                          |
|         |             |                 |                          |            |            | 216.1023 (c-NH$_3$, 5%)         |         |                          |
|         |             |                 |                          |            |            | 213.1602 (d, R$_2$: C$_3$H$_7$, R$_3$: C$_3$H$_8$N, 2%) |         |                          |
|         |             |                 |                          |            |            | 204.1022 (f, 4%)                |         |                          |
|         |             |                 |                          |            |            | 185.1652 (d-CO, R$_2$: C$_3$H$_7$, R$_3$: C$_3$H$_8$N, <1%) |         |                          |
| 11      | 9.70        | 583.2906        | C$_{36}$H$_{38}$N$_4$O$_8$| 17.5       | −1.5       | 555.2964 ([M + H-CO]$^+$, 83%) | 210;    | xylopyrine B (Ib)       |
|         |             |                 |                          |            |            | 538.2357 ([M + H-NH(CH$_3$)$_2$]$^+$, 5%) | 280     |                          |
|         |             |                 |                          |            |            | 436.2233 ([M + H-C$_9$H$_9$NO]$^+$, 44%) |         |                          |
|         |             |                 |                          |            |            | 408.1902 (c, R$_1$: C$_7$H$_7$, 100%) |         |                          |
|         |             |                 |                          |            |            | 380.1961 (c-CO, R$_1$: C$_7$H$_7$, 17%) |         |                          |
|         |             |                 |                          |            |            | 233.1329 (c, 5%)                |         |                          |
|         |             |                 |                          |            |            | 216.1068 (c-NH$_3$, 5%)         |         |                          |
|         |             |                 |                          |            |            | 204.1058 (f, 5%)                |         |                          |
| 12      | 10.00       | 648.3742        | C$_{38}$H$_{40}$N$_3$O$_6$| 14.5       | −2.1       | 515.2862 ([M + H-C$_6$H$_7$N]$^+$, 1%) | 217;    | palurine-D (Ib)         |

*Continued*
| Peak No. | $t_R$ (min) | $m/z$ [M +H]$^+$ | Elemental composition$^2$ | RDB Equiv. | Error (ppm) | MS$^2$ peaks $m/z$ (relative intensity in %) | UV (nm) | Annotation (CPA type) Ref. |
|---------|-------------|------------------|--------------------------|------------|------------|---------------------------------------------|---------|--------------------------|
| 13      | 10.06       | 662.3893         | C$_{27}$H$_{51}$N$_5$O$_6$ | 14.5       | −2.8       | 487.2913 ([M+H-C$_{10}$H$_{11}$NO]$^+$, 6%) 281; |         | paliurine-C (Ib)         |
|         |             |                  |                          |            |            | 402.2018 ([M+H-C$_{13}$H$_{22}$N$_2$O]$^+$, <1%) |         |                          |
|         |             |                  |                          |            |            | 374.2074 (c, R$_3$: C$_4$H$_9$, 100%)         |         |                          |
|         |             |                  |                          |            |            | 346.2125 (c-CO, R$_3$: C$_6$H$_9$, 46%)        |         |                          |
|         |             |                  |                          |            |            | 275.1756 (d, R$_2$: C$_4$H$_8$, R$_3$: C$_6$H$_{12}$N, 31%) |         |                          |
|         |             |                  |                          |            |            | 247.1806 (d-CO, R$_2$: C$_4$H$_8$, R$_3$: C$_6$H$_{12}$N, 26%) |         |                          |
|         |             |                  |                          |            |            | 233.1286 (c, 9%)                               |         |                          |
|         |             |                  |                          |            |            | 216.1021 (c-NH$_3$, 9%)                        |         |                          |
|         |             |                  |                          |            |            | 204.1021 (f, 10%)                              |         |                          |
| 14      | 10.30       | 682.3585         | C$_{30}$H$_{57}$N$_5$O$_6$ | 18.5       | −2.1       | 515.2852 ([M+H-C$_{10}$H$_{13}$N]$^+$, <1%) 214; |         | nummularine-H (Ib)       |
|         |             |                  |                          |            |            | 487.2914 ([M+H-C$_{11}$H$_{13}$NO]$^+$, 3%)    |         |                          |
|         |             |                  |                          |            |            | 374.2075 (c, R$_3$: C$_4$H$_9$, 44%)           |         |                          |
|         |             |                  |                          |            |            | 346.2126 (c-CO, R$_3$: C$_6$H$_9$, 29%)        |         |                          |
|         |             |                  |                          |            |            | 289.1912 (d, R$_2$: C$_4$H$_8$, R$_3$: C$_6$H$_{14}$N, 100%) |         |                          |
|         |             |                  |                          |            |            | 261.1963 (d-CO, R$_2$: C$_4$H$_8$, R$_3$: C$_6$H$_{14}$N, 57%) |         |                          |
|         |             |                  |                          |            |            | 233.1287 (c, 5%)                               |         |                          |
|         |             |                  |                          |            |            | 216.1021 (c-NH$_3$, 5%)                        |         |                          |
|         |             |                  |                          |            |            | 204.1022 (f, 6%)                              |         |                          |
| 15      | 10.37       | 696.3736         | C$_{40}$H$_{40}$N$_5$O$_6$ | 18.5       | −2.9       | 549.2710 ([M+H-C$_{11}$H$_{13}$N]$^+$, 4%) 220; |         | jubanine-A (Ib)         |
|         |             |                  |                          |            |            | 521.2760 ([M+H-C$_{10}$H$_{11}$NO]$^+$, 40%) 282; |         |                          |
|         |             |                  |                          |            |            | 374.2076 (c, R$_3$: C$_4$H$_9$, 100%)           |         |                          |
|         |             |                  |                          |            |            | 346.2127 (c-CO, R$_3$: C$_6$H$_9$, 53%)        |         |                          |
|         |             |                  |                          |            |            | 309.1600 (d, R$_2$: C$_4$H$_8$, R$_3$: C$_6$H$_{12}$N, 71%) |         |                          |
|         |             |                  |                          |            |            | 281.1651 (d-CO, R$_2$: C$_4$H$_8$, R$_3$: C$_6$H$_{12}$N, 61%) |         |                          |
|         |             |                  |                          |            |            | 233.1287 (c, 5%)                               |         |                          |
|         |             |                  |                          |            |            | 216.1022 (c-NH$_3$, 8%)                        |         |                          |
|         |             |                  |                          |            |            | 204.1022 (f, 9%)                              |         |                          |
| 16      | 10.48       | 716.3424         | C$_{43}$H$_{43}$N$_5$O$_6$ | 22.5       | −2.6       | 583.2556 ([M+H-C$_{10}$H$_{11}$N]$^+$, 3%) 217; |         | nummularine-O (Ib)       |
|         |             |                  |                          |            |            | 555.2603 ([M+H-C$_{10}$H$_{11}$NO]$^+$, 38%) 30 |         |                          |

(Continued)
| Peak No. | $t_R$ (min) | $m/z$ [M + H]$^+$ | Elemental composition$^2$ | RDB Equiv. | Error (ppm) | MS$^2$ peaks $m/z$ (relative intensity in %) | UV (nm) | Annotation (CPA type) Ref. |
|---------|-------------|-------------------|-----------------------------|------------|------------|--------------------------------|--------|--------------------------|
| 17      | 10.57       | 730.3583          | C$_{48}$H$_{47}$N$_{5}$O$_6$ | 22.5       | −2.1       | 408.1917 (c, R$^1$: C$_{7}$H$_7$, 100%) 380.1971 (c-CO, R$^1$: C$_{7}$H$_7$, 31%) 309.1600 (d, R$^2$: C$_{7}$H$_7$, R$^3$: C$_{6}$H$_{12}$N, 45%) 281.1651 (d-CO, R$^2$: C$_{7}$H$_7$, R$^3$: C$_{6}$H$_{12}$N, 37%) 233.1288 (e, 4%) 216.1023 (e-NH$_3$, 6%) 204.1023 (f, 3%) | 280; 315 | jubaranine-B (lb) |
alkaloid 1 it is suggested to be a hitherto unknown hydroxyl derivative of sanjoinine-F.

For alkaloid 6 the loss of the styrylaminine moiety leads to a different a-fragment ion at \( m/z \) 400.2591 (calcd. for \([C_{23}H_{34}N_3O_3]^+ m/z \) 400.2595, R\(^1\): C\(_4\)H\(_9\), R\(^2\): C\(_{11}\)H\(_{14}\)NO, R\(^3\): C\(_3\)H\(_7\), Table 1) compared to \( m/z \) 416.2541 detected for alkaloid 3 (with fragment a, calcd. for \([C_{23}H_{34}N_3O_4]^+ m/z \) 416.2544, R\(^1\): C\(_4\)H\(_9\)O, R\(^2\): C\(_{11}\)H\(_{14}\)NO and R\(^3\): C\(_3\)H\(_7\), Table 1). Therefore, it was deduced that the OH group is absent in R\(^1\) of alkaloid 6, which is also corroborated by the subsequent fragmentation leading to \( m/z \) 287.1755 (b, calcd. for \([C_{17}H_{23}N_2O_2]^+ m/z \) 287.1755, R\(^2\): C\(_{11}\)H\(_{14}\)NO, R\(^3\): C\(_3\)H\(_7\), Table 1). Thus, the fragmentation pattern of alkaloid 6 is in accordance with the known CPAs sanjoinine-A or lotusanine-A,\(^{21,24}\) which are isomers with leucine or isoleucine residual chains as R\(^1\), respectively. In general, leucine and isoleucine residues cannot be distinguished using collision-induced fragmentation experiments during mass spectrometric analysis.

Alkaloid 2 (t\(_R\) 8.10 min, \( m/z \) 521.3113 [M+H]\(^+\) calcd. for \([C_{30}H_{41}N_4O_4]^+ m/z \) 521.3123, Table 1) features a type-Ia2 CPA core due to the presence of different a and b fragments at \( m/z \) 386.2440 (a, calcd. for \([C_{22}H_{32}N_3O_3]^+ m/z \) 521.3123, Table 1) and \( m/z \) 273.1600 (b, calcd. for \([C_{16}H_{21}N_2O_2]^+ m/z \) 521.3123, Table 1), which indicate a phenylalanine residual moiety at R\(^3\). The fragmentation pattern (Figure S4) led to the annotation of the known mauritine-L or nummularine-D,\(^{19,20}\) position isomers with isoleucine or leucine residual chains as R\(^3\), respectively.

The twelve remaining alkaloids possess a type-Ib CPA core, characterized by a meta-substituted aromatic ring (from the styrylaminine moiety) and with a proline residual chain attached (Figure 2). The characterization is based on the high number of fragment ions arising in the MS\(^2\) spectra, which are typical for this type of CPA (Figure 3B). In particular, the fragments e (\( m/z \) 233), e − NH\(_3\) (\( m/z \) 216) and f (\( m/z \) 204) confirmed the type-Ib CPA core in alkaloids 4, 5, and 7 to 16.

Alkaloid 4 (t\(_R\) 8.62/8.68 min) shows a protonated ion peak of \( m/z \) 608.3068 [M+H]\(^+\) (calcd. for \([C_{32}H_{42}N_5O_7]^+ m/z \) 608.3079, Table 1). The MS\(^2\) spectrum displayed a fragmentation of the side chain attached to the proline residue resulting in fragment c at \( m/z \) 424 (c, R\(^1\): C\(_7\)H\(_7\)O, Figure 3B) followed by the loss of H\(_2\)O, leading to \( m/z \) 406 (c − H\(_2\)O R\(^1\): C\(_7\)H\(_6\), which was attributed to an OH group attached to R\(^1\) (phenylalanine residual chain). The fragment ions at \( m/z \) 318 (c − C\(_7\)H\(_7\)O) and \( m/z \) 590 ([M +H-H\(_2\)O]\(^+\)) confirmed the presence of an OH group at the suggested position. After full evaluation of the MS\(^2\) fragments (Figures S7 and S8), alkaloid 4 was suggested to be a new hydroxylated type-Ib CPA derivative of either nummularine-B or nummularine-N (alkaloid 7), isomers in R\(^3\) with either N-methylalanine or N,N-dimethylglycine side chains, respectively.

Alkaloid 7 (t\(_R\) 9.13 min, annotated as nummularine-B or nummularine-N,\(^{25}\) presented a protonated ion peak of \( m/z \) 592.3114 [M+H]\(^+\) (calcd. for \([C_{32}H_{42}N_4O_6]^+ m/z \) 592.3130, Table 1). The mass difference of 16 amu in comparison to alkaloid 4 is due to the absence of an oxygen atom. The MS\(^2\) spectrum shows the characteristic peaks at \( m/z \) 408 (c, R\(^1\): C\(_7\)H\(_6\), Table 1) and 380 (c − CO R\(^1\): C\(_7\)H\(_5\), Table 1). The fragmentation of the side chain led to fragments d (\( m/z \) 185) and d − CO (\( m/z \) 157), that even with low intensity (<1%) were helpful to assign the side chain (Figure S14).
Alkaloid 5 (tR 8.86 min), m/z 558.3284 [M+H]+ (calcd. for [C_{29}H_{44}N_{5}O_{6}]^{+} m/z 558.3287, Table 1), in contrast to alkaloids 4 and 7, showed a MS² fragment ion at m/z 374 (c, R1: C_{4}H_{9}), which indicates an isoleucine or leucine residual chain coupled to C-5 (R') of the CPA core. The fragmentation of the side chain resulting in m/z 473.2764 [M+H-C_{4}H_{7}NO]^{+} (calcd. for [C_{25}H_{37}N_{4}O_{5}]^{+} m/z 473.2759, Table 1) infer the presence of either N-methylalanine or N,N-dimethylglycine in R³. Therefore, the fragmentation of alkaloid 5 is in accordance with the three known isomers, sativanine-C, sativanine-H and nummularine-P²²,²³ (Figure S10), which cannot be differentiated by means of MS¹ and MS² analysis due the presence of isomeric side chain amino acids.

Compound 8 (tR 9.20 min), m/z 487.2791 [M+H]+ (calcd. for [C_{27}H_{39}N_{2}O_{6}]^{+} m/z 487.2803) exhibited only one MS² fragmentation peak of m/z 148.1122 (calcd. for [C_{10}H_{14}N]^{+} 148.1121) (Figure S15). The structural formula and fragmentation pattern are not in accordance with either known CPAs or

![Figure 3. Proposed fragmentation mechanism of type-Ia (A) and type-Ib (B) cyclopeptide alkaloids. The characteristic styrylaminine moiety is highlighted in bold in the core structures.](image-url)
any known natural product. However, the base peak fragment at $m/z$ 148 might suggest N,N-dimethylphenylalanine as the terminal amino acid residue. Therefore, it was not possible to propose any putative compound for this peak.

Alkaloid 9 ($t_R$ 9.28 min), $m/z$ 586.3587 [M+H]$^+$ (calcd. for [C$_{31}$H$_{48}$N$_5$O$_6$]+ $m/z$ 586.3600, Table 1) displayed, like alkaloid 5, a MS$^2$ peak of $m/z$ 374 (c, R$^1$: C$_2$H$_5$) indicating the presence of a leucine or isoleucine residual chain attached to C-5. The side chain attached to the proline residual chain was determined by a peak of $m/z$ 501 ([M+H-C$_6$H$_{11}$NO]$^+$, Table 1), also observed in alkaloid 7, suggesting the presence of a phenylalanine residual chain as R$^1$. The fragment peak of $m/z$ 473.2759, [M+H-C$_6$H$_{11}$NO]$^+$ (calcd. for [C$_{25}$H$_{37}$N$_4$O$_5$]+ $m/z$ 473.2759, Table 1), which corroborates the presence of a valine residual chain as R$^2$ (Figure S17).

Thus, alkaloid 9 was suggested as a yet unknown type-Ib CPA, with the molecular formula C$_{31}$H$_{47}$N$_5$O$_6$.

Alkaloid 10 presented a [M+H]$^+$ ion at $m/z$ 620.3431 [M+H]$^+$ (calcd. for [C$_{34}$H$_{46}$N$_5$O$_6$]+ $m/z$ 620.3443, Table 1). Its MS$^2$ spectrum shows a fragment ion at $m/z$ 408 (c, R$^1$: C$_7$H$_7$N, Table 1), also observed in alkaloid 7, suggesting the presence of a phenylalanine residual chain as R$^1$. The fragment peak of $m/z$ 507.2607 (calcd. for [M+H-C$_6$H$_{11}$NO]$^+$, $m/z$ 507.2602) suggests the loss of a N-methylvaline residual chain (R$^3$) and also corroborates the presence of a valine residual chain as R$^2$ due to the mass difference of 99 amu resulting in the ion at $m/z$ 408. Therefore, alkaloid 10 is proposed as a previously not described type-Ib CPA based on the high-resolution MS$^1$ and MS$^2$ spectra (Figures S18 and S19).

Table 2. Antibacterial and Cytotoxic Activity of Crude Methanolic Extract From the Stem of Z. joazeiro.

|               | Antibacterial assays | Cytotoxic assays  |
|---------------|----------------------|------------------|
|               | Inhibition [%] | Survival [%] | PC-3/MTT | PC-3/CV | HT-29/MTT | HT-29/CV |
| B. subtilis   | A. fischeri          |                  |
| Conc.         |                     |                  |
| MeOH extract  | 500 µg/mL           | 50 µg/mL         |
| 36.0 ± 29.0   | 100.0 ± 0.0         | 4.1 ± 18.2       | 7.0 ± 9.1 | 2.6 ± 24.7 | -0.3 ± 6.2 |
| 99.0 ± 1.0$^1$ | 99.5 ± 0.1$^1$   | 0.6 ± 8.9$^2$    | -0.1 ± 17.6$^2$ |

1100 µM chloramphenicol.
2125 µg/mL digitonin.
For alkaloid 11, a protonated ion peak of \( m/z \) 583.2906 \([M + H]^+ \) (calcd. for \([C_{66}H_{168}N_{34}O_{36}]^+ \) \( m/z \) 583.2915, Table 1) was determined. Typical fragment ions of type-Ib CPA were observed along with \( m/z \) 538.2357 (calcd. for \([M + H-NH\{CH_3\}_3]^+ \) \( m/z \) 538.2337), formed by a \([1,3]\)-proton rearrangement under elimination of a \( N,N \)-dimethyl moiety (Figure S20). The fragment \( m/z \) 408 (c, \( R^1 \): C-H), resulting from the loss of C1-HN(NO), suggests again the presence of a phenylalanine residual chain as \( R^1 \), and the presence of a \( N \), \( N \)-dimethylphenylalanine residual chain attached to the proline site. Alkaloid 11 was extensively analyzed, and it was suggested to be the known xylopyrine-B.26

Alkaloids 12 (\( m/z \) 648.3742 \([M + H]^+ \), calcd. for \([C_{36}H_{40}N_{34}O_{36}]^+ \) \( m/z \) 648.3756, Table 1) and 13 (\( m/z \) 662.3892 \([M + H]^+ \) calcd. for \([C_{37}H_{42}N_{34}O_{36}]^+ \) \( m/z \) 662.3913, Table 1) present very similar fragmentation patterns (Figures S22 and S28), with some equal fragments. Both exhibited a c-fragment ion at \( m/z \) 374 (\( R^1 \): C-H), suggesting leucine or isoleucine attached to the CPA core. Ions detected at \( m/z \) 487 in both alkaloids can be related to the presence of a second leucine or isoleucine residual chain in \( R^2 \). The differentiation of both alkaloids was possible due to the evaluation of peaks \( m/z \) 515 \([M + H-C_6H_11N]^+ \) in 12 and \([M + H-C_{10}H_{13}N]^+ \) in 13, arising from the loss of a \( N \)-methylphenylalanine residual chain in 12, and \( N \)-\( N \)-dimethylphenylalanine in 13. This was further confirmed by ions detected at \( m/z \) 275 (d in 12 and at \( m/z \) 289 (d in 13 formed during the loss of the side chain attached to the proline. The mass difference of 14 amu indicates one additional CH2 as a \( N \)-methyl group in 13. Further loss of CO afforded ions at \( m/z \) 247 (d – CO) in 12 and \( m/z \) 261 (d – CO) in 13. Based on MS data, alkaloids 12 and 13 could be putatively identified as nummularine-A or paluirine-D, and mucronine-D or paluirine-C, respectively.27,35

Alkaloids 12 and 13, the major CPAs detected in the alkaloidal extract, were isolated by means of HPLC, submitted to NMR analysis (see experimental), and compared to literature models.27 NMR spectra of both compounds (Figures S24–S27 and S30–S34 for compounds 12 and 13, respectively) presented signals that corroborate the presence of two isoleucine moieties at C-5 and C-20. In 13, four signals of shielded methyl groups were detected with \( \delta_{\text{H}}/\delta_{\text{C}} \) 10.9/0.86 (t, 7.3 Hz, CH3-5'), 11.1/0.85 (t, 7.3 Hz, CH3-17), 15.2/0.79 (d, 6.8 Hz, CH3-6') and 16.1/0.94 (d, 6.8 Hz, CH3-18). These data, along with their long-range heteronuclear, and homonuclear coupling (1HMB, COSY, Tables S1 and S2), were helpful to assign isoleucine instead of leucine present in the alkaloidal structure. Altogether, the evidence observed in the MS fragmentation and NMR spectra led to the unequivocal identification of alkaloid 13 as paluirine-C. The NMR data of 12 show great similarities to those of 13, with exception of positions 2' and the NMe moiety indicative for the \( N \)-methylphenylalanine residual chain in 12. Thus, compound 12 was identified as paluirine-D.27

Alkaloids 14, \( m/z \) 682.3585 \([M + H]^+ \), calcd. for \([C_{40}H_{50}N_{34}O_{36}]^+ \) \( m/z \) 682.3600, Table 1), and 15, \( m/z \) 696.3736 \([M + H]^+ \), calcd. for \([C_{40}H_{50}N_{34}O_{36}]^+ \) \( m/z \) 696.3756, Table 1) (Figures S35 and S37) also showed a very similar fragmentation pattern. Both alkaloids presented a fragment peak of \( m/z \) 521 resulting from the loss of \( N \)-methylphenylalanine in the residual chain R3 in 14, and \( N \), \( N \)-dimethylphenylalanine in 15. The occurrence of \( m/z \) 374 (c, \( R^1 \): C-H) in both MS2 spectra confirm the presence of isoleucine/leucine residual chains in R3, and the comparison with \( m/z \) 521 supports the assignment of a phenylalanine residual chain attached to R3. Fragments d and d – CO of 14 (\( m/z \) 309 and 281) and 15 (\( m/z \) 323 and 295) were also helpful to assign the difference of one methyl group at the phenylalanine residual chain (R3), due to a difference of 14 amu. Thus, alkaloid 14 was putatively identified as nummularine-H and 15 as jubanine-A.28 It is worth pointing out that the presence of a leucine side chain as \( R^3 \) is also a possibility, and in that case this alkaloid was not described before.

Alkaloid 16 showed a protonated ion peak of \( m/z \) 716.3424 \([M + H]^+ \), calcd. for \([C_{42}H_{46}N_{34}O_{36}]^+ \) \( m/z \) 716.3443). The mass difference of 14 amu to alkaloid 17, \( m/z \) 730.3583 \([M + H]^+ \), calcd. for \([C_{42}H_{46}N_{34}O_{36}]^+ \) \( m/z \) 730.3600), indicates again the difference of one methyl group (Figures S39 and S41). In both MS2 spectra, peak \( m/z \) 408 (c, \( R^3 \): C-H) suggests the presence of a phenylalanine residual chain as \( R^3 \). Alkaloid 17 showed the fragment \( m/z \) 685 \([M + H-NH\{CH_3\}_3]^+ \) resulting from the loss of NH(CH3)2, which is common for alkaloids containing \( N \)-\( N \)-dimethyl groups as the N-terminus of peptides. The peak \( m/z \) 555 observed in both MS2 spectra was useful to assign an \( N \)-methylphenylalanine in 15 \([M + H-C_{10}H_{11}N]^+ \) and \( N \), \( N \)-dimethylphenylalanine in 16 \([M + H-C_{11}H_{13}N]^+ \). This hypothesis was endorsed by the fragmentation of the side chain attached to the proline site, resulting in peaks \( m/z \) 309 (d in 16 and \( m/z \) 323 (d in 17, which also generate daughter peaks \( m/z \) 281 (d – CO) and \( m/z \) 295 (d – CO), respectively (Figures S40 and S42). Due to the mass differences (14 amu), it was possible to confirm one extra CH3 group in 17. Extensive analyses of MS1 and MS2 spectra of alkaloids 16 and 17 lead to the putative identification of nummularine-O and jubanine-B, respectively.29

The extract of stems from Z. joazeiro exhibited significant activity (at 500 \mu g/mL) against the Gram-negative bacterium Allivibrio fischi but influenced the growth of the Gram-positive test organism Bacillus subtilis only to a minor extent (Table 2). Furthermore, substantial cytotoxic activity (at 50 \mu g/mL) was detected when using the human cancer cell lines HT-29 and PC-3. Antibacterial activity is frequently reported for CPAs37-39 and is in accordance with the traditional application of this species. The observed anticancer effects might be attributed to the characteristic triterpenoids reported for Z. joazeiro.40,41

Conclusions

Sixteen cyclopeptide alkaloids were detected and characterized in the crude alkaloid fraction from the stems of Ziziphus joazeiro.
by means of UHPLC coupled to high-resolution mass spectrometry. The putative identification of several cyclopeptide alkaloids was possible, while others escaped exact constitutional analysis due to the presence of residues of isomeric amino acid side chains (esp. Leu/Ile). The present method demonstrates an effective way to determine CPAs in alkaloidal extracts, and can be easily reproduced as required, e.g., for phytopharmaceutical quality control and adulteration analyses. Collision-induced fragmentation of type-Ia and -Ib CPA show many similarities in their fragment ions within the classes and some key fragments which can be used to identify related compounds in alkaloid rich extracts. This can guide the way to identify new cyclopeptides with yet to be determined configurations.

**Experimental**

**Plant Material**

Stems of *Ziziphus joazeiro* Mart. (Rhamnaceae) were collected on the campus of the Federal Rural University of Rio de Janeiro (Soropédica – RJ, Brazil, GPS coordinates: −22.762988, 43.695338). The species was identified by Prof. MSc. Marilena de Menezes Silva Conte from the Botany Department of the Federal Rural University of Rio de Janeiro and a voucher specimen was deposited in the Herbarium of this University under register number: RBR 39319.

**Extraction Procedure and Sample Preparation**

Stems of *Z. joazeiro* (1.72 kg) were dried, milled and macerated with methanol (5 × 2 L). After removal of the solvent under reduced pressure, 118 g of crude extract was obtained. The extract was suspended in H2O (1 L) and acidified with HCl (2 M) to pH 1 to 2. The solution was then extracted with EtOAc (3 × 300 mL). The acidified residue was basified with ammonia solution to pH 8 to 9 and the basified solution was partitioned with CHCl3 (3 × 300 mL). The organic layer was concentrated under reduced pressure affording 523 mg of the crude alkaloidal fraction. Ten mg of the crude alkaloidal fraction was solubilized in 1 mL of methanol (spectroscopic grade, Chromasolv™, Honeywell Riedel-de Haën™, Germany) and filtered through a Chromabond C18 SPE cartridge (Macherey-Nagel, Germany), eluting with 3 × 1 mL of methanol.

**UHPLC-ESI-Orbitrap-HR-MS Analysis**

The positive ion high resolution ESI mass spectra were obtained from an Orbitrap Elite mass spectrometer (Thermofisher Scientific, Bremen, Germany) equipped with a HESI electrospray ion source (spray voltage 4.5 kV; source heater temperature: 300 °C; capillary temperature 300 °C; FTMS resolution 30,000). Nitrogen was used as the sheath and auxiliary gas. The MS system was coupled with an ultra-high-performance liquid chromatography (UHPLC) system (Dionex UltiMate 3000, Thermofisher Scientific), equipped with a RP-C18 column (1.9 μm; 50 × 2.1 mm; Hypersil Gold; Thermofisher Scientific; column temperature: 40 °C), and a photodiode array detector (PDA, Thermofisher Scientific). For UHPLC a gradient system was used, starting from H2O:CH3CN 95:5 (each of them containing 0.1% formic acid, isocratic for 2 min) raised to 5:95 within 15 min and then held on 5:98 for a further 3 min; flow rate 400 μL/min; injection volume 2 μL. The wavelength range of the PDA measurements was 190 to 600 nm. The CID mass spectra (buffer gas: helium, FTMS resolution 15,000) were recorded in data dependent acquisition mode using a normalized collision energy of 45%. The instrument was externally calibrated by the Pierce® LTQ Velos ESI positive ion calibration solution (product number 88323, Thermo Fisher Scientific, Rockford, IL, 61105 USA). The data were evaluated with Xcalibur software 2.2 SP1 (Thermo Fisher Scientific).

**Isolation and HPLC Separation of Major CPAs**

An aliquot of the crude alkaloidal fraction (474 mg) was fractionated by column chromatography on Sephadex LH20, eluting with MeOH/CHCl3 (9:1), followed by CC of 73 mg on silica gel eluting with CHCl3/MeOH (9:1). The separation was monitored by TLC (silica gel, CHCl3/MeOH [9:1]) and fractions containing Dragendorff positive spots (Rf 0.63) were combined. Final purification was performed on an Agilent 1260 HPLC system (quaternary pump, autosampler, column oven, DAD) equipped with a fully end capped RP C18 column (5 μm, 150 × 4.6 mm YMS, ODS-A). Due to the low amount of compounds, the separation was performed on an analytical scale applying an isocratic solvent mixture of 20% acetonitrile/80% H2O containing 0.1% formic acid, flow rate 1 mL/min, detection at 280 nm) to give compounds 12 (tR 12.04 min) and 13 (tR 12.33 min).

**NMR Analysis**

1D NMR spectra were obtained with an Agilent DD2 400 NMR spectrometer at 399.915 and 100.569 MHz for 1H and 13C, respectively. 2D NMR spectra were recorded on an Agilent 600 NMR spectrometer (599.831 MHz). TMS was used as an internal standard for 1H NMR analysis.

**Biological Activities**

The crude methanol extract of *Z. joazeiro* stems (50 and 500 μg/mL) was tested against Gram-negative (*Aliivibrio fischeri*) and Gram-positive (*Bacillus subtili*); bacteria, as well as against the human tumor cell lines PC-3 (prostate cancer) and HT 29 (colon adenocarcinoma), as reported previously.42
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Supplemental Material
Supplemental material for this article is available online, and all data (NMR and MS) will be made available upon request.

Author Contributions
CHCS conceived the study, performed experimental work, wrote the manuscript draft. AL performed MS measurements. KF contributed to data curation and manuscript writing. All authors contributed to evaluation of MS fragmentation patterns. MGC and LW were involved in supervision, guidance of the work and raising the funding. All authors took part in editing and correcting the final draft and read and approved the final manuscript.

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Ethical Approval
Not applicable, because this article does not contain any clinical trials.

Informed Consent
Not applicable, because this article does not contain any human or animal subjects.

Trial Registration
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